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Preface

Our knowledge of the biochemistry and biophysics of dinitrogen fixation has developed rapidly in the 15 years since the first N_2-fixing enzyme system was successfully extracted from a bacterium. This period has produced a literature that now describes the N_2 fixation reaction and the nitrogenase enzyme itself in sophisticated terms, though a detailed reaction mechanism at the chemical level has not yet emerged. It is the purpose of the present monograph to present an in-depth review, analysis, and integration of this research as is possible with a non-contributed publication and to relate this work to considerations of N_2 fixation that reach beyond the confines of the biochemist's laboratory.

The first section is directed as much toward the general science reader as toward the specialist. It covers the agricultural origins of man's interest in N_2 fixation and also pertinent areas of taxonomy, physiology, and evolution. Ecological aspects of the subject include a comprehensive evaluation of the nitrogen cycle leading to a substantially greater estimate of the rate of global N_2 fixation than previous ones. The treatment is of a survey fashion, in part to provide a general over-view of N_2 fixation and in part to provide context for the biochemistry and biophysics that follow in the second section. This section begins with a brief chronological review of the biochemical and biophysical research, then treats individual areas of interest topically. These center mainly on the nitrogenase enzyme and the reactions it catalyzes, but also deals with enzyme systems and metabolic activity associated with nitrogenase activity.

Biochemical and biophysical research in N_2 fixation has been characterized by productive interactions with a variety of other disciplines, ranging from agriculture and ecology to transition metal chemistry. The debt owed by the laboratory scientist in this field to his more agriculturally oriented associates has been paid at least in part by the development of the acetylene reduction assay that has made possible extensive evaluations of N_2 fixation in the field and enabled a dramatic expansion in laboratories exploring all phases of N_2 fixation research. The unique character of the nitrogenase enzyme and related proteins such as ferredoxin has attracted the interest of enzymologists as well as specialists in such fields as Mössbauer and nuclear magnetic resonance spectroscopy. As intriguing as the enzyme are the reactions it catalyzes. These include unique triple bond reductions, H_2 formation, and ATP hydrolysis, and when these electron transfer and hydrolitic reactions are fully understood, they will certainly provide an interesting chapter in the study of enzyme mechanisms and kinetics. Increasingly, the biological scientist and his abiological counterpart track each other in their respective N_2 fixation studies, and non-enzymic catalytic systems, some closely mimicking the enzyme reaction, have already been developed. Both the biol-

ogical and abiological work have obvious potential to upgrade and expand agricultural production. In today's world of pending food crises, few research areas are more relevant to human needs. We hope that the current volume will serve to stimulate further work at all levels of N_2 fixation research.

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Part I. The Diazotrophs*

* The term diazotroph is introduced here and defined as any N_2-fixing organism or symbiotic association.

Chapter 1

Recognition

I. Historical Roots*

A. The Agricultural Imperative

Through most of the world legumes have played a major role in the history of man's efforts to produce food. Nutritional merit alone would probably have earned these plants priority consideration, but what must have assured them the greatest respect among farmers was their unique ability to enrich the soil. The prevalence of various pulse seeds in archeological diggings attests to the early origins of legume culture, though one may only guess when man began to realize that these plants could maintain the fertility of his fields. However, it is not likely that a cause-effect relationship of such economic significance would remain unnoticed for long in the early empirical approach to agriculture. The soil enriching quality of legumes was clearly appreciated by the early Romans, who developed the concept of crop rotation in which legumes figured prominently. This practice persists today as a major instrument in soil management throughout the world. In developed countries the advent of cheap nitrogen fertilizer has currently lessened the importance of legumes, but increasing concern over possible contribution of fertilizer nitrogen to water pollution has stimulated a renewed interest in extending biological N_2 fixation.

The fact that legumes "did better" than other plants was not lost on the 19th century experimentalists who attempted to construct a scientific rationale for plant nutrition. For example, Sir Humphrey DAVY observed in his book Agricultural Chemistry in 1813 that "peas and beans in all instances seem well adapted to prepare the ground for wheat.....it seems that the azote which forms a constituent part of (their) matter is derived from the atmosphere." This knowledge exerted a continuing pressure which motivated considerable theorizing and experimentation, and it was the need to explain this unique property of the legumes that led finally to the recognition of dinitrogen fixation as a biological process.

B. Early Experimentation

In the early history of dinitrogen fixation research the work of HELLRIEGEL and WILFARTH (303) in the 1880's was unquestionably the decisive single contribution. By means of a relatively straightforward series of nutritional experiments on some common agricultural plants,

* Informative accounts of the historical aspects of N_2 fixation the early research developments are found in books by FRED, BALDWIN and McCOY (238) and McKEE (460) and in articles by WILSON (714, 715, 716, 718) and AULIE (25). The factual aspects of the brief history reported here have been distilled largely from these sources.

as in Table 1-1, the two German experimentalists showed unequivocally that:

1. legumes were different from other plants; specifically, they could utilize atmospheric N_2,
2. this utilization, or fixation, depended on the active participation of certain microorganisms in a legume-microorganism symbiosis, and
3. the root nodules were the active centers of N_2 fixation.

