PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Where to Now? [and Discussion]

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Where to now?

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This paper is concerned with looking into the future and trying to discern the shape of the directions nitrogen fixation research will take. Accordingly, much of it may be proved incorrect or impracticable; this is the danger for anyone who makes forecasts.

It seems clear that, although rapid progress is being made in our theoretical understanding of the nitrogen fixation process, little of that progress has yet been applied in a practical sense to improve crop production. Our future directions need to encompass this phase of application. One of the dilemmas is to decide how to use our techniques: to forge new nitrogen-fixing systems or associations, or to improve existing ones, or to pursue some combinations of the two. In the legume systems, there is still much slack in technology to be taken up across the world. Simple problems in production, such as widespread boron deficiency in Thailand, remain to be corrected. Some questions to be considered include the following:

(i) The ability to manipulate expression of *sym* and *nif* genes exists; what are we going to do with it?

(ii) Acid tolerance in legume bacteria remains a major problem. What conditions such tolerance, and how can it be recognized and exploited?

(iii) Nitrogen fixation in legume nodules depends on dicarboxylate supplies from the plant, apparently because the legume controls what the nodule bacteroids receive. Would a greater supply of dicarboxylates improve nitrogen fixation? Would making other classes of substrates available to bacteroids in larger amounts have beneficial effects?

(iv) 'Alternative' nitrogenases are now known; can they be used beneficially in existing or new systems?

To consider a hundred years' studies on nitrogen fixation, and project even a short time forward into the future, is a tall order. The topics which I can examine will be limited, and the views personal and possibly controversial. I hope that I offend nobody if I leave whole areas of the topic of nitrogen fixation untouched. The way of the prophet in science is at least as hazardous as that of the Biblical one, with a very high probability of being proved wrong in either the short or the long term. Although the scientific prophecies are perhaps more difficult in that they can be so comprehensively proved incorrect, the penalties for being wrong are, fortunately, less final.

It seems appropriate after 100 years to take note of a sentence in the first part of Hellriegel's paper of 1886, where he said (in my rough translation) 'correct scientific knowledge of a natural process forms the best foundation for the useful, practical application of it.' A century later, we have a great deal of scientific knowledge (which we hope is correct) about the natural process of dinitrogen fixation. How do we measure up in terms of having applied that knowledge in useful and practical ways?

It is not my brief to dwell on the chemical fixation of N_2, but because little has been said so far on this topic it seems fair that a few comments should be made. Progress in this area

seems to fit into several phases. The mid-1950s saw interest centred on the conditions necessary for the production of metal complexes of dinitrogen, and their properties. A second phase has involved the working-out of the conditions necessary for that complexed dinitrogen to be reduced to ammonia or other products, such as hydrazine. There now seems to be a vast array of metal–N_2 complexes in the literature; some of them can be reduced to produce NH_3. The attraction of a chemical process analogous to the biological one in being able to work at low temperature and pressure and under mild conditions is, of course, considerable. But which way will the enormous amount of chemistry learned over the past 30 years now go? Will it be heading towards a rival of the Haber process for the large-scale manufacture of nitrogenous fertilizers, or towards a low-technology process able to be applied in a decentralized way more appropriate for developing countries?

One electrolytic method for the cyclic formation of a complex and its reduction (Pickett & Talarmin 1985) to give NH_3 is an obvious milestone along the road towards a cheap and simple small-scale way of adding ammonia to an agricultural system. Let us hope that there will be further progress along that road, but recognize that the achievement of a working system will be difficult, not only because of the scientific problems, but because of the way industry in developed countries operates. It seems highly improbable that the larger chemical industries are going to be interested in spending money on development or manufacture of devices which will compete with their existing investments, and whose sale to Third World countries is unlikely to generate large profits. Who, then, is going to be interested in doing the development work, and particularly in financing it?

