La Química en el siglo XX y lo que nos depara el siglo XXI

A Brief History of Nitrogen Fixation

*G.J. Leigh**

Nitrogen fixation has been a subject of great interest to chemists for the best part of a century. As a chemical problem it is possibly unique because the research has really been led by biologists for most of this time. Currently it is being studied by a wide range of scientists of many different stripes, and the interplay between them lends fascination to the topic. Even more, nitrogen fixation has been used by humans for at least two thousand years even though the primitive agriculturalists could not have been aware of what was going on. This essay is rather Eurocentric, in part because of my own limitations, but also because I do not know whether agriculture, sophisticated as it was in some respects, such as with the use of the floating chinampas by the Aztecs, ever generally passed beyond the stage of slash and burn as practiced by the Maya and in much of pre-Columbian America. I would appreciate receiving more information on this subject.

The technique of using soil to grow crops for a period, and then allowing it to "rest" while it recovered its fertility was employed in Europe by the Romans though I do not know when the practice started, or where it originated. It was not necessary in Egypt or Mesopotamia, where annual river floods effectively replenished the soil. Two- and three-crop rotations, one stage of the rotation involving leaving the fields fallow for a season, was apparently used in England from at least the eleventh century and considerably later(1). What happened to the soil in the fallow years was not understood, and indeed, could not have been understood at that time. First the nature of the chemical elements had to be established.

In about 1620 the Dutch scientist Van Helmont (2) undertook a marvellous experiment. Like those contemporary scientific giants, Isaac Newton and Robert Boyle, Van Helmont was also an alchemist, interested in transmuting elements, and especially into gold. He grew a willow tree, initially weighing five pounds in a pot containing two hundred pounds of earth. After 15 years he recovered the tree, now weighing one hundred and sixty nine pounds from the soil. The soil had lost two ounces, although the tree had gained one hundred and sixty four pounds in weight. Since he had added only water to the pot in which the tree was growing, he reasoned that this was proof that the element water could be changed into plant material. This was true enough, but only part of the story. Not only were his measurements too crude to assess the significance off very small changes in weight, but he did not have the scientific background to interpret his observations correctly.

During the seventeenth century and later, very complicated rotations were introduced into English agriculture, some extending over periods as long as ten years. The use of turnips as animal feed, imported from Flanders (3), helped to consolidate an agricultural and social revolution. First it produced a reliable supply of animal feed that could be used throughout the winter, so that it was no longer necessary to slaughter most of the farm animals at the end of the summer. Second, the introduction of the animals into the turnip fields for them to feed meant that the fields were fertilised efficiently. Indeed, the value of manure as a fertiliser was established very early times. An account of 1694 describes (4) the use of turnips in this way, and even refers to the involvement of a material called nitre, nowadays potassium nitrate, though it is not clear what the name implied at that time. The involvement of nitrogen in all this could only have been realised once the nature of elementary nitrogen had been established, and that was still to come.

By about 1800 elementary nitrogen was realised to be a constituent of air. It was clearly an unreactive species, so much so that Davy for one doubted whether it could be made to react. Even Liebig did not believe that atmospheric nitrogen could be converted to ammonia. One of Leibig's great achievements was to lay down the basis of rational application of manures and fertilisers in agriculture (5), but he could not accept that plants or animals might be able to activate dinitrogen. He even carried out careful experiments at Giessen to show not only that ordinary rain water contained traces of ammonia, but that there was enough ammonia circulating through the biosphere via plant and animal decay,

* School of Chemistry, Physics and Environmental Science, University of Sussex, Brighton BN1 9QJ, UK.

release of ammonia, precipitation in rain and uptake by growing organisms, to account for all the fixed nitrogen in all the plants and animals that lived. The ammonia cycle was shown to be closed.

In 1798, an English clergyman, Robert Malthus published his Essay on the Principle of Population, in which he claimed to show that populations always grow to outstrip the resources that support them. For human beings that indicated inevitable famine, and indeed this is precisely what some scholars believe caused the collapse of the Maya civilisation. In Europe there was a great desire to find ways of preventing such a disaster. A compendium of agricultural lore, the Library of Agricultural and Horticultural Knowledge, published in 1834 (6), contains a footnote explaining that agricultural productivity had been so increased in recent years that there was no doubt that rational agriculture would be able to meet the demands of rapidly increasing population. The spectre of Malthus was dismissed.