Table 1-1. Typical data of HELLRIEGEL and WILFARTH (303), showing superiority of legumes at low soil nitrogen levels

Pot No.	Initial Nitrogen in calcium nitrate	Oats Average weight of grain and straw	Peas Average weight of seed and vines
	gm.	gm.	gm.
1	None	0.390	4.380
2	0.056	5.876	4.128
3	0.112	10.961	9.132
4	0.168	15.997	-----
5	0.224	21.357	9.725
6	0.336	30.175	11.352

Their experiments accomplished several objectives which had eluded all predecessors. Most significantly, they proved to a skeptical scientific community that biological N_2 fixation did in fact exist. Further, they identified the requirements of N_2 fixation in legumes and clarified why legumes "did better" than other plants under adverse nutritional conditions. Finally, they explained why earlier experimentation by others had failed. Thus the past was explained and the future course was pointed out. Clearly a fresh page had been turned.

In the 50 to 60 years leading up to this work the question of N_2 utilization by plants had passed a tumultuous gestation. It engaged the minds of some of the century's most prominent scientists with the result that a confusing, quarrelsome and equivocal literature on the subject had accumulated. By the time HELLRIEGEL and WILFARTH reported their results, the concept of N_2 fixation lay in official disrepute for almost thirty years. However, their data were so convincing that the legitimacy of N_2 fixation was almost immediately accepted and the concept never again placed in serious doubt.

Why was the definitive experimentation so long in coming? Certainly credit must be given to the authors, WILFARTH and particularly HELLRIEGEL. Yet these men were not of the scientific caliber of LIEBIG or numerous others who had considered but failed to solve the question. Part of the answer is found in the general status of science in the 19th century and in a consideration of N_2 fixation within this context. The success of the German workers rested on crucial inputs from such varied disciplines as agronomy, plant physiology, microbiology and chemistry. Their experimentation was truly a multidisciplinary accomplishment which could hardly have occurred before these different fields had reached an appropriate maturity.

The possibility that plants might be able to utilize N_2 began to receive consideration soon after RUTHERFORD discovered in 1772 that N_2 was a major atmospheric component. By the end of the eighteenth cen-

tury the major components of the atmosphere had been correctly identified. The remarkable involvement of O_2 and CO_2 in life processes was rapidly established by INGEN-HOUSZ, SENEBIER, SAUSSURE and PRIESTLEY, whose findings led to the concept of an aerial nutrition for plants. Dinitrogen appears to have been loosely included in this concept, its participation assumed by PRIESTLEY, INGEN-HOUSZ and DAVY. Though this assumption lacked experimental support, it must have appeared quite logical; the ubiquity of nitrogen in living matter was becoming well established, and if the carbon needs of life could be met with the 0.03% CO_2 present in the atmosphere, the massive concentration of N_2 in the atmosphere could reasonably be expected to satisfy nitrogen needs.

The first recorded experimental work appears to be tests conducted around 1806 by SAUSSURE. This work was done with potted plants and formed part of SAUSSURE's pioneering efforts to extend chemical techniques and quantitation into biology; the tests indicated that the soil, not the atmosphere, was the source of plant nitrogen. Others who considered the question of aerial dinitrogen assimilation included GAY-LUSSAC and HERMSTÄDT, but none of these early experimentalists developed a detailed or extended investigation of the question.

A more enduring commitment to this problem was initiated by Jean Baptiste BOUSSINGAULT in France. BOUSSINGAULT was among the first to pursue the quantitative chemistry of field crops, and the question of nitrogen nutrition occupied a major place in his research. Through his own early experimentation and from more preliminary analyses by VAUQUELIN and FOURCROY, BOUSSINGAULT was aware that the superiority of legumes over cereals was due to the high level of nitrogen in the legumes. Based on analyses of crop rotations which included clover and which extended for 4 to 5 years on hectare size plots, he concluded that the atmosphere supplied nitrogen to the plants, and he announced this conclusion to the French Academy in 1838 after obtaining supporting data from pot experiments with clover and wheat. His early experimentation showed that nitrogen was the key component of added fertilizers and that legumes enriched the soil by introducing nitrogen from the air; the experiments did not define the nature of the nitrogen as specifically N_2, airborne organic particulates or gaseous ammonia.