The single largest input from biological nitrogen fixation into agricultural production is made by the legumes, with the nodules that Hellriegel recognized as the agents of a nitrogen-input process special to those plants. The technology to achieve nodulation of appropriate hosts with appropriate rhizobia is well developed, and well applied in a number of countries. Yet in the developing countries of Africa and southeast Asia the technology gap in the use of legumes can be appalling. Let me illustrate with a few very simple examples within my own experience. Thailand, arguably one of the better developed countries of southeast Asia, has an excellent fermentation plant for the production of legume inoculants in its Department of Agriculture in Bangkok. Yet there is no organized system for cool storage and transmission of such inoculants through the country. Many other countries of the region have neither fermentation plant nor transmission system, but are trying to introduce new crops into areas where they have not been grown previously. This sort of technology gap is something which must really attract our attention and be solved if we are to apply the principle noted by Hellriegel. Trace elements for legume nutrition are obvious areas where a very small investment in fertilizer is accessible even to peasant farmers in developing countries, and can produce very large dividends in production. Thailand, for example, is proving to have very large areas which are boron-deficient for peanut production. The deficiency is not manifest as a general yield reduction, but in a 'hollow heart' in the kernel, reducing its saleability and viability. How many similar production or quality limitations are there for which the appropriate technology already exists?

In terms of the legume root nodule and its bacteroids, progress in understanding the system seems to be accelerating. Others have described the genetic organization of the *nod* and *nif* genes in these organisms, and given exciting insights into the way such genes are controlled (A. W. B. Johnston, this symposium). Elucidation of the biochemical activities of the various gene products is as usual lagging behind the molecular biology, and that lag will clearly need

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

to be made up. Identification of the roles the plant itself plays in the processes of initial nodulation and later nodule differentiation are obviously areas to which more attention will need to be devoted. That the *nod* genes are switched on by flavonoid compounds (Peters *et al.* 1986; Redmond *et al.* 1986) from the legume simply underlines the two-way nature of the establishment and maintenance of symbiosis.

There are, however, many areas of the biology of rhizobia which remain obscure, and yet which need to be established if the legumes are to be exploited optimally. From the production point of view, one naively simple question is to define what makes a rhizobial strain an agriculturally useful one. The literature contains lists of factors which may be of importance, though some statements of such properties are descriptions rather than understandings. Saprophytic competence, for example, is a phrase designed to describe an organism which establishes itself in the soil, nodulates effectively, persists and does all the right things by the legume in question. The concept is valuable, but raises a number of questions. Why does it succeed when others fail? What specific biochemical properties does it have which are special enough to let it succeed?

One biochemical factor which seems likely to be of importance in the soil phase is versatility of substrate use. In the periods between active plant growth, common in agricultural systems with pronounced wet and dry seasons, rhizobia are presumably in competition for the very limited carbon resources coming from the organic fraction of soils. Much of this organic matter is aromatic in chemical character, and so the ability to cleave the aromatic ring may be important to rhizobia. Not surprisingly, most of them have the ability to grow on some aromatic compounds (Parker *et al.* 1977; Muthukumar *et al.* 1982; Parke & Ornston 1984). But the variability in the scope of their activities is considerable. The two most common pathways in aromatic metabolism – the catechol and protocatechuate branches of the 3-oxoadipate pathway – are both present in many rhizobia (Parke & Ornston 1986); some have only the protocatechuate pathway (Chen *et al.* 1984). Some have at least the first enzyme of the alternative *meta*-cleavage pathway for catechol, although the presence of the rest of the pathway is not established. Does the possession of one, two or three pathways for aromatic degradation mean that some organisms have access to more aromatic products of soil organic matter than others? Does it matter? Clearly this requires some carefully designed experiments with isogenic mutants before any ecological relevance can be established.

The regulation of the aromatic pathways also appears to be very different between the rhizobia and the bradyrhizobia, at least for the few strains so far studied (Parke & Ornston 1986). The rhizobia seem to have a sequential induction system responding to substrate addition, whereas the bradyrhizobia seem to maintain constitutive levels of a number of the enzymes and derepress one or two key ones in response to substrate availability. It seems that if we could understand the importance of the basic biochemical makeup of the strains we might gain some clues about what is critical for success in the soil and what is not.

The success of inoculation of legumes at their first introduction into a soil devoid of rhizobia is legendary. What happens to soil populations of these organisms afterwards is not really very clear. Does a single dominant type of organism arise and essentially take over? Are there a limited number of types of strains which have particular groupings of high-value characteristics? How much genetic interchange, of chromosomal and/or plasmid material, goes on in the soil environment? How much influence does the presence of a legume crop or pasture have in selecting strains and so distorting what might be a different balance if soil conditions alone

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

determined it? If we ever do succeed in identifying all the plant and bacterial genes required for a symbiosis and N_2 fixation, and in transferring them to other crop plants, these questions will need answers. If we were to want one strain of symbiotic bacteria to function with a cereal crop and another with a subsequent legume crop we might have something of a problem.

