NITROGEN FIXATION

Volume II:
Symbiotic Associations &
Cyanobacteria

Edited by
William E. Newton
&
William H. Orme-Johnson

Nitrogen Fixation

Volume II: Symbiotic Associations and Cyanobacteria

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University Park Press
Baltimore

UNIVERSITY PARK PRESS
International Publishers in Science, Medicine, and Education
233 East Redwood Street
Baltimore, Maryland 21202

Typeset by American Graphic Arts Corporation.

Manufactured in the United States of America by
The Maple Press Company.

Library of Congress Cataloging in Publication Data

Kettering International Symposium on Nitrogen Fixation, 3d, Madison, Wis., 1978.
Nitrogen fixation.

Includes index.
1. Micro-organisms, Nitrogen-fixing—Congresses. 2. Nitrogen—Fixation—Congresses. I. Newton, William Edward, 1938- II. Orme-Johnson, William H. III. Title.
QR89.7.K47 1978 589 79-28285
ISBN 0-8391-1560-1 (v.1)
ISBN 0-8391-1561-X (v.2)

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Preface

These volumes constitute the Proceedings of an international symposium that was held on June 12–16, 1978, in Madison, Wisconsin (USA). Three principal benefactors made this Symposium possible. The Steenbock Symposia Committee of the Department of Biochemistry, the University of Wisconsin–Madison, provided a grant as part of a regular program of annual symposia established in honor of the eminent biochemist, the late Professor Harry F. Steenbock, under the benefaction of Mrs. Evelyn Steenbock, whom we sincerely thank. The meeting was thus the Seventh Harry F. Steenbock Symposium. The Charles F. Kettering Foundation, Dayton, Ohio, and the Kettering Research Laboratory, Yellow Springs, Ohio, were established by the renowned scientist-inventor, Charles F. Kettering, and have organized several previous gatherings devoted to plant science. This meeting was the Third Kettering International Symposium on Nitrogen Fixation, the previous two being held in Pullman, Washington, in 1974 and Salamanca, Spain, in 1976. We thank Mr. Robert G. Chollar, President and Chairman of the Board of the C. F. Kettering Foundation, for his continued support of the concept of an interdisciplinary meeting in the area of nitrogen fixation. Finally, the Chemical Development Division of the Tennessee Valley Authority, under its programmatic interests in industrial nitrogen fixation and fertilizer economics, also provided support. Our thanks are extended to Mr. Charles H. Davis and his colleagues at TVA for their timely interest and generosity.

We are particularly pleased to note that three names widely associated with the application of science and technology to the betterment of mankind's condition have joined in this enterprise, which we hope has stimulated (and will continue to do so) interdisciplinary discussion on this vitally important contemporary problem.

The organization of this meeting was in the hands of a series of committees under our overall direction. We thank our colleagues on the International Program Committee, M. J. Dilworth (Murdoch University, Australia), R. W. F. Hardy (DuPont Company, USA), and J. R. Postgate (A.R.C. Unit of Nitrogen Fixation, UK), for their invaluable advice and counsel. R. H. Burris and W. J. Brill (University of Wisconsin-Madison) and H. J. Evans (Oregon State University) also contributed to this part of the endeavor, as did the many participants who wrote with programming suggestions. All are thanked.

The hard work of local arrangements and day-to-day organization fell in varying proportions to our colleagues on the Local Committee, Karen Davis, Vicki Hudson Newton, Bill Hamilton, Cynthia Touton, Nanette Orme-Johnson, Catherine Burris and Bob Burris, without whose efficiency and fidelity we would have been lost.

The interest in and importance of this area of research is demonstrated by the increasing demand for participation in these meetings. In 1974, about 200 scientists were in attendance, whereas in 1976 nearly 300 people participated. Attendance in 1978 was in excess of 400. This attendance trend is paralleled by the enormous progress made in the science in the four years since the Kettering-Pullman Symposium. These volumes speak very clearly to this point. We hope that this record of an outstanding symposium will benefit all persons interested in this area of research endeavor.

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Nitrogen Fixation

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Section I
Leguminous Associations

Nitrogen Fixation, Volume II
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Host and *Rhizobium* Contributions to the Physiology of Legume Nodules

M. J. Dilworth

This paper concentrates on the following areas of the legume nodule symbiosis: 1) the biology of leghemoglobin; 2) the control of nitrogenase production and activity in *Rhizobium*, both free-living and symbiotic, and its relation to the enzymes of ammonia assimilation; 3) the evidence concerning DNA and RNA contents and stability in nodule bacteroids, and their implications for bacteroid viability; 4) the consequences of H_2 evolution and uptake by nodule bacteroids; and 5) the evidence regarding ammonia assimilation into amino acids in the bacteroid and plant fractions of nodules.

