A TREATISE ON DINITROGEN FIXATION

SECTION III

BIOLOGY

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Section III: Biology

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Preface

During the last quarter of the twentieth century, the world's population will increase from 4 billion to 6 or 7 billion, and the demand for food will more than double. Provision of an adequate supply of fixed nitrogen is central to the successful meeting of this food challenge. The supplementation of biological dinitrogen fixation with 40 million tons of fertilizer nitrogen at a cost of 8-10 billion U. S. dollars in 1975, and the estimated need for a supplement equivalent to 160 million tons of fertilizer nitrogen in 2000 A.D. indicates the magnitude of the problem. Moreover, the growing awareness of environmental quality and limitations of nonrenewable resources may introduce additional constraints.

Ignited by the extraction of the dinitrogen-fixing enzyme from a bacterial cell and abiological fixation of dinitrogen under ambient conditions, research on dinitrogen fixation entered a new log phase of growth about 15 years ago, and this remarkable phase continues to grow in both accomplishments and person power. Our objective is to provide the first comprehensive interdisciplinary reference work on dinitrogen fixation combining these recent advances with earlier work. Section I of this work on Inorganic and Physical Chemistry is edited by Frank Bottomley, Section II on Biochemistry is edited by Richard C. Burns, Section III on Biology is edited by Warren S. Silver, and Section IV on Agronomy and Ecology is edited by Alan H. Gibson, with Ralph W. F. Hardy as general editor for all sections. We hope that this coverage will facilitate research to explore biological and abiological dinitrogen fixation and to develop technologies for provision of fixed nitrogen.

Biological dinitrogen fixation has contributed to production in natural and agricultural habitats from an early stage in the development of living matter on earth. Until the late 1880s, none of the causal agents had been identified positively, although early writing, as far back as 5000 years ago, testify to the appreciation of legumes as contributors to soil fertility. Since the pioneering studies of Boussingault,

Hellriegal, Winogradsky, and Beijerinck, a very considerable understanding of the various diazotrophic forms, the factors affecting their activity, and their contribution to the nitrogen economy of many habitats, has been achieved. However, this understanding is far from complete, and as we stand at the threshold of an era of intensive investigation in all aspects of dinitrogen fixation, it is timely that existing knowledge be reviewed and collated.

Two major reasons can be given for the necessity to intensify our studies of dinitrogen fixation. The first is the increasing need to understand the earth's biological environment, to understand the inputs and outputs from all habitats, and to appreciate how perturbation of any elements of existing ecosystems will influence the behavior of all component parts. Developed and developing soils, lakes, rivers, tundras, and oceans have been and are receiving close attention, and with the availability of new techniques, additional diazotrophic forms are being identified. Many of these diazotrophs are free-living procaryotes (mainly bacteria or blue-green algae), but increasingly, facultative, and more particularly, associative symbiotic systems are being described from many habitats (e.g., marine, tundra, rhizosphere of cereals and dicotyledonous species). In many ways the associative symbiotic systems are essentially unknown with regard to our understanding of the diazotroph involved, of the factors involved in the association, and of the nitrogen contribution to the host or to the habitat.

The principal need for a deeper understanding of biological dinitrogen fixation is the urgent requirement to increase agricultural production for a seemingly ever-expanding human population. Although all forms of biological dinitrogen fixation ultimately contribute to such production, increases in the short term are likely to depend on the greater and more efficient utilization of legume-*Rhizobium* associations. The grain and pulse legumes are foremost in current research approaches to increasing protein production, although pasture and forage legumes, both in temperate areas and in the tropics, and green manure legumes, make significant contributions to animal production and the soil's nitrogen supply, respectively. The problems of increasing food supply are exacerbated by difficulties in producing nitrogenous fertilizers. In the short term the high capital cost of building fertilizer plants prevents many countries from

producing the quantities of fertilizer required, whereas in the long term there is grave concern about the supplies of natural gas required to manufacture these fertilizers. In addition, the inefficiency of uses of fertilizer nitrogen by agricultural crops, potential pollution of groundwater by unused nitrogen, denitrification loss of fertilizer nitrogen, possible destructive effects of denitrification products on atmospheric ozone, and transportation, storage and application costs for fertilizer nitrogen are limitations of our current technology. The nature and the magnitude of these problems require that intensive efforts be made to increase biological nitrogen fixation, not only from legumes but from blue-green algae and other procaryotes, either in the free-living state or in some form of symbiosis with a macrosymbiont driven by solar energy.

Because of the current importance of the legumes, as well as for convenience, there is a broad division of the subject into the legume and "nonlegume" systems. The latter comprises the bacterial and blue-green algal diazotrophs that function either in the free-living state or as microsymbionts associated with lower plants or higher plants, other than legumes. Section III, the biological section, is devoted to a consideration of the broader aspects of dinitrogen fixation — the organisms involved, pertinent physiological processes of the microbe and the host plant, and where appropriate, certain phytogeographical facets. The current importance of the leguminous symbiosis is reflected in the six chapters devoted to this topic, and three chapters on the genetic aspects of dinitrogen fixation attest to its increasing importance as a possible approach to potentiating and extending the process of dinitrogen fixation by genetic manipulation.