In the pre-nodulation process, are motility and chemotaxis important in determining which strains successfully reach the legume rhizosphere? Several factors that are essential for motility in the laboratory (Ca^{2+}, pH above certain limits, lack of excess heavy metals) (Bowra & Dilworth 1981) have at various times also been shown important in successful inoculation. Coincidence? Motile cells of both *Rhizobium trifolii* (H. Mellor, unpublished) and *R. meliloti* (Ames & Bergman 1981) show a large advantage over non-motile mutant derivatives in forming nodules even when both are directly placed in the rhizosphere together. The reason for this difference in the immediate inoculation zone is not at all clear. Chemotaxis may be important in leading the organism from a soil microcolony to the rhizosphere, but although several studies have shown rhizobia to be chemotactic to a wide range of compounds (Bowra & Dilworth 1981; Gaworzewska & Carlile 1982) and root exudates (Currier & Strobel 1976; Gaworzewska & Carlile 1982) the role of chemotaxis (if any) is really still to be established as a field phenomenon. With the demonstrated importance of flavones in inducing the *nod* genes, their function in eliciting chemotaxis would also be of interest.

The role of acid-tolerance in rhizobia is becoming an area of considerable interest, particularly in Australia. There, a steadt decrease in pH in soils fertilized for many decades with superphosphate to overcome general phosphorus deficiency is associated with a decline in productivity of legume-based pastures grown on them. The argument about the cause of the acidification (superphosphate or nitrification of symbiotically fixed nitrogen) remains unresolved, but the problem, which appears to be associated with less effective nodulation, has to be faced. What do we do now? Lime application on a massive scale on an extensive basis is economically unattractive or impossible. Can we define what makes one rhizobial strain more tolerant of acid soils than another, and can we transfer this property to other strains? The genetic variability certainly exists within rhizobia, as shown by the successful introduction of strains of *R. meliloti* from Sardinia into West Australian soils that have proved too acid for previous commercial inoculants (Howieson & Ewing 1986). The first problem is that we do not have the correct scientific knowledge of this natural process to define what makes an organism acid-tolerant.

Dinitrogen fixation in the legume root nodule depends for its electron supply and its energy supply on the photosynthates transferred from the plant top. The nodules of different species of legumes contain a wide variety of organic solutes, including considerable quantities of C_{12} and C_6 sugars, organic acids, amino acids and polyols. Which of these many apparently available carbon sources are used by the symbiotic bacteroids, and for which purposes? The earliest answers implicated the C_4-organic acids (malate, fumarate, succinate) because isolated bacteroids proved capable of oxidizing these readily although they were apparently unable to utilize most other substrates. In confirmation of these results, mutants with specific metabolic blocks have delivered the same message: those with impaired systems for sugar oxidation remained infective and effective, whereas those with lesions in organic acid uptake (dicarboxylate transport, *dct*) were infective but did not fix N_2. The use of mutants of these types is fraught with some danger, since by their very nature they result in the accumulation of metabolic intermediates in most cases. If that were to result in very slow growth or metabolism,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

one might get the wrong idea about the importance of some substrate. There is also a need to treat with caution results with a mutation causing the organism to become a metabolic 'cripple', such as a succinate dehydrogenase mutation (Gardiol *et al.* 1982). The message is, however, surprisingly clear: only C_4-dicarboxylates will support N_2 fixation in nodule bacteroids. This has now been demonstrated for the clover–*R. trifolii* (Ronson *et al.* 1981), pea–*R. leguminosarum* (Finan *et al.* 1983; Arwas *et al.* 1985) and cowpea *Rhizobium*–snake bean (S. Boyer, unpublished) systems.

An important corollary is that substrates other than the C_4-dicarboxylates must be adequate for infection and initial bacteroid development to occur (Ronson *et al.* 1981). However, even double mutants blocked in dicarboxylate utilization and cut off from all C_6 and C_{12} sugars still nodulate, though of course ineffectively (Arwas *et al.* 1986).