The identity of the functional atmospheric species appeared to be resolved in favor of ammonia as a result of the strong support for this source provided by LIEBIG in his influential "Chemistry and Its Application to Agriculture and Physiology", published in 1840. Ammonia had been recognized as a possible source by almost all who had considered the question of plant nitrogen nutrition, and though LIEBIG offered no new data, his persuasive arguemnt led now to general acceptance of the ammonia theory. Less well received, however, was LIEBIG's mineral theory of plant nutrition in which he dismissed nitrogen as a trivial component of fertilizers and acknowledged minerals to be the only effective components. Though this concept denied BOUSSINGAULTS's work and conclusions, BOUSSINGAULT did not take issue with LIEBIG, and it was rather LAWES and GILBERT at the Rothamsted Experiment Station who challenged this theory. Extensive field experiments involving crop rotation had convinced the Rothamsted workers that BOUSSINGAULT's contributions were valid and the mineral theory incorrect; these views became generally accepted in the 1850's following more than 10 years of heated debate in the literature.

Meanwhile BOUSSINGAULT had begun a second series of experiments on the source of nitrogen. These appear to have been prompted by the work of Georges VILLE, who in 1850 began to report considerable evidence in support of N_2 fixation by potted plants. Both workers reportedly grew their plants in closed systems from which ammonia was excluded, and most significantly both used calcined soil. BOUSSINGAULT observed consistent negative results and concluded that plants could not use N_2. In contrast VILLE continued to report substantial N_2 fixation; however, his results could not be verified by LAWES, GILBERT and PUGH at Rothamsted in a series of experiments in 1857, 1858, and 1861, even using apparatus sent to them by VILLE. In retrospect it is difficult to rationalize the positive results of VILLE with his use of calcined soil. This practice of exhaustively roasting the soil before use, which clearly doomed their best experimental efforts to failure, was apparently never considered to be an important consideration by any of the early experimenters.

In the course of his work during this period BOUSSINGAULT undertook extensive analyses to determine whether the atmosphere contained sufficient ammonia to sustain plant requirements. He concluded that it did not, and his results were verified in London in 1856 by WAY, who extended the analysis to include atmospheric nitrate and showed that even the combined sources were inadequate. Attempts to implicate atmospheric sources of nitrogen in plant nutrition effectively ceased following publication of the Rothamsted report of 1861, and were not resumed for almost 25 years. With the atmospheric sources apparently discredited and with the mineral theory now in general disrepute, attention was logically turned to the soil as the most likely source of nitrogen during this period.

Though little direct experimentation on N_2 fixation was undertaken during this time, the information which would contribute to a full understanding of N_2 fixation was in fact being accumulated through other apparently unrelated findings. LACHMANN, in investigations of the legume root nodules which were so consistently overlooked by the chemists, established the presence therein of "vibro-like" bodies in 1858, and WORONIN in 1866-67 spoke of these as "bacterial-like". In 1862 JODIN reported N_2 fixation by microorganisms based on growth in nitrogen-free media and on an observed uptake of N_2, but the work was not pursued after an unfavorable reception by a French Academy Committee of Examination; BOUSSINGAULT's studies on soil nitrogen during the period of 1860-1876 led to the clarification of the major chemical features of nitrification (p. 40), and showed LIEBIG's early theory of this phenomenon to be essentially correct. In 1877 SCHLOESING and MÜNTZ established the role of bacteria in nitrification, and initiated the novel concept of participation by micro-organisms in soil chemistry. That they might also play a role in reactions with N_2 was implicit in JODIN's early results (above), and was further indicated by BERTHELOT who observed an increase in soil nitrogen that could be prevented if the soil were first heated at 100°C.

Persisting throughout this period, as before, was the certain knowledge among agriculturists that under field conditions legumes "did better" and possessed definite soil-enriching properties. LAWES and GILBERT in fact pointedly observed that legumes in the field in contrast to the legumes grown under their rigidly controlled experimental conditions contained unaccounted for nitrogen. Others, including SCHULTZ-LIPITZ in Germany and ATWATER in the U.S.A., made similar observations and pointed out the inadequacy of current dogma to account for their findings. It was in this atmosphere of extreme un-

certainty about the existence of N_2 fixation that Hermann HELLRIEGEL, independently at first and then with WILLFARTH, conducted the experiments which yielded the long sought solution. Unlike all predecessors they examined the significance of root nodules in the nitrogen nutrition of plants, and this of course proved to be the key piece of information leading to an explanation of N_2 fixation by legumes.