Fred, Baldwin, and McCoy, in introducing their monumental monograph "Root Nodule Bacteria and Leguminous Plants" in 1932, expressed their concern with the volume of material at their disposal, and the adequacy of its communication, by quoting W. J. Humphrey: "Many investigations are lost for years, if not forever, in the jungle of journals and the tangle of tongues." How much more relevant such comment is today! The contributing authors have not attempted to provide a complete citation of all references; rather they have concentrated on citing more recent work and landmark studies in particular

areas, and through such references, providing access for interested readers to earlier studies. This, we believe, has allowed them to provide completeness of cover of their topics, to present current thinking on the subject, and to indicate their thoughts on the most profitable areas for future investigation.

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June 1976

SPECIAL NOTE

The Plant and Soil, Special Volume which is referred to in many chapters is catalogued in some libraries as Biological Nitrogen Fixation in Natural and Agricultural Habitats, T. A. Lie and E. G. Mulder, Eds., Martinus Nijhoff, The Hague, 1971.

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SECTION III
Biology

CHAPTER 1

Perspectives in Biological Dinitrogen Fixation

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1.1 RETROSPECT

The aim of this chapter is to place biological N_2 fixation in perspective as a natural phenomenon and as an essential part of the ecosystem. It concludes by asking questions that beg answers.

Excellent books and reviews are available on the subject of dinitrogen (N_2) fixation, so that readers requiring a more detailed historical treatment are referred to these (1-8).

Professor Perry Wilson kindly sent me a copy of Aulie's historical article on "Boussingault and the Nitrogen Cycle" (9). It makes a good starting point, for Boussingault's long engagement (1836-1876) with the problem of the source of nitrogen in plants which will strike a sympathetic chord in many contemporary minds. Boussingault's field evidence and biological observations with legumes were so much at variance with his careful and controlled chemical evidence, that he finally abandoned his earlier ideas about N_2 utilization by legumes, and came to view the soil as a chemical system capable of supplying the nitrogen needs of all plants.

The enigma of leguminous behavior was solved when Hellriegel and Wilfarth proved N_2 fixation by nodulated legumes in 1887, the year of Boussingault's death. This was followed in 1888 by the isolation of the first nodule bacteria by Beijerinck (1). Winogradsky reported in 1893 the isolation of a soil bacterium, Clostridium pasteurianum, which fixed N_2 in the test tube (3). Beijerinck isolated the aerobic diazotroph Azotobacter in 1901. Contradictory reports appeared in the years 1889-1928 about N_2 fixation in pure cultures of blue-green algae (2).

In 1932 Fred, Baldwin, and McCoy published their monograph (1) which collated, reviewed, and analyzed existing information on legume symbiosis (including a section of other N_2-fixing plants). They achieved a biological synthesis that had a profound influence on subsequent theory and practice. The monograph published by P. W. Wilson in 1945 achieved a similar but more biochemical synthesis and provided a blueprint for future research strategy.

For the next two decades Wilson and Burris with their colleagues and students, employing the new technique of mass spectrometry with $^{15}N_2$ to trace reactions, dominated the scene on the biochemistry of N_2 fixation. In a series of brilliantly conceived experiments, based on considerations of enzyme theory and the likely pathways of N_2 fixation (10) they traced the pathway of fixation through ammonia to glutamic acid and, thence, to other amino acids.

Finally they obtained enriched juvenile ammonia directly from Clostridium in 1951, and in 1953 achieved the same result with Azotobacter (11).

A new impetus to biochemical studies occurred when extracts capable of fixing N_2 were prepared from Clostridium by Carnahan, Mortenson, Mower, and Castle in 1960 (12, 13). Cell-free extracts of other diazotrophs were soon prepared (14), and it became possible to investigate the nature of "nitrogenase," uncomplicated by growth, nutrition, or respiration. The enzyme itself has now been purified and and resolved into two components (15). Exciting recent research shows that "nitrogenase" activity can be transferred from N_2-fixing Klebsiella to nonnitrogen-fixing mutants (16, 17) and to E. coli (18).

Meanwhile, through the use of $^{15}N_2$, a large number of diazotrophs were added to the list (19, 20) while others were confirmed or removed (21). Symbiotic systems have been confirmed and extended (8, 22-26) and some challenged (27). We can now be confident that N_2-fixing symbioses are present in certain members of the Coriariales, Rosales, Myricales, Fagales, Casuarinales, and Rhamnales, as well as Leguminales. All have bacterial root-nodule endophytes (8, 22).