How then do we visualize the nodule functioning in biochemical terms? Is the bacteroid treating the materials in the nodule as a smorgasbord from which it is selecting what it needs for particular purposes? Or is the plant calling the tune by only allowing the bacteroid to have access to certain substrates despite the wide range it may have available?

Nodule architecture is important in this connection. Each bacteroid or group of bacteroids is enclosed by a peribacteroid membrane of plant origin, the orientation of which is such that the bacteroid is essentially on the outside as far as the plant is concerned. The properties of this peribacteroid membrane in terms of its permeability are largely unknown. Yet the peribacteroid membrane, in combination with the bacteroid membrane, may well regulate what goes in and what comes out.

Two lines of evidence suggest that the peribacteroid membrane does indeed control materials entering the bacteroid. In the pea system with *R. leguminosarum* MNF3841, cells growing on C_4-dicarboxylates in the laboratory derepress a fructose bisphosphate aldolase and a phosphoenolpyruvate carboxykinase (PEPCK) for gluconeogenesis, but the synthesis of these enzymes is repressed by relatively low concentrations of sugars such as sucrose (no detectable PEPCK at 0.4 mM sucrose in chemostat cultures). Yet the pea bacteroid contains significant levels of PEPCK, suggesting that, despite the quantity of sugars known to be present in pea nodules, very little actually reaches the bacteroid (McKay *et al.* 1985).

With the cowpea *Rhizobium* strain NGR234, many of the sugar-catabolic enzymes are induced only by growth on sugars, and not by growth on C_4-dicarboxylates. Thus invertase is induced by growth on sucrose, and fructokinase by growth on fructose or sucrose, whereas both glucose-6-phosphate dehydrogenase and the Entner–Doudoroff enzymes are induced by growth on C_6-sugars. In the snake bean nodule, none of these enzymes is detectable in the bacteroids, once again suggesting that only very small amounts of sugars are entering the bacteroid (Saroso *et al.* 1986). This interpretation is dependent on the assumption that regulation of catabolism in the bacteroid is similar to that in the laboratory culture. In the succinate uptake system for cowpea *Rhizobium* NGR234, which is inducible by C_4-dicarboxylates in the laboratory, the bacteroid is definitely derepressed in the nodule (Saroso *et al.* 1984) where the ineffective phenotype of a *dct* mutation indicates that dicarboxylate uptake is vital for N_2 fixation. At least this aspect of regulation seems to be the same.

The peribacteroid membrane may therefore have a major regulatory role in carbon supply to bacteroids. Several further questions obviously arise. Why does the supply of dicarboxylates to bacteroids seem to be a requirement for N_2 fixation in all the nodule systems so far examined? Admittedly the number of legumes is small, but the plants are relatively diverse, and the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

dicarboxylate story is still the same. Is this simply because nodule tissue, in all legumes, has only a very low partial pressure of O_2 and therefore favours malate accumulation by nearly anaerobic plant tissue (De Vries *et al.* 1980)? But why just dicarboxylates, when such nodules also contain considerable quantities of free sugars?

However, as mentioned before, the formation of nodules by *dct* mutants unable to take up dicarboxylates tells us that other substrates must get across the peribacteroid membrane, but does not tell us what these are. Preparation of totally intact bacteroids surrounded by peribacteroid membranes may provide some urgently needed answers to such questions, but getting such wholly intact preparations is a major problem, and even small contaminations with damaged membrane envelopes could give seriously misleading answers.

This apparent control of substrate flux by the peribacteroid membrane raises the question of what would happen if we could engineer a situation where all the other substrates in the nodule were available to the bacteroid. Would this result in enhancement of N_2 fixation or is the regulation of the *nif* system such that only dicarboxylate metabolism will do? More questions to which there are at present no answers.

Although it has been obvious for some years that dicarboxylate metabolism is important for N_2 fixation, the route that it follows has not been defined. A mechanism is required for converting malate to pyruvate and allowing for dicarboxylate oxidation via the tricarboxylic acid cycle. A mutant lacking such a system should therefore be ineffective.