However, then as now, not everyone was convinced that Malthus was completely in the wrong, and the search for new sources of fixed nitrogen went on. One result was the rapid exploitation of the deposits of guano and nitrate in Chile and Peru. Although European agriculture most certainly benefited from this exploitation, there seems to have been little long-term profit to either of these two Latin American countries. In fact, there was a war between them for the control of the assets that were literally vanishing. Peru lost a considerable amount of territory as a consequence of this dispute (7).

The opinion of Liebig that atmospheric nitrogen is not convertible by plants to ammonia was not accepted everywhere. Agriculturalists in at least Britain and France were trying to prove definitively that nitrogen could be fixed by plants. The names of Boussingault (8) and of Gilbert and Lawes (8) are associated with some of the most significant work. The proof that biological nitrogen fixation is indeed a reality was finally established by about 1886 by Hellriegel and Wilfarth (8). With the unification of Germany under Prussia in 1870 and the realisation that the new nation could only challenge Britain and France as an imperial power by using every asset available, and especially the new sciences, it is perhaps not surprising that the real advances in nitrogen fixation research at that time came from Germany.

Hellriegel and Wilfarth (9) really adapted the experiment of Van Helmont, but they operated on a much larger scale and using the appropriate intellectual and technical equipment. They also showed that the fixation of nitrogen had something to do with the nodules that they observed to grow on the roots of nitrogen-fixing plants. They were even able to show that these nodules were a result of the infection of the plant root hairs by a micoorganism and they were eventually able to characterise the organism responsible for the fixation (8, 9). It is now believed that no plants fix nitrogen unaided, though some, principally legumes, can do so by acting as symbiotic partners with microorganisms called *Rhizobia* that are the actual nitrogen-fixing agents (10).

The realisation that legumes can mediate the fixation of nitrogen finally explained what was happening during the time that fields were allowed to remain fallow during crop rotations. It did not relieve all the worries about shortage of nitrogen for agriculture, and the problem of feeding Europe's growing population. In 1898 Sir William Crookes made his famous address to the British Association in which he again raised the Malthusian spectre and called upon chemists to meet the challenge of fixing nitrogen industrially. It must have been quite clear by then that the chemistry being used by microorganisms to fix nitrogen was very different from anything known to the chemists. In truth, it may well still be, but in 1898 the ability to fix nitrogen artificially and industrially was already close to being achieved. The solution came again for Germany.

Carl Bosch started work at BASF on the problem of nitrogen fixation in 1900 and Fritz Haber, then teaching at Karlsruhe, became involved in 1904 (11). It was already believed in 1900 that ammonia could be synthesised from its constituent elements at high temperatures and in the presence of iron filings, and Ostwald had apparently also believed that the reaction was possible (11). The converse, the breakdown of ammonia into dinitrogen and dihydrogen, had also been established by 1884 (12). Several industrial processes for the fixation of atmospheric nitrogen were under development around that time, including the cyanamide process (patented in 1900) and the Norwegian Arc process. Although these were used industrially to produce ammonia or nitric acid, neither survived past the 1940's. Haber's work involved the investigation of a large number of catalysts and a wide range of reaction conditions. Indeed, not only were the Haber Group exploring new chemistry, they were also forced to develop a new kind of technology, chemistry at very high pressures. With Bosch in the lead, BASF took the development under

its wing and advanced the process to an industrial scale, which involved not only the establishment of a viable method of combining dinitrogen and dihydrogen at high temperatures and pressures on a large scale, but also of producing dihydrogen reliably from coal and steam. BASF took out a patent for the process in 1909 (13). Nowadays, of course, the preferred source of dihydrogen is the steam reformation of methane (14).

The Haber process appeared just in time to help satisfy Germany's need for fertiliser and explosives during the First World War. By the end of this war, BASF had plants capable of producing more than 100,000 tonnes of ammonia per year, and Haber received the Nobel prize in 1918. Currently a large ammonia plant can produce about 2,000 tonnes of ammonia per day. The process is highly efficient. Ammonia is plentiful and cheap, though the industrial installation to produce it is very expensive. The availability of ammonia fertilisers to poor subsistence farmers is not a scientific problem, but political and financial.

