# The Chemistry and Biochemistry of Nitrogen Fixation

Edited by J. R. Postgate

# The Chemistry and Biochemistry of Nitrogen Fixation

Edited by

J. R. Postgate

*University of Sussex,*
*Falmer, Brighton BN1 9QJ,*
*Sussex.*

PLENUM PRESS · London — New York · 1971

Plenum Publishing Company Ltd.
Davis House,
8 Scrubs Lane,
Harlesden,
London NW10 6SE.

U.S. Edition published by
Plenum Publishing Corporation,
227 West 17th Street,
New York, New York 10011.



SBN: 306-30459-7

Library of Congress Catalog Card Number: 70-161303

Printed in Great Britain by
William Clowes & Sons Limited
London, Colchester and Beccles

## CONTRIBUTORS


R. H. Burris — *Department of Biochemistry, University of Wisconsin, Madison 6, Wisconsin 53706, U.S.A.*

J. Chatt — *A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton BN1 9QJ, Sussex.*

H. J. Evans — *Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon, U.S.A.*

G. J. Leigh — *A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton BN1 9QJ, Sussex.*

J. R. Postgate — *A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton BN1 9QJ, Sussex.*

R. L. Richards — *A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton BN1 9QJ, Sussex.*

S. A. Russell — *Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon, U.S.A.*

W. S. Silver — *Department of Life Sciences, Indiana State University, Terre Haute, Indiana 47809, U.S.A.*

P. W. Wilson — *Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin 53706, U.S.A.*

M. G. Yates — *A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton BN1 9QJ, Sussex.*

# Preface

Understanding of biological nitrogen fixation has advanced with impressive rapidity during the last decade. As befits a developing area of Science, these advances have uncovered information and raised questions which will have, and indeed have had, repercussions in numerous other branches of science and its applications. This 'information explosion', to use one of to-day's cant idioms, was initiated by the discovery, by a group of scientists working in the Central Research laboratories of Dupont de Nemours, U.S.A., of a reproducibly active, cell-free enzyme preparation from a nitrogen-fixing bacterium. Full credit is due to them. But subsequent developments, albeit sometimes quite as impressive, have too often been marked by that familiar disorder of a developing field of research—the scramble to publish. It is a scramble which, at its best, may represent a laudable desire to inform colleagues of the latest developments; yet which too easily develops into an undignified rush for priority, wherewith to impress one's Board of Directors or Grant-giving Institution. This, in miniature, is the tragedy of scientific research to-day: desire for credit causes research to be published in little bulletins, notes and preliminary communications, so that only those intimately involved in the field really know what is happening (and even they may well not see the forest for the trees). Those outside the field, or working in peripheral areas, may glean something of what is going on from reviews and fragments presented at meetings, but the broad pattern of development is often elusive.

This book is an attempt to correct the situation within its own particular field. An explanation (some might even say an apology) is to-day necessary from everyone who presumes to add to the mounting deluge of scientific publications; for this book I offer the view that it is intended for the informed outsider. I asked contributors to stand back a little from the subject and to describe what the *real* advances of the last decade had been. What were they? What was their impact on our understanding of this particular subject? How did they influence our background knowledge of chemistry,

biochemistry or physiology? The book was intended to be complementary to existing works on nitrogen fixation: an advanced but readily comprehensible survey of the last decade's innovations for students, teachers and research workers who would be reasonably well-informed about its background. Contributors, all recognized authorities in their particular fields, were asked specifically not to write reviews: there was no obligation to cite all known references bearing on a given aspect of the subject; historical continuity could be ignored and even the desire to be in all ways up-to-date could be resisted. Painstaking and exhaustive reviews of nitrogen fixation, in its biochemical, chemical and biological aspects, exist in plenty; contributors were asked to present their material as a survey which, while in no way 'talking down', would be useful and comprehensible to scientists whose training and interests would be broader and might range from purely chemical to wholly biological.

I thank my fellow contributors for interpreting my intentions so effectively. I am aware that some have not been able to resist adding their latest 'stop-press' item; that others, having been aware of an unseemly squabble for priority, may have felt it necessary to describe the history of a certain advance in a degree detail which may seem strange to those not involved; yet others have been overtaken by events (my own contribution contains information about heterocysts and nitrogen fixation by blue-green algae which became obsolete in press; fortunately Professor Burris's chapter came in later and amends it).* I have left these human touches intentionally, in the hope that the synoptic view taken by the book as a whole will compensate for the occasional idiosyncracy. (And what could be more tedious than contributions devoid of idiosyncracy?)