The importance of the uptake hydrogenase system to aerobic N_2-fixers has already been discussed in principle (Evans *et al.*, this symposium). Its importance to legume nodule function and legume productivity has also been addressed quite intensively over recent years. Despite the fact that the presence of this energy-conserving system should, on theoretical grounds, give rise to quite large differences in amounts of nitrogen fixed, such yield increases have been relatively difficult to demonstrate in the field. Now that they have been, one has to ask if possession of this particular property results in sufficient gains in the field to merit its inclusion as a desirable criterion for strain selection? Are there any deleterious effects on soil performance resulting from its presence in a strain?

Another area of rhizobial metabolism which has received little attention is that of mineral nutrition. When one looks at a whole functioning legume, one often needs to know how a mineral nutrient deficiency affects the performance of the plant itself and that of its symbiotic rhizobia, to identify the primary lesion. Two extreme situations have already been established. One is where the element is of no value to the plant itself and where the bacteroid can be deficient despite the presence of excess element in the plant; this is exemplified by cobalt (Dilworth *et al.* 1979; see also p. 287). The other is where the plant itself is deficient in the element for its own purposes, but where the bacteroid nevertheless receives adequate or luxury levels, a situation typical of phosphorus (Smart *et al.* 1984). How about all the other essential elements? These are areas of some applied importance, but answers to them have important basic implications for all microbial systems, where knowledge of mineral element uptake and function is not extensive.

On the plant side of the legume symbiosis, it now appears probable that the bacteroid is held in an O_2-limited state by its plant partner. If the proposal that the plant controls the diffusion resistance of the nodule to oxygen (Minchin *et al.* 1985) is true, then it appears that the plant can adjust the oxygen-diffusion rate to the bacteroids in response to external oxygen pressure, acetylene and possibly carbohydrate supply. If the plant is in fact operating a type

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

of insurance policy against too much or too little oxygen, what will happen if we tinker with the regulation of such a system? Is it already optimized or is there scope for improvement in this way?

Another question which has recently come to the fore is that of alternative nitrogenases, a question whose resolution has helped to explain a variety of apparently discordant observations over the past twenty years or so. If ever there was a case of a requirement for scientists not to close their minds to possible alternative explanations of data, this must surely be it. If established dogma has it that N_2 fixation involves only molybdenum, then it becomes only too easy to dismiss any other sort of system proposed as heresy, faulty experimentation, inappropriate interpretation or some combination thereof.

At least in *Azotobacter chroococcum*, it is now clear that deletion strains lacking *nifHDK* synthesize a vanadium-containing nitrogenase protein corresponding to the Mo–Fe protein of the classical system, as well as an analogue of the Fe protein (Robson *et al.* 1986). In *Azotobacter vinelandii*, where the original nonconformist idea of an alternative nitrogenase originated (Bishop *et al.* 1980), similar deletion strains can be shown to synthesize an alternative nitrogenase under Mo-deficient conditions, although the metal involved (if any) in this system has yet to be identified. The evidence for the involvement of vanadium is compelling, and a variety of interesting questions, chemical, biochemical and physiological, promptly emerge, we hope with some haunting shadow of a practical usefulness lurking in the background.

The *Azotobacter* strains appear to synthesize the alternative nitrogenases only under conditions of Mo-starvation, either directly imposed or generated by addition of tungstate as an antagonist. With *A. chroococcum*, addition of vanadium is required for the synthesis of the alternative nitrogenase; in *A. vinelandii* the absence of Mo seems to be the only condition required (Bishop *et al.* 1986). Whether trace contamination of another metal or utilization of a metal already added to the medium is required remains to be determined.

In broad terms, the vanadium nitrogenase appears to be relatively inefficient compared with its molybdenum counterpart†. Dinitrogen, presumably but not necessarily the *raison d'être* for the enzyme, is a poor substrate as judged by its limited ability to prevent the evolution of H_2. Acetylene, which for the molybdenum enzyme is a very effective substrate in preventing H_2 evolution, is nowhere nearly as effective with the vanadium enzyme. The inefficiency appears both *in vivo* and *in vitro*; uptake hydrogenase negative (*hup*$^-$) strains show the same poor suppression of H_2 evolution by N_2 or by C_2H_2 as does the enzyme isolated from them. Does this mean that the vanadium enzyme is the natural prototype, which has been subsequently improved on with the development of the molybdenum system? Is its survival an indication that those organisms with both sets of nitrogenase structural genes have retained the alternative system as an insurance policy against Mo deficiency? Does this have any practical value for those agricultural soils which are found to be molybdenum-deficient for plant growth but not necessarily for bacteria?