The current industrial process is, in its essentials, still that which Haber developed. The story of how the British obtained the details of this German technology is diverting (11), and the involvement of Haber in the development of gas warfare provides an ironic counterpoint to his life (15). Haber was responsible both for a chemical invention that might reasonably be claimed to have benefited mankind more than any other, and also for the inception of one of the most horrifying kinds of chemical warfare.

What was now clear from the Haber process and from the other industrial processes in use in the period after the First World War was that the chemistry of nitrogen-fixing bacteria must be very different. Again biology led the way. The German microbiologist Bortels showed (16) that either molybdenum or vanadium were requirements for biological nitrogen fixation in *Azotobacter vinelandii*. The vanadium requirement was overlooked for more than fifty years. Thereafter, research on nitrogen fixation tended to be on the scale of whole organisms. The real breakthrough was in 1960, with the isolation of the first cell-free extracts of nitrogenase, the enzyme responsible for the conversion of dinitrogen to ammonia, from *Clostridium pasteurianum* (17). Once this had happened the field developed rapidly. The optimal stoichiometry for the biological reaction was established. In the equation below, ADP and ATP carry their usual meanings, and P_i represents inorganic phosphate. The reaction clearly requires the hydrolysis of large amounts of atp, and consequently a large input of energy. Consquently, organisms do not fix nitrogen unless they must do so.

$$N_2 + 8\,H^+ + 8\,\text{electrons} + 16\,\text{ATP} \rightarrow 2\,NH_3 + H_2 + 16P_i + 16\,\text{ADP}$$

The nitrogenase enzyme itself was shown to be a complex of two air-sensitive metallo-proteins, one of molecular weight *ca.* 60,000 and containing an Fe_4S_4 cluster, and the other of molecular weight of about 222,000 and containing the active site (supposedly a molybdenum atom in an $MoFe_7S_9$ cluster) as well as a slightly unusual Fe_8S_8 cluster (18). The smaller protein acts as a specific electron-transfer agent to the larger, and a mechanistic scheme was developed to explain the observed reaction rates (19). What the study of the enzymes has never fully explained is why so much dihydrogen is always evolved, often wastefully, and why so much energy is required.

Just after the production of the first cell-free extracts, the chemistry of nitrogen fixation suddenly came to life. In 1964, Volpin and Shur described (20) systems of transition metal compounds plus a strong reducing agent that react with dinitrogen, presumably to form uncharacterised metal nitrides. It was only in 1995 that Laplaza and Cummins reported (21) a simple complex, $[Mo\{N(C_6H_3Me_2)^tBu\}_3]$ able to do this and produce a mononuclear nitrido-complex, $[MoN\{N(C_6H_3Me_2)^tBu\}_3]$. The Volpin nitrides yielded ammonia on treatment with protic acids, but the systems are not cyclable, so this cannot provide a catalytic route to ammonia.

The first dinitrogen complex $[Ru(NH_3)_5(N_2)]^{2+}$ was described in 1965 (22), and was discovered by Allen and Senoff while trying to synthesise $[Ru(NH_3)_6]^{2+}$ by a published (23) method. It is in fact probable that this dinitrogen complex was first prepared unwttingly, along with $[Ru(NH_3)_6]^{2+}$, perhaps ten years earlier. Speculation about the possible existence of dinitrogen complexes had been evident ever since the chemistry of carbon monoxide complexes had flowered in the 1930's (24), but the spectroscopic methods required to detect dinitrogen-binding were not widely available until the 1960's. There are now hundreds of dinitrogen complexes in the literature (25), several of them with dinitrogen binding simultaneously to more than one metal ion. There are also protic systems that can catalyse the conversion of dinitrogen to ammonia (26) though

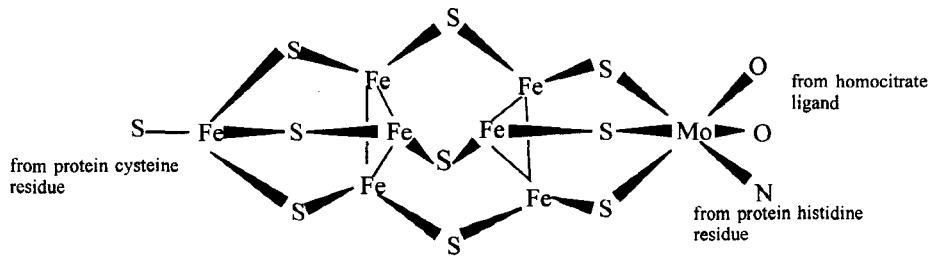

Figure 1. The structure of the principal cluster in the larger protein of the molybdenum-iron nitrogenases.

the reaction mechanisms have not been unequivocally established.