A final word about the first contributor. In 1940 Perry Wilson published 'The Biochemistry of Symbiotic Nitrogen Fixation', a seminal work which served as a springboard for a great deal of meaningful work in this field. The decade which the present volume covers ended with the thirtieth anniversary of Wilson's book and, at an early stage, we contributors had intended to dedicate our book to Perry Wilson as a kind of *festschrift.* Illness led to a re-shuffle of contributors and I found myself calling upon Perry, not to stand and be admired, but to do some more work.

So our book is graced by Perry's opening chapter. Our intention to honour him may have been thwarted but our esteem for his monumental contribution to the subject remains unaltered. We

* Compare pages 177 and 149

wished to do him formal honour; in the event we could not. But it is a great pleasure, and in some ways it feels more natural, to have him here with us.

*John Postgate*
*University of Sussex*
*January, 1971*

*Acknowledgement.* Four contributors (G. J. Leigh, J. Chatt, J. R. Postgate, M. G. Yates) are employees of the British Agricultural Research Council at its Unit of Nitrogen Fixation, University of Sussex. They acknowledge permission to contribute and emphasize that all opinions and interpretations expressed are their own and in no way commit the Council.

# Contents

# CHAPTER 1

# The Background

PERRY W. WILSON

*Department of Bacteriology*
*University of Wisconsin, Madison,*
*Wisconsin 53706, U.S.A.*



## 1.1 INTRODUCTION

### *1.1.1 The 1886-1906 period*

Beginning in the middle 1830's the roll-call of chemists interested in answering the important theoretical and practical question of whether green plants can use atmospheric $N_2$, includes such famous names as Boussingault and Ville in France, Lawes and Gilbert in England, Liebig in Germany and Atwater in the United States

(Wilson, 1957). When Hellriegel and Wilfarth in 1886-1887 reported their classical researches, interest of the chemist languished except for a few who undertook the rather thankless chore of confirming and extending the observations. The discoveries of Berthelot, Winogradsky and Beijerinck of biological nitrogen fixation by free-living soil bacteria and by Hiltner and others that even nonlegumes such as the alder in association with an appropriate endophyte can also use $N_2$ provided a backlog of basic science that could be exploited in agricultural practice. Such application was carried out appropriately in agricultural colleges and experimental stations; the research, dealing with laboratory, greenhouse and field experiments designed for solving very practical problems, was published as station bulletins and reports in agricultural journals. Studies concerned with more basic aspects usually appeared in an appropriate (i.e., nonmedical) journal of microbiology although many would have been acceptable in the chemical literature.

The indices of the basic chemical journals for the next forty years do contain references under the entry *nitrogen fixation,* but few of these refer to the biological process. For example, the total was four in the *Journal of the American Chemical Society* from 1886 to 1906, a period during which workers in the United States at many of the State agricultural experiment stations were actively engaged in research (Wilson, 1963). In the first of these, Coates and Dodson (1896) argued:

> 'As the cotton plant is highly nitrogenous in character, and as there seems to be no reason why the leguminosae should have a preemptive claim on the absorption of free nitrogen, it was decided to undertake certain experiments in the hope that something important would be discovered.'

Whether they regarded the negative results obtained as important is not stated.

Weber (1898) described an ingenious technique for demonstrating $N_2$-fixation by dwarf peas grown in an aerated water culture: another confirmation of Hellriegel and Wilfarth. Voorhees and J. G. Lipman (1905) contributed a lengthy paper dealing with nitrogen fixation in the soil, a subject typical of the material appearing in the agricultural bulletins, and Hopkins (1902), in a paper that was essentially a preprint for an Illinois agricultural bulletin, described nitrogen fixation by the alfalfa plant in the midwest prairies.

These are cited to suggest that the lack of publication in the professional journal did not reflect a snobbish editorial policy. There is additional evidence that the chemists were interested, philosophically at least, in the field even though they chose not to work there. The parochial coverage implicit in the title of *The Review of American Chemical Research* did not prevent this forerunner of *Chemical Abstracts* from publishing an abstract of Berthelot's paper in the *Bulletin Société Chimique de France* that told of the discovery of asymbiotic nitrogen fixation in the soil. Moreover, Friedburg (1890) furnished the journal a detailed translation of a contribution by A. Petermann describing nitrogen fixation in the lupine which originally appeared in the memoires of the Royal Academy of Belgium.

Finally, Wiley (1894) in his presidential address to the Society on the conservation and waste of plant foods reported with satisfaction that:

> 'Winogradsky and Warington have shown that an organism can grow in a sugar solution and excluding all nitrogenous matter save the free nitrogen of the atmosphere which it is capable of oxidizing and assimilating . . .'

He used this an an example in support of his optimistic prediction that:

> 'With the aid of scientific agriculture, with the help of the agricultural chemist, we may safely say that a thousand million people will not crowd our (i.e., USA) means of subsistence . . . the death of humanity is not to come from starvation but from freezing and many a geological epoch will come before this planet dies of cold.'