The breadth of microorganisms with alternative systems remains to be defined. Probing the genomes of a variety of prokaryotes will no doubt tell us how widespread the phenomenon may be, but it already seems likely to include the cyanobacteria (Sahay & Sankaram 1968) and the clostridia (Jensen & Spencer 1947). Importantly, does it also include the rhizobia? The answer to that question appears likely to be negative. Vanadium is reputedly more generally and generously distributed than molybdenum and plant tissues commonly contain

† But see the first two pages of the general discussion.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

0.5–1.0 p.p.m. (by mass). If rhizobia were to have a vanadium nitrogenase system, one might expect that Mo deficiency would result in derepression of any such vanadium system in the plant. Practical experience suggests that this does not occur, but of course it is not possible to say whether that vanadium (generally regarded as useless to the plant and present only by accident) is actually available to the nodule bacteriods; but Mo-deficient plants usually produce vast numbers of small, white, ineffective nodules and remain as N deficient as if they had no nodules at all.

The apparent inefficiency of the vanadium nitrogenase, and indeed of the alternative nitrogenase in *A. vinelandii*, may help to explain another legendary problem in nitrogen fixation. It has been notoriously difficult to make calculations of N_2 fixed in natural systems based on the acetylene reduction assay. If there is a significant contribution to N_2 fixation being made by alternative nitrogenases and these vary in the efficiency with which they reduce N_2 and C_2H_2, then it will be a grand guessing game to try to extrapolate from C_2H_2 reduction to N_2 fixation. In all fairness, however, there already appear to be sufficient variables preventing that extrapolation (Knowles 1980) without blaming alternative nitrogenases.

While on that theme, another biological curiosity may have an explanation in an alternative nitrogenase. It has always been puzzling why *Beijerinckia* species appear to require high partial pressures of C_2H_2 in acetylene-reduction assays to saturate their nitrogenase activities (Spiff & Odu 1973; MacRae 1977). If these organisms were to operate with an alternative nitrogenase, for which such values are already known, these odd reports might be resolved. The future for this prediction, however, is not good; Becking's survey of organisms which could fix N_2 in the absence of molybdenum if given vanadium did not report *Beijerinckia* strains among the successful ones (Becking 1962).

Although molecular biological methods can tell us whether particular genes are present and being expressed in particular organisms, it would be extremely useful for physiological experiments if an assay were available which would measure the alternative nitrogenase in the presence of the conventional one. The obvious answers are substrates for nitrogenase which either do not act as substrates for one or other nitrogenase, or which give different products for the different enzymes. Such an assay might allow estimations of the function of alternative nitrogenases in natural systems, and perhaps of how significant their activities may be.

The establishment of an alternative nitrogenase system as a reality brings up a variety of questions of the genetic and physiological control of such a system. Even though the structural genes are clearly different, are the metal-processing systems the same, in whole or in part? How does the cell organize the switch-on of the alternative genes in the absence of molybdenum? From the fact that in some organisms tungstate antagonizes the uptake of molybdenum but not of vanadium, the vanadium-uptake system must clearly be different from the molybdenum one, but the mechanics of such an uptake system and its control should prove interesting.

If we leave aside the intrinsic scientific interest of the alternative systems, and the slightly enhanced employment opportunities for nitrogen-fixers, of what general use are they likely to be? From the point of view of a chemical mechanism for N_2 reduction by the enzyme, the presence of a second way of doing it must in the long run increase our chance of understanding the process. We have a vast number of experiments we could repeat with the vanadium (and perhaps other) nitrogenases, in the hope of defining the key differences in the way the enzymes behave. Comparing them with the way that the chemical systems involving these elements work to reduce N_2 may indicate just how the enzyme operates.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

So where now? Why should we continue striving for an understanding of N_2 fixation? In the first place, N_2 fixation is a biological process which is as important to the biosphere as photosynthesis, and research directed by curiousity about how it happens is entirely justifiable. In the second, the fact that such research may well point out how the process can be better exploited in agricultural systems must also be taken into account. What we need to keep in mind is that while we are gaining Hellriegel's 'correct scientific knowledge of a natural process' we should not forget to look for the 'useful, practical application of it'.

