THE BIOLOGY OF NITROGEN FIXATION

edited by A. QUISPEL

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General preface

The aim of the publication of this series of monographs, known under the collective title of '*Frontiers of Biology*', is to present coherent and up-to-date views of the fundamental concepts which dominate modern biology.

Biology in its widest sense has made very great advances during the past decade, and the rate of progress has been steadily accelerating. Undoubtedly important factors in this acceleration have been the effective use by biologists of new techniques, including electron microscopy, isotopic labels, and a great variety of physical and chemical techniques, especially those with varying degrees of automation. In addition, scientists with partly physical or chemical backgrounds have become interested in the great variety of problems presented by living organisms. Most significant, however, increasing interest in and understanding of the biology of the cell, especially in regard to the molecular events involved in genetic phenomena and in metabolism and its control, have led to the recognition of patterns common to all forms of life from bacteria to man. These factors and unifying concepts have led to a situation in which the sharp boundaries between the various classical biological disciplines are rapidly disappearing.

Thus, while scientists are becoming increasingly specialized in their techniques, to an increasing extent they need an intellectual and conceptual approach on a wide and non-specialized basis. It is with these considerations and needs in mind that this series of monographs, '*Frontiers of Biology*' has been conceived.

The advances in various areas of biology, including microbiology, biochemistry, genetics, cytology, and cell structure and function in general will be presented by authors who have themselves contributed significantly to these developments. They will have, in this series, the opportunity of bringing together, from diverse sources, theories and experimental data, and of integrating these into a more general conceptual framework. It is unavoidable, and probably even desirable, that the special bias of the individual authors will become evident in their contributions. Scope will also be given for presentation of new and challenging ideas and hypotheses for which complete evidence is at present lacking. However, the main emphasis will be on fairly complete and objective presentation of the more important and more rapidly advancing aspects of biology. The level will be advanced, directed primarily to the needs of the graduate students and research worker.

Most monographs in this series will be in the range of 200–300 pages, but on occasion a collective work of major importance may be included somewhat exceeding this figure. The intent of the publishers is to bring out these books promptly and in fairly quick succession.

It is on the basis of all these various considerations that we welcome the opportunity of supporting the publication of the series '*Frontiers of Biology*' by North-Holland Publishing Company.

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CHAPTER 1

General introduction

A. QUISPEL

All life on earth depends on the use of the energy of sunlight, the only source of energy reaching the earth continuously in such a way that it can be used for the reduction of carbon dioxide to organic substances. This process of primary production depends not only on the sunlight itself, but especially on the quantity of green organisms, lower and higher plants, that develop the apparatus for photosynthesis.

The growth of these green organisms is, of course, dependent on the use of carbon dioxide in photosynthesis but other elements are also essential for their production just as they are essential for all other living organisms. A shortage of any one of these other essential elements therefore will limit the possibilities of primary production and the growth of all organisms, including man, that depend on it. Such limitations may play a role in natural communities as well as in the man-controlled communities of agri- and sylviculture.

The element which is considered to be the most common limiting factor for primary production is nitrogen. Unlike other growth-limiting elements, whose total amount in water or soil is limited so that shortage can only be replenished by better forms of recycling, no shortage of nitrogen should ever occur as the atmosphere contains abundant amounts of it (table 1.1). However, only relatively few organisms are able to use these reserves of elementary nitrogen.

Table 1.1
Distribution of nitrogen (from Delwiche 1965).

Region	g/cm^2
Atmosphere	755
Biosphere	0.036
Hydrosphere (excl. dissolved N_2)	0.033
Earth crust (rough estimation)	2,500

In natural ecosystems and in old forms of agriculture still based on natural processes, the nitrogen cycle was essential and sufficient for the maintenance of the necessary amounts of nitrogen in the soil. In such natural cycles fixation of atmospheric nitrogen plays an essential role in the replenishment of nitrogenous substances leached out from the soil, and especially in counteracting the loss of nitrogen to the atmosphere during dissimilatory nitrate reduction.