BIOLOGY OF LEGHEMOGLOBIN

Numerous earlier reports based on direct light microscopy (Bergersen, 1966), electron microscope autoradiography (Dilworth and Kidby, 1967) and cytochemical staining of leghemoglobin through its pseudoperoxidase activity (Truchet, 1972; Bergersen and Goodchild, 1973; Gourret and Fernandez-Arias, 1974) indicated that leghemoglobin was localized in the space between the peribacteroid membrane of plant origin and the bacteroid cell wall. Two recent reports throw doubt on this localization. When extensively washed, thin sections of fixed soybean nodules were treated with ferritin-coupled antibody to leghemoglobin, ferritin was found only in cytoplasmic locations and not in the space between bacteroid and plant cytoplasm (Verma and Bal, 1976). The failures by others to find leghemoglobin in the cytoplasm were attributed to inadequate fixation with consequent migration during processing. However, electron microscope autoradiography

would have, but did not, detect migration of labeled leghemoglobin out of the tissue (Dilworth and Kidby, 1967). This reasoning does not explain the specific staining with diaminobenzidine seen by Bergersen and Goodchild (1973), Gourret and Fernandez-Arias (1974), and Truchet (1972). Furthermore, why should all leghemoglobin move from plant cytoplasm to membrane envelope? The result of Verma and Bal (1976) could be explained by the removal of surface-reactive leghemoglobin from all locations except where it was attached to plant ribosomes. Translation of globin messenger RNA undoubtedly occurs on plant cytoplasmic ribosomes (Verma, Nash, and Schulman, 1974; Verma and Bal, 1976).

In a second study (Robertson et al., 1978), membrane envelopes still containing bacteroids were isolated by sucrose density gradient centrifugation, after gentle rupture of nodules, and found to be free of leghemoglobin. To demonstrate that membranes had not ruptured during isolation and then resealed, ferritin was added to the isolation medium. Because ferritin was found inside only 1% of the membranes examined, their conclusion was that leghemoglobin must be located in the plant cytoplasm. However, the possibility of leakage through the peribacteroid membrane has not been excluded. In *Bacillus amyloliquefaciens*, simple cold shock to 16°–18°C causes complete leakage of ribonuclease inhibitor (molecular weight about 12,000) from the cells (Smeaton and Elliott, 1967), so leakage of leghemoglobin during isolation must be taken seriously. Simple techniques that do not involve fixation or tissue disruption are urgently needed to solve this localization problem.

The number of leghemoglobin (Lb) components found in any particular nodule type continues to increase. Better resolution techniques (Dilworth, 1969) applied to soybean Lb reveal that both Lb*c* and Lb*d* have two subcomponents (Appleby et al., 1975) and Lbc_2 has now been sequenced (Hurrell and Leach, 1977). Equal in length to Lb*a*, alignment required one COOH-terminal addition and two internal deletions, as well as a minimum of 14 substitutions, all conservative. The biological significance of these different Lb molecules remains to be determined. Thus far, only differences in O_2 affinity (Appleby, 1962) and in the pK for the conversion of CO-bound Lb from a neutral to an acid form (Fuchsman, unpublished data) have been reported. The changing ratio between different components during nodule development in soybean possibly suggests different physiological functions, different intracellular localization, or some combination of these (Fuchsman et al., 1976). No physiologically significant differences have been demonstrated, and when the leghemoglobin sequence variation between plants is considered (Dilworth and Appleby, 1977) for Lb with ostensibly the same function, genetic variability without physiological significance remains quite possible. Immunological studies on a variety of Lb (Hurrell et al., 1977) have

produced a "family tree" of Lb that is, so far, parallel to the normal botanical relatedness of the plants, with the possible exception of serradella.