The herbaceous angiosperm Gunnera (Haloragidaceae), which carries blue-green algae in stem cortical tissues (24), fixes appreciable amounts of N_2 (26). Associations with blue-green algae as the diazotrophs are widespread, involving lichens, liverworts, ferns, cycads, as well as higher plants (24, 23). Associations between blue-green algae and some protozoa, e.g., Cyanophora paradoxa (24), and green algae (23) are known but the function of the blue-green algae is poorly understood.

Confirmation of ammonia as the key intermediate in N_2 fixation had important ecological and biochemical implications. Wilson and Burris (10) and Bayliss (28) had calculated from thermodynamic data that in the reduction of N_2 to NH_4^+, using glucose as the source of electrons, energy might become available and not be required from external sources to "drive" the reaction.

What, then, was to prevent asymbiotic diazotrophs from exploiting their enzymic advantage in competition for organic carbon? Again, since N_2 could be considered as a major alternative oxidant, the question of the relationship between O_2 and N_2 in aerobic diazotrophs was reviewed (29). Evidence that these

gases may in fact compete for substrate electrons was reported by Parker and Scutt (30) and supported by Dalton and Postgate (31).

In legumes also, ammonia was shown by Kennedy (32, 33) to be the key intermediate in N_2 fixation, and this was confirmed by Bergersen (34). Kennedy also implicated bacteroids as the site of nitrogen fixation (33, 35-37), but it remained for Bergersen and Turner (38), and Koch, Evans, and Russell (39) to demonstrate this proposal by obtaining fixation in bacteroids separated from nodule tissues. Glucose and/or fructose were found to be the likely substates for bacteroids in Ornithopus and Lupinus nodules (40).

Recognition of the ability of nitrogenase to reduce substances with C ≡ C, C ≡ N or N ≡ N structures began with the report on N_2O reduction by Mozen and Burris in 1954 (41). A wealth of chemical information for comparison with the inorganic complexes of dinitrogen has followed (see II.4). Perhaps most useful was that showing that "acetylene and N_2 reduction are analogous processes, catalysed by the same enzyme system" (42), which led to the proposal by Hardy and Knight for the use of acetylene reduction as a sensative assay for nitrogenase and its widespread application in many laboratory and field studies (see IV.12).

1.2 THE NITROGEN ECONOMY OF THE BIOSPHERE

1.2.1 Sources of Nitrogen

An excellent discussion on the bio-geochemistry of nitrogen is given by Alexander (43), who considers sources, sinks, states, and amounts of nitrogen on Earth (also see IV.1). "To maintain a steady-state level of nitrogen in the biosphere, losses must equal gains, and the leakage from the biosphere is apparently made up by the unique organisms that bring about nitrogen fixation..." (43).

1.2.1.1 Rainfall

Allison (44) lists from many parts of the world rainfall nitrogen values ranging from 1.8-22 kg/ha·yr. In northern Australia Wetselaar and Hutton (45) conducted careful experiments to estimate the annual

accession of rainfall N to the soil, and to judge its origin. The annual average rainfall at the site is 925 mm, the area is subject to frequent electric storms, and is remote from industrial or urban activity. They found annual increments of nitrate plus ammonium nitrogen amounting to 1.5 kg/ha, the concentration of ions becoming lower as the season progressed. The authors concluded from consideration of the ionic balance that neither the ocean nor the atmosphere could be major contributors, and that "most of the material in the rain water is part of a terrestrial cycle and not a true accession."

Thus it appears that rainfall makes minor contributions of "new" fixed nitrogen to the biosphere.

1.2.1.2 Autotrophic Microorganisms

N_2 fixation in aquatic environments by photosynthetic bacteria (46, 47) or by blue-green algae (48) has been estimated to vary from a few kilograms to 60-70 kg/ha in paddy soils. In arid areas algal crusts may contribute meaningful amounts of nitrogen to the ecosystem (62, 48). Marine gains from N_2 fixation are about one-third of the terrestrial gains (43).

1.2.1.3 Heterotrophic Bacteria

Certain bacteria in the genera Clostridium, Bacillus, Klebsiella, Azotobacter, Derxia, Beijerinckia, and Mycobacterium fix N_2, either anerobically or more efficiently at low pO_2 (49). Their activity can be measured using $^{15}N_2$ or acetylene, but their numbers are likely to be grossly underestimated by laboratory counting methods (50, 51).

Nitrogen accumulation in unsupplemented soils growing grass without legumes has been reported (52, 53). Recent evidence indicates fixation of N_2 in the rhizosphere of grass (54, 55) and herbs (56), the quantities ranging from a few kilograms up to 90 kg nitrogen/ha·yr. The microbial agents appear to be embedded in the mucigel on the root surface (55). This looser type of symbiosis, termed associative by Hardy and Holsten (88), may be of considerable importance in tropical soils.

The importance of N_2 fixation in the phylloplane (57) is more difficult to assess. High pO_2 would exclude most N_2-fixing bacteria from activity, and