This latter process is generally underestimated as it occurs only under anaerobic conditions. This does not mean that losses of nitrogen by reduction of nitrates to gaseous forms of nitrogen are limited to wholly anaerobic ecosystems like muds. Even in well-aerated soils dissimilatory reduction of nitrates may be considerable, e.g. in the rhizosphere where anaerobic niches may occur due to oxygen consumption by aerobic organisms (Woldendorp 1963, Stefansen 1972).

While the fixation of elementary nitrogen is therefore indispensable for the mere maintenance of the already existing levels of bound nitrogen at those sites where they are needed for primary production, a marked increase of available nitrogen is essential if the growing demands of food production for the increasing world population are to be met. Although everyone must agree that this population increase must be stopped to prevent a world-wide catastrophe, the increased use of nitrogenous compounds for plant growth is essential to combat famine in the population existing at this very moment.

From ancient times the use of biological nitrogen fixation played a dominant role in agricultural production. The empirical usefulness of Leguminosae as green fertilizers was mostly based on their symbiotic nitrogen fixation in the root nodules, though this was only scientifically established by the classical work of Hellriegel and Wilfarth (1888).

Recent times have shown a gradual decrease in the use of leguminous plants in agriculture and instead a rapid rise in the use of industrial fertilizers, produced by some form of chemical fixation of nitrogen. According to recent estimates the annual production of commercial nitrogenous fertilizers was about 30 million tons of nitrogen in 1968, and now equals the amounts that were fixed by biological nitrogen fixation before the advent of modern methods of agriculture. A recent estimation of the world nitrogen balance sheet is given in table 1.2.

Yet there are indications that a renewed interest in biological nitrogen fixation is developing (Becking 1971). For many years the use of artificial nitrogenous fertilizers has been advocated on the grounds that plants could conserve the energy otherwise expended in fixing nitrogen as this had already been done in the factory. Even though this argument is fallacious in most cases, the reduction of nitrate using practically the same amount of energy as the reduction of elementary nitrogen (Minchin and Pate 1973), it is be-

Table 1.2
Data for a nitrogen balance on earth (based on Delwiche 1970; Hardy and Holsten 1972).

	Area ha × 10^6	Kg N_2 fixed per ha × yr	Metric tons per yr × 10^6
Biological fixation			
legumes	250	55 –140	14–35
non-legumes	1,015	5	5
rice fields	135	30	4
other soils and vegetations	12,000	25 –30	30–95
marine	36,100	0.3–1	10–36
Industrial fixation			30
Atmospheric fixation			7.6
Juvenile addition			0.2
Denitrification			
terrestrial	13,400	3	43
marine	36,100	1	40
Loss to sediments			0.2

coming realized that the energy from limited amounts of fossil fuels must not be used for purposes that can be served by solar energy. In the many poor countries where increases in food production are most needed it is highly erroneous to spend considerable parts of the national income for the purchase of fertilizers that could be obtained at negligible cost by a more efficient use of nitrogen fixing organisms. Of course, we think about leguminous plants first, but other organisms as well may be important. The inoculation of moist rice fields with blue-green algae occasionally increased crops by 20%. The introduction of new high-yielding grain varieties with their extreme demands for high levels of nitrogen will make such heavy demands on nitrogen that one may doubt whether enough artificial fertilizers could be produced to meet these demands.

Though it would be unwise to state that to-day nitrogenous fertilizers could be entirely replaced by biological nitrogen fixation, it has been widely recognized that more attention should be paid to the possibilities of the use of atmospheric nitrogen by living organisms. This was highly stimulated by the 'International Biological Programme' which in its section 'Production Processes' selected the problems of biological nitrogen fixation as one of the key processes for study.