In chemical terms, the most fruitful dinitrogen complexes have been those of molybdenum and tungsten, such as $[Mo(N_2)_2(Ph_2PCH_2CH_2PPh_2)_2]$ and $[W(N_2)_2(Ph_2MeP)_4]$ (27). These react with protons to produce ammonia in favourable cases (28), and they also react with organic radicals (29). Such systems can even be made to cycle using electrochemical reduction of a suitable tungsten complex (30). Reactions such as that shown below

$$[W(N_2)_2(Ph_2MeP)_4] + 6\ HCl \rightarrow 2\ NH_3 + N_2 + W^{VI}$$

led to the proposal that in nitrogenase itself the dinitrogen attaches itself to molybdenum and then receives electrons form the metal as it picks up protons from solution.

$$Mo_{(reduced,\ in\ protein)} + N_2Mo(N_2) + H^+_{(from\ solution)} \rightarrow Mo_{(oxidised,\ in\ protein)} + NH_3$$

The discovery of the vanadium nitrogenase (31) so clearly implied by the work of Bortels, did not really change this picture other than allowing us to replace molybdenum in this reaction sequence by vanadium. The discovery of a third nitrogenase, based upon iron alone, does imply that there may yet be secrets to uncover (31). Nevertheless, until about eight years ago, many chemists, including myself, were reasonably convinced that we knew how nitrogenase fixes nitrogen. We are much less sure now.

The biological fixation of nitrogen is a complex process. Molecular biologists have been able for some time to transfer genes from one organism to another and have been able to express that gene in the foreign environment. Similarly, they have been able to transfer the ability to fix nitrogen from one bacterium to another (32), but there are upwards of twenty genes involved in the nitrogen fixation process, and to get these to function in an environment as alien as a plant cell, for example, in order to develop a nitrogen-fixing wheat or rice, would be an achievement indeed (32). As it is, the detailed information we now have is not easily rationalised by chemists. For example, the mutation of amino acid residues apparently far removed from the apparent active site can substantially modify the reactivity of the enzyme (33). However, the most puzzling problem arose with the determination of the crystal structures of the molybdenum nitrogenase itself, something that was expected to make everything clear (34). We now know how the two nitrogenase proteins interact, we know something about how the structures change upon reduction, and we know quite a lot about how the ATP is hydrolysed. But how the dinitrogen is reduced is as mysterious as ever.

Figure 1 represents the structure of the molybdenum iron cluster presumed to contain the nitrogenase active site. If it is the molybdenum, then we have to explain how it is that dinitrogen can bind to this particular metal ion, even accepting that the structure represented corresponds to an oxidation level of the protein that does not interact with dinitrogen. The metal is presumably in oxidation state IV, but there must be reduction and a considerable reorganisation before it can bind dinitrogen. Reduction does seem to diminish some of the metal-metal separations within the cluster, but apparently it does not affect the structure greatly. No spectroscopic technique has yet detected a state of the enzyme in which there is clear evidence of dinitrogen-binding, though the binding of carbon monoxide has been detected by IR and EPR methods. The carbon monoxide appears to bind to the iron (35).