II. Detection of Diazotrophs and Nitrogenase

Other scientists were quick to build on the foundation laid by HELLRIEGEL and WILLFARTH, and a period of important productivity and discovery followed their work. In 1888 BEIJERINCK isolated the nodule bacterium and named it *Rhizobium radicicola*, subsequently changed to *R. leguminosarum*. NOBBE suggested N_2 fixation by a nodulated non-legume, *Elaeagnus angustifolia*, in 1892, and N_2 fixation by free-living microorganisms was established by WINOGRADSKY's demonstration in 1893 that *Clostridium pasteurianum* could fix N_2. The momentum that appeared to be building up in N_2 fixation research, however, was not sustained. An increasing flow of reports describing N_2 fixation by various free-living microorganisms began to appear in the literature, but the lack of a sufficiently sensitive assay, and often the lack of sufficiently pure cultures, made N_2 fixation and the N_2-fixing agents difficult to establish with certainty. It became lamentably clear, as WILSON pointed out, that "Nature failed to provide a bright-hued signal comparable to chlorophyll to mark the nitrogen fixers" (713). More significantly, existing technology was unable to overcome this oversight of Nature, and interest in N_2 fixation research declined as investigators turned to fields that could be more readily exploited.

Ostensibly, the qualification test for a candidate diazotroph should be simple and direct: either an organism would grow in the presence of N_2 and absence of fixed nitrogen, or it would not. In practice, however, such a direct approach seldom met with success. The difficulties encountered proved to be both considerable and at least partly unexpected. The extraordinary ability of numerous microorganisms to scavenge fixed nitrogen was not well appreciated, and the resultant growth caused considerable problems; these difficulties were frequently compounded by the impure nature of available reagents or the presence of atmospheric ammonia in minisculé but decisive amounts. Furthermore, it is now apparent that N_2 fixation is not characterized by a high degree of taxonomic continuity, but rather by considerable variation in specificity with respect to family, genus and even species, and these inconsistencies precluded an effective phylogenetic approach to the identity of the diazotrophs.

The general ignorance of the conditions which best supported - or, in fact, were absolutely required for - N_2 fixation imposed additional obstacles; for example, the repressive effect of fixed nitrogen on nitrogenase was not understood, nor was the need for anaerobic conditions by facultative diazotrophs known. Even today the inability of a particular isolate to fix N_2 in the laboratory may simply reflect the investigator's inability to cater to what may be very exacting requirements, as suggested in recent reports on the importance of reduced pO_2 (p. 102) for certain aerobic diazotrophs and the apparent beneficial effect of certain non-N_2-fixing organisms on the fixation rates of known diazotrophs. A pertinent case is the recent demonstration of N_2 fixation in *Aphinosomen flos-aquae* (661): evidence for fixation was obtained using C_2H_2 reduction and ^{15}N analyses,

whereas earlier ^{15}N assays indicated no fixation (711). The failure to provide the most favorable conditions for N_2 fixation for a given agent is probably also at the heart of the apparently great variability in the facility with which known diazotrohs actually fix N_2, though much intrinsic variation also no doubt exists. Whatever the cause, certain organisms, e.g., *Azotobacter* and *Clostridium*, are well known to be high activity fixers, while numerous others are not. An interesting case is that of the bacterium *Derxia gummosa*, reported variously as fixing N_2 with "unusually high efficiency" and subsequently as being a "typical awkward N_2 fixer" (308, 562).

An additional difficulty associated with early investigations was the lack of a suitable analytical method for much of this work. In fact, little real progress was made until analytical methodology advanced. The Kjeldahl method for determination of nitrogen content was introduced in 1883, just prior to the experimentation which established that N_2 fixation did in fact exist. This method was better than the Dumas procedure for most applications, and it formed the basis for determination of N_2 fixation for the next fifty years. While unquestionably a good quantitative method, the Kjeldahl nitrogen determination method had serious limitations for detection of N_2 fixation. The amount of nitrogen fixed, as determined by the Kjeldahl method, is measured as the difference between total nitrogen found in the test material before and after growth on N_2; since this frequently meant accepting a very small difference between two large values as evidence for N_2 fixation, small variations in sampling or techniques easily led to erroneous conclusions. It is not surprising then that as late as 1948 only the *Rhizobium*-legume associations (obligatory symbioses), *Clostridium* and *Azotobacter* (free-living bacteria) and *Nostoc* (a blue-green alga) were critically accepted as agents of N_2 fixation, though many other candidates were promoted with variable merit and enthusiasm.

As research in N_2 fixation and analytical techniques advanced, a variety of assay methods for N_2 fixation were developed. These are listed in Table 1-2, which also includes the applicability and principle of each method. The most commonly used are discussed below.

Table 1-2. N_2 fixation assays

Assay method and principle	N_2-fixing system		
	Nitrogenase *in vitro*	Organisms in culture	Field and natural systems
Growth and morphology			
Increase of biomass or optical density in N-free medium		++	++
Heterocyst detection (algae) by observation		++	++
Nitrogen-based methods			
Increase in N content after growth on N_2 -Kjeldahl or Dumas		++	++
^{15}N or ^{13}N enrichment in NH_4^+, cell components after exposure to $^{15}N_2$ or $^{13}N_2$			
-mass spectrometry (^{15}N)	++	++	++
-optical mass emission (^{15}N)		+	+
-radioactive counting (^{13}N)	+	+	