The importance of an increased use of nitrogen-fixing organisms might be considered from still another point of view. If nitrogenous fertilizers are not carefully applied, they can add another contribution to pollution by leaching into natural waters. This does not need to be an important aspect: careful estimations have shown that the contribution of agriculture to eutrophication of natural waters may be small as compared to other sources of contamination. In the Netherlands, Kolenbrander (1969) concluded from lysimeter experiments that from heavy soils no leaching of NO_3 occurred during the summer. Leaching was possible, however, in spring or autumn, and on lighter soils in summer as well. Of course this does not assure that under less controlled conditions, under other edaphic and climatic circumstances, leaching of excess nitrates might not lead to serious problems. Here the fixation of nitrogen by biological systems has the great advantage that it is more or less subjected to natural feed-back regulations. In many cases the enzyme nitrogenase is not active if enough bound nitrogen is present in the environment, while in symbiotic nitrogen fixation the initiation and development of effective root nodules may be inhibited. Though these feed-back regulations certainly do not always guarantee the absence of eutrophication, they will regulate the increase of nitrogen in a better way than inorganic nitrogen fertilizers that can only be regulated by the self control of farmers. Only biological nitrogen fixation offers opportunities for nitrogen fertilization with built-in regulation.

The conviction that a better and more efficient use of biological nitrogen fixation must be stimulated has already led to some far reaching thoughts. During the introduction to the Symposium World Food Supply at the XIth International Botanical Congress in 1969 the chairman of the symposium, J. van Overbeek, said 'I would urge botanists to tackle the problem of nitrogen deficiencies that exist in the living world with boldness. We need to devise more efficient ecosystems, put root nodules on cereal crops, and put nitrogen fixing chloroplasts in their leaves ... If we do succeed in developing cereal grains that are fed from the nitrogen of the air rather than from nitrogen of fertilizer, we shall have achieved a whole new step in sophistication in food production'.

On April 5 and 6, 1971 the Rockefeller Foundation was inspired by comparable motives when organizing a conference on extending symbiotic nitrogen fixation to increase man's food supply. The participants of this conference agreed that a real extension of e.g. nitrogen fixing root nodules to plants like cereals will not be a practicable goal for many years to come, if it ever could be realized. On a more modest scale, however, much can be expected from an intensification of research in this field which may lead to a more sophisticated use of the potentialities of nature.

It is evident from the considerations given above that the practical importance of biological nitrogen fixation is considerable so that research in this field must be stimulated. Certainly such research is not only important because of its practical aspects but it is moreover extremely awarding from a purely biological point of view. Studies on biological nitrogen fixation confront the investigator with a wealth of problems related to many of the most fundamental aspects of present-day biology, ranging from molecular biology, biochemistry and cell biology to morphogenesis, whole plant physiology, physiological plant pathology and ecology.

Important progress has been made in our understanding of the biochemical mechanism of biological nitrogen fixation (ch. 13). Moreover, these biochemical results led to the discovery of entirely new methods for the determination of the potentialities for nitrogen reduction e.g. the use of C_2H_2 reduction for assays of nitrogenase activity (ch. 2). These results therefore opened wide perspectives for progress in research on the biological aspects of nitrogen fixation. It is the consideration of these biological aspects which forms the main purpose of this book.

All available evidence indicates that the enzyme nitrogenase is limited to certain prokaryotic organisms: heterotrophic (ch. 3) and photoautotrophic bacteria (ch. 6) and blue-green algae (ch. 7). This restriction of a certain type of metabolic reactions to prokaryotes is not exceptional. The occurrence in only a limited number of species indicates that only these species possess the genetic information for the synthesis of the enzyme nitrogenase.