Loss of Lb has been implicated as a possible cause of the inhibitory effect of nitrate on N_2 (C_2H_2) fixation in pea nodules (Bisseling, van den Bos, and van Kammen, 1978). Pea nodule N_2 fixation was markedly inhibited by NH_4NO_3, $(NH_4)_2SO_4$, or NH_4Cl, but not by KNO_3, indicating that the effects were due to ammonium ion rather than nitrate, which contrasts with the results of Mahon (1977), where KNO_3 and NH_4NO_3 were equally inhibitory per unit of nitrogen. Bisseling, van den Bos, and van Kammen (1978) showed that, although activity declined, the amount of nitrogenase present (measured by the amount of $^{35}SO_4$ incorporation) remained constant. Since nodule heme content declined in parallel with nitrogenase activity, lowered nitrogenase activity was explained as Lb loss. In the pea, however, darkening is sufficient to cause Lb degradation (Roponen, 1970), probably due to decreased carbohydrate supply. Since nitrate will divert carbohydrate toward active growth sinks, low carbohydrate supply to pea nodules probably lowers their energy charge, thus lowering nitrogenase activity (Ching et al., 1975). Lb degradation and decline in nitrogenase activity would then have a common trigger rather than a causal relationship. Nitrite may also affect nitrogenase activity and Lb (Rigaud and Puppo, 1977). Nitrite inhibits nitrogenase activity in both bacteroids and extracts from soybean nodules (Rigaud et al., 1973; Kennedy, Rigaud, and Trinchant, 1975) and has now been shown to cause autoxidation of ferrous-oxyLb to the ferric form unable to transport O_2. However, the attempt to implicate nitrite as an intermediate in nitrate effects awaits the demonstration of significant nitrite concentrations in nodules.

Heme biosynthesis has been investigated, particularly in relation to the role of the bacteroid. Aminolaevulinate (ALA) synthetase activity is restricted to bacteroids in soybean nodules (Nadler and Avissar, 1977) and serradella nodules (Godfrey, Coventry, and Dilworth, 1975); however, ALA dehydratase activity occurs only in bacteroids in effective soybean nodules. Whereas Godfrey, Coventry, and Dilworth (1975) suggest that plant ALA dehydratase may function in heme synthesis for Lb, Nadler and Avissar (1977) consider that the time course of ALA dehydratase production and its absence in ineffective bacteroids indicate that heme synthesis for Lb is wholly a bacteroid property. This view must remain tentative until ALA synthesis and metabolism in the plant fraction can be established qualitatively and quantitatively. During microaerophilic growth of *Rhizobium japonicum*, a tenfold derepression of ALA synthetase and ALA dehydratase occurred and proto- and coproporphyrin were excreted into the media (Avissar and Nadler, 1978).

CONTROL OF NITROGENASE PRODUCTION AND ACTIVITY

With the discovery of rhizobial strains that would derepress nitrogenase in laboratory cultures, it became possible to examine the control mechanisms for nitrogenase. Until recently, the property appeared to be restricted to a few slow-growing *Rhizobium* strains (Pagan et al., 1975) and caution needed to be exercised in generalizing from such a few strains. Now, the regulation of nitrogenase production by O_2 concentration and by various nitrogen sources, notably ammonia, glutamate, and glutamine, are the key problems.

In liquid media, a very low O_2 concentration was clearly required for nitrogenase production (Tjepkema and Evans, 1975; Evans and Keister, 1976; Keister and Evans, 1976). On solid media, however, the highest nitrogenase activities were obtained with atmospheric O_2 concentrations (Pagan et al., 1975). This apparent contradiction proved to be a result of the assay time used to measure nitrogenase response to O_2. After 16-hr incubations with varying O_2 concentrations in the gas phase, nitrogenase derepression occurred and the optimal O_2 concentration was very low (Dilworth and McComb, 1975). In glutamine-limited continuous culture, nitrogenase activity appeared when dissolved oxygen concentration fell below 3 μM (Bergersen et al., 1976). If respiratory activity was lowered by stopping the supply of an easily respirable substrate (succinate), dissolved O_2 increased sharply and nitrogenase disappeared. Exposure to O_2 resulted in irreversible inactivation of nitrogenase and restoration under low O_2 concentration took much longer than inactivation. Addition of 5 mM ammonia to N_2-fixing continuous cultures resulted in partial loss of nitrogenase activity, not complete loss as found with other N_2-fixing bacteria (Postgate, 1974).

The response of respiration to O_2 concentration in such cultures showed an interesting contrast to nodule bacteroids. Bacteroids possess two distinct oxidase systems, one tightly coupled to ATP production and one more loosely coupled (Appleby, Turner, and Macnicol, 1975; Bergersen and Turner, 1975). The latter appears to be a flavoprotein oxidase located in the cell membrane (Appleby, unpublished data) that apparently offers "respiratory protection" for nitrogenase. Chemostat cultures of 32H1 grown at 30 μM or more O_2 had no nitrogenase but had both types of respiratory system. Cultures grown at around 1 μM O_2 had nitrogenase activity but not the flavoprotein oxidase. Tubb (1976) also observed in batch cultures ammonia-mediated decrease in nitrogenase activity, an effect that could be partially prevented by glutamate. Mutants of *Rhizobium trifolii* selected for the ability to derepress nitrogenase in the laboratory also showed this effect (O'Gara and Shanmugam, 1977).

Excretion of ammonia is a very important point of similarity between