### *1.1.2 The 1906-1928 period*

If the appearance of speciality journals heralds the birth of a new discipline, a fairly reliable estimate of the date can be set for biochemistry: 1906. Three journals appeared: *Biochemical Journal* (1906); *Biochemische Zeitschrift* (1906), and *Journal of Biological Chemistry* (1905-6). Their existence, however, did not appear to stimulate interest among the professionals in biochemical investigations of $N_2$-fixation. In the first 22 volumes of *Biochemical Journal*, only one paper was published directly related to the field. Florence Mockeridge (1915) investigated soil organic matter as a source of energy for the azotobacter. She reported fixation of 6 to

10 mg $N_2$/g substrate not only for glucose and sucrose but also for butyric acid, starch, dextrin, gum arabic and gum tragancanth. The most impressive result was obtained with ethylene glycol, but only one experiment was possible owing to the scarcity of this source of carbon. She stated that 20 or more days were required for the exhaustion of the one gram of substrate.

During this same period, *Biochemische Zeitschrift* published 202 (somewhat smaller) volumes, but papers on biological $N_2$-fixation were extremely rare. Kossowicz (1914) reported that, although he had claimed fixation by yeasts and fungi two years before, more careful experiments (chiefly filtering the air supply through acid and alkali) had failed to confirm the results. As a part of his extensive studies of the influence of radioactivity on plants, Stoklasa and his associates (1922, 1926, 1928) included trials on nitrogen fixation by *Azotobacter chroococcum*. Their chief interest was the effect of radiation; they alleged that $N_2$-fixation was stimulated by radium emanations and uranyl nitrate. Two other minor elements were also included: iodine stimulated, selenium inhibited. Of greater significance for the future developments in the field was a contribution from the Valio Laboratory in Finland by A. I. Virtanen (1928).

*The Journal of Biological Chemistry* had two entries: one by C. B. Lipman (1911) claiming fixation by yeast and other fungi, and finally, a paper from Wisconsin describing nitrogen fixation in fermenting manure (Tottingham, 1916).

## 1.2 PROPERTIES OF THE ENZYME SYSTEM IN *AZOTOBACTER*

### *1.2.1 The method*

A paper by Meyerhof and Burk (1928) ushered in an era that saw a sharp revival of interest in the basic chemistry of biological $N_2$-fixation. Although their laboratories were often in agricultural colleges or experiment stations, the research workers attracted to the field, were trained in chemistry or biochemistry and bacteriology and published in the professional journals of these disciplines rather than station bulletins. Intact cells, either growing or 'resting', were the usual experimental material, but the goal always was verification and extension of the findings with cell-free preparations. This was accomplished with several of the auxiliary systems, e.g. the electron transport systems, including hydrogenase, but fixation of $N_2$ by such preparations remained elusive until 1960.

The contribution by Meyerhof and Burk was two-fold. The introduction of a new technique: the use of a microrespirometer for

indirect measurement of $N_2$ fixed; and the development of experimental concepts that would allow critical examination of the properties of a specific enzyme system in growing cells. The instrument not only allowed experiments to be completed in hours that had required weeks with the traditional cultural methods but also provided a much more sensitive method for test of an hypothesis. It provided an essential technical back-up for the conceptual plan of attack on problems long neglected in this field.

Burk (1934) stated the basic assumptions as:

> 'Azotase is the enzyme system or complex in the aerobic organism Azotobacter that catalyzes the change of gaseous $N_2$ from a free to a fixed state at ordinary temperatures and pressures. Like the zymase complex, it consists of one or more specific enzymes, and of auxiliary substances of low molecular weight and relative greater stability. Nitrogenase is the specific enzyme within the azotase system that combines directly with $N_2$ with characteristic affinity.'

The task of the experimenter was to define the properties of the enzyme, nitrogenase, and to identify the auxiliary substances. To succeed, several essential criteria must be met in the experiments (Burk, 1937). The two most important were: (a) the use of appropriate velocity constants in measurement of the rate of reaction; (b) comparison of these constants when the cells are grown on both free and fixed forms of nitrogen. The appropriate velocity constant could be readily determined by observing the increase in rate of respiration with time during the period of exponential growth of the organism. During the 1930's the Fixed Nitrogen Laboratory group in the Bureau of Chemistry and Soils of the USDA (Burk, Allison, Lineweaver, Horner) performed the essential experiments; their significant findings concerned with the enzyme system are summarized here, but it is emphasized that equally important contributions were made dealing with other physiological aspects of both asymbiotic and symbiotic biological nitrogen fixation.