The vanadium nitrogenases are presumably of very similar structure. If vanadium does really bind dinitrogen, coordination chemistry seems to imply that it must be in the oxidation states II or III (36), since these are the highest oxidation states of vanadium that bind dinitrogen These are isoelectronic with plausible comparable molybdenum oxidation states, III and IV. However, the iron-only nitrogenase presents a problem. Even assuming that the iron-

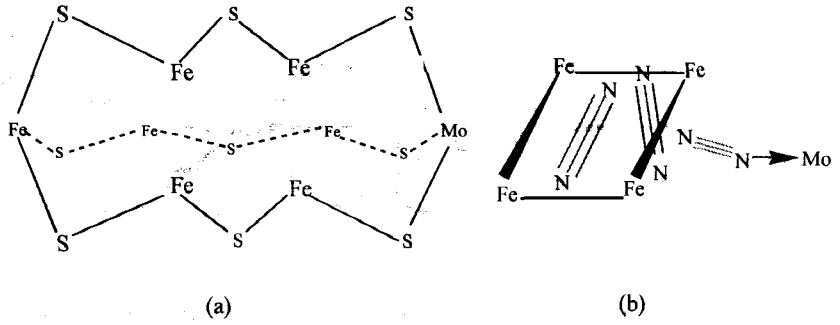

Figure 2. Representation (a) of the iron-molybdenum-sulfur core of the larger nitrogenase proteins and (b) three suggested cluster-dinitrogen interactions.

only nitrogenase is structurally similar to these molybdenum and vanadium species, it seems unlikely that the active iron ion would be isoelectronic with either. This raises the as yet unanswered question of whether these metal ions really do constitute the site at which dinitrogen is bound and reduced.

The molybdenum-iron cluster is situated in a void in the protein structure that is filled with several hundred organised water molecules. The cluster itself is of a structure unique in both biology and chemistry. There are no comparable model compounds, and it has yet to be synthesised in the laboratory. It is bound into the protein only at either end (Figure 1). It is not at all clear how such a species will interact with substrates, and it is probably unwise to assume that there is a single site at which all substrates interact. There may be more than one reactive site. What makes the problem much more difficult is that there is, as yet, no example of a cluster of the kind that we know exists in nitrogenases, a metal-sulfur cluster, able to bind dinitrogen, let alone reduce it. This has let the theoreticians have a field day speculating on how biological nitrogen fixation actually occurs.

Figure 2(a) shows a more stylised representation of the structure, so that the essentially planar arrangement of four iron atoms on the face of the cluster can be recognised. It has been suggested that the dinitrogen may bind entirely within the cluster and essentially to the molybdenum as shown in Figure 2(b), dinitrogen molecule at right, (37). Other proposals illustrated in Figure 2(b) are that the dinitrogen binds across the face with one nitrogen atom inside the cluster and one outside (in the middle) (38), and that the dinitrogen binds in the plane of the four iron atoms (at left) (39). The last suggestion seems to the author to be the most appealing, especially as the author also presents a very plausible reaction mechanism for the protonation of the dinitrogen. However, there is little empirical evidence to support these specific proposals, or any of several others that are in the literature. Consideration of how a dinitrogen molecule might migrate inside a cluster, and how the ammonia might exit are generally ignored. The only detailed, empirically based description of the protonation of coordinated dinitrogen is still that related to the molybdenum and tungsten dinitrogen complexes mentioned above, and it is much too soon to dismiss molybdenum as the active site in the molybdenum-iron nitrogenases.

So the chemists have a problem. And now the biologists have one, too. A completely new kind of nitrogenase has recently been described (40). The organism containing it is thermophilic, and this nitrogenase, though still consisting of two principal proteins, seems to be structurally distinct from the molybdenum-iron nitrogenases described hitherto. The nitrogenase actually uses dioxygen as an electron carrier, it oxidises CO to CO_2, and it uses much less ATP to produce a single electron than any other nitrogenase type so far described. It reduces dinitrogen, but it cannot reduce ethyne, hitherto regarded as a reaction typical of all nitrogenases, and a property assumed to be characteristic of all nitrogenases. This prompts the thought that we may have been so concentrated on air-sensitive molybdenum-iron systems and their occurrence and properties that we have overlooked other forms of biological nitrogen fixation. The process may be much more widespread than currently believed.

At the end of a century that has seen nitrogen fixation move from a wildly improbable reaction to a commonplace, that has seen an immense effort expended by a host of researchers of many different disciplines, biological nitrogen fixation is still a mystery. That Nature has been able to develop a method of fixing nitrogen so complex though superficially so simple is a matter for wonder, and is also very chastening. It may still be another generation before the puzzle is finally solved. ◩

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