However, the situation appears to be far more complicated. The actual reduction of nitrogen depends not only on the potential formation of the specific enzyme but even more on the fulfillment of certain prerequisites for its formation and activity (ch. 15). Here aspects of regulations come to the fore, the dependence on other metabolic processes, on cellular and intercellular regulation and on environmental circumstances, abiotic as well as biotic. The role of biotic influences is of the utmost importance in symbiotic nitrogen fixation in the root nodules of leguminous plants (ch. 9). Here the symbiotic relation with bacteria of the genus *Rhizobium* leads to a situation which offers the essential prerequisites for the reduction of nitrogen which are evidently never fulfilled in free living Rhizobia (ch. 15.4). As these Rhizobia generally occur in soil and can be easily cultivated without showing any signs of nitrogen fixation one wonders whether still other organisms might possess the potentiality for nitrogen fixation without ever having the opportunity to realize them, because they lack the capacities for entering into the appropriate symbiotic relationship.

Even in organisms that are known to fix nitrogen in pure cultures, the proximity of other organisms may stimulate the fixation of nitrogen. These types of symbiosis may range from rather disjunctive effects of other microorganisms in the same biocoenosis, in rhizosphere (ch. 4) and phyllosphere (ch. 5), to real conjunctive symbioses as found in lichens with blue-green algal partners (ch. 8). A better understanding of such symbiotic stimulation of nitrogen fixation may yield valuable information for regulation phenomena in nitrogen fixing organisms in general and the more advanced cases of symbiotic nitrogen fixation in particular.

When speaking about the prerequisites for biological nitrogen fixation in free living organisms, one may consider the physiological and ecological conditions, the intra-, and extracellular relations that permit the functioning of the nitrogenase system and possibly also stimulate its activity (ch. 15). In the symbiotic systems another group of problems is equally important: the infection and subsequent development of effective nodules as a result of the complicated interplay of actions and reactions between the host plant and the microorganisms. Here a comparison between the Rhizobium symbioses, which are limited to the Leguminosae, and the so-called Actinomycete symbioses, whose host plants are scattered among the plant kingdom, may yield valuable information (ch. 10). An inclusion in this comparison of related nodular structures, though most probably not leading to nitrogen fixation, may yield information that is thought-provoking for the study of the real nitrogen fixing symbiotic relations (ch. 12). Of course the study of symbiotic relations, based on the occurrence of specific infections leads us into the realm of the problems of physiological plant pathology. In fact the situation in leguminous and non-leguminous root nodules reflects one of the most highly developed phases in the mutual relations between two different organisms, leading to an organized new structure with a definite function (ch. 11).

These problems finally lead to the problem which is the most essential for our understanding of the biology of nitrogen fixation: what are the prerequisites for nitrogen fixation to occur in a living cell. This problem can never be solved without good knowledge of the biochemical reactions. As so many excellent reviews of the biochemistry appeared in recent years (e.g. Hardy and Burns 1968, 1972; Hardy et al. 1972; Postgate 1971, 1972; Bergersen 1971), the discussion of the biochemistry remained limited to a general and comparative introduction (ch. 13) though some aspects are discussed in somewhat more detail for the different biological systems in ch. 15. The first prerequisite for nitrogen fixation is the presence of genes to form the essential enzymes (ch. 14), while the final realization of the genetic potentialities depends on regulation phenomena, either with regard to induction or repression of the enzyme formation, or to the activation and regulation of their activity (ch. 15).

Our central theme is the biology of nitrogen fixing systems. The understanding of this biology and the evaluation of our present state of knowledge and ignorance needs a survey of the data now available. These data are only relevant if they are related to the different problems of research in this field. It is hoped that this book will especially emphasize these problems, show their importance for our general understanding, demonstrate how they have been solved, how it has been attempted to solve them, or how they might be solved in the future. From this it may be concluded where the most exciting developments are going on, where the most important progress is to be expected and where future research must be especially stimulated.

In this respect the book can be used complementary to older reviews e.g. the classical books of Fred et al, (1932), and Wilson (1940), the articles of Allen and Allen (1958), Wilson (1958), Raggio and Raggio (1962), Virtanen and Miettinen (1963), a more recent book of Stewart (1966) and some translations of books by Sowjet authors which give a comprehensive account of the Russian work in this field (Fedorow 1952; Mishustin and Shil'nikova 1971).

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