### *1.2.2 The $pN_2$ function*

Experiments that measured the rate of $N_2$-fixation as a function of the $pN_2$ led to an estimate of 0.21 atm. for the Michaelis constant of fixation. From these experiments and others, Burk (1934) calculated the free energy of dissociation of $N_2E$, the heat of activation of $N_2E$ association and other physical constants. However, in most of the

experiments $H_2$ was used as the inert gas to replace the $N_2$. As will be discussed later, this led to a serious error and a controversy with the group at Wisconsin.

A byproduct of the research, however, has been of more significance than the precise evaluation of the $K_{N_2}$. Treatment of the extensive data gathered for its estimation resulted in an independent discovery of a method, earlier suggested by Barnett Woolf to Haldane (1957), that transforms the data so that a linear rather than a hyperbolic function results: the Lineweaver-Burk plot. This transformation, together with other methods suggested in their paper (Lineweaver and Burk, 1934) has played an important role in the development of enzyme kinetics. It is worth remembering that the extensive data gathered for estimating the $K_{N_2}$ for nitrogen fixation provided the raw material for illustration of, together with the, often overlooked, appropriate statistical treatment, the usefulness of the method (Lineweaver, Burk and Deming, 1934).

### *1.2.3 The $pO_2$ function*

The experiments described in the 1928 paper of Meyerhof and Burk dealt with the relationship between $N_2$-fixation in the azotobacter and the $pO_2$. In one experiment, the following values for $\mu$gN fixed/10 ml at the indicated $pO_2$ were obtained: 0.21 atm., 6; 0.13, 6; 0.07, 14; 0.04, 26; 0.013, 21. Although maximum fixation occurred at a $pO_2$ of 0.04 atm., the efficiency of fixation measured by (moles $N_2$ fixed)/(moles $O_2$ used) was greater at a $pO_2$ of about 0.01 atm. Burk (1930) confirmed these observations, but since the $pO_2$ functions with respect to respiration, growth and efficiency of growth were the same when the organism was grown on either free or fixed nitrogen, he concluded that 'they offer no indication of the chemical mechanism of nitrogen fixation'. Be that as it may, later investigators did establish definite possible roles for $O_2$. For example, the experiments of Parker and Scutt (1960) indicating competitive inhibition between $O_2$ and $N_2$, suggested that the two gases competed as terminal hydrogen (electron) acceptors. These questions are discussed more extensively in Chapter 5.

### *1.2.4 Role of minerals*

Using the traditional stagnant culture method with long incubation times, Bortels (1930) reported that the growth of *Azotobacter chroococcum* was increased 2-3 fold through the addition of 0.0005% sodium molybdate. The microrespirometric technique was ideally suited for detailed study of this effect; Burk and his associates

examined not only the effect of molybdenum but also a large number of other trace minerals using different strains and species of azotobacter (Burk, 1934, 1937). Their conclusions were that molybdenum (replaceable in part by vanadium) and calcium (replaceable by strontium) were specific requirements for $N_2$ fixation by the azotobacter (i.e. were auxiliary factors of azotase), but the requirement for iron was nonspecific.

However, in a 1941 review, his last contribution to the field, Burk stated:

> '. . . although all nitrogen-fixing organism require molybdenum (or vanadium), iron and calcium (or strontium), in no case—regardless of earlier indications—can it not be regarded as probable that these elements are specifically required in fixation as distinguished from general nitrogen assimilation . . . The only qualitative fixation specificity that can be regarded as definitely established at present is hydrogen inhibition . . .' (Burk and Burris, 1941).

Other investigators have not accepted such a limited definition, and the cell-free work has established that both iron and molybdenum are constituents of the nitrogen-fixing enzyme system in essentially the sense that Burk originally defined their role. The term azotase, however, has been dropped in favour of nitrogenase for designation of the complete system. The specific role of calcium, if any, remains obscure (Jakobsons, Zell and Wilson, 1962).

## 1.3 THE PROPERTIES OF THE ENZYME SYSTEM IN LEGUMINOUS PLANTS

### *1.3.1 The method*

In 1929 the departments of Agricultural Bacteriology and of Agricultural Chemistry at the University of Wisconsin received a grant from the Herman Frasch Foundation to investigate the biochemistry of symbiotic $N_2$-fixation. As greenhouse experiments involving the complicated two membered plant system obviously promised to be time-consuming as well as difficult to interpret, initial studies were directed toward attempts to obtain $N_2$-fixation by the bacteria when grown alone. When such efforts failed (Wilson, Hopkins and Fred, 1932), attention was turned to the intact plant system; a technique was developed that, it was hoped, would provide results similar to those found for the azotobacter using the microrespirometer. Briefly, clover plants were grown in a closed