References

Ames, P. & Bergman, K. 1981 Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. *J. Bact.* **148**, 728–729.

Arwas, R., Glenn, A. R. & McKay, I. A. 1986 Properties of double mutants of *Rhizobium leguminosarum* which are defective in the utilization of dicarboxylic acids and sugars. *J. gen. Microbiol.* **132**, 2743–2747.

Arwas, R., McKay, I. A., Rowney, F. R. P., Dilworth, M. J. & Glenn, A. R. 1985 Properties of organic acid utilization mutants of *Rhizobium leguminosarum* strain 300. *J. gen. Microbiol.* **131**, 2059–2066.

Becking, J. H. 1962 Species differentiation in molybdenum and vanadium requirement and combined nitrogen utilization by Azotoacteriaceae. *Pl. Soil* **16**, 171–201.

Bishop, P. E., Jarlenski, D. M. L. & Hetherington, D. R. 1980 Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7342–7346.

Bishop, P. E., Premakumar, R., Dean, D. R., Jacobson, M. R., Chisnell, J. R., Rizzo, T. M. & Kopczynski, J. 1986 Nitrogen fixation by *Azotobacter vinelandii* strains having deletions in structural genes for nitrogenase. *Science, Wash.* **232**, 92–94.

Bowra, B. J. & Dilworth, M. J. 1981 Motility and chemotaxis towards sugars in *Rhizobium leguminosarum*. *J. gen. Microbiol.* **126**, 231–235.

Chen, Y.-P., Glenn, A. R. & Dilworth, M. J. 1984 Uptake and oxidation of aromatic substrates by *Rhizobium leguminosarum* MNF841 and *Rhizobium trifolii* 7A1. *FEMS microbiol. Lett.* **21**, 201–205.

Currier, W. W. & Strobel, G. A. 1976 Chemotaxis of *Rhizobium* spp. to plant exudates. *Pl. Physiol.* **57**, 820–823.

De Vries, G. E., In't Veld, P. & Kijne, J. W. 1980 Production of organic acids in *Pisum sativum* root nodules as a result of oxygen stress. *Pl. Sci. Lett.* **20**, 115–123.

Dilworth, M. J., Robson, A. D. & Chatel, D. L. 1979 Cobalt and nitrogen fixation in *Lupinus angustifolius*. II. Nodule formation and function. *New Phytol.* **83**, 67–79.

Finan, T. M., Wood, J. M. & Jordan, D. C. 1983 Symbiotic properties of C_4-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. *J. Bact.* **154**, 1403–1413.

Gardiol, A., Arias, A., Cervenansky, C. & Martinez-Drets, G. 1982 Succinate dehydrogenase mutant of *Rhizobium meliloti*. *J. Bact.* **151**, 1621–1623.

Gaworzewska, E. T. & Carlile, M. J. 1982 Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J. gen. Microbiol.* **128**, 1179–1188.

Govers, F., Gloudemans, T., Moerman, M., van Kammen, A. & Bisseling, T. 1985 Expression of plant genes during the development of pea root nodules. *EMBO J.* **4**, 861–867.

Hellriegel, H. 1886 Welche Stickstoff-quellen stehen der Pflanze zu Gebote? *Z. Ver. dt. ZuckInd* **36**, 863–877.

Howieson, J. & Ewing, M. A. 1986 Acid tolerance in the *Rhizobium meliloti–Medicago* symbiosis. *Aust. J. agric. Res.* **37**, 55–64.

Jensen, H. L. & Spencer, D. 1947 The influence of molybdenum and vanadium on nitrogen fixation by *Clostridium butyricum* and related organisms. *Proc. Linn. Soc. N.S.W.* **72**, 273–286.

Knowles, R. 1980 The measurement of nitrogen fixation. In *Current perspectives in nitrogen fixation* (ed. A. H. Gibson & W. E. Newton), pp. 327–333. Canberra: Australian Academy of Science.

McKay, I. A., Glenn, A. R. & Dilworth, M. J. 1985 Gluconeogenesis in *Rhizobium leguminosarum* MNF3841. *J. gen. Microbiol.* **131**, 2067–2073.

MacRae, I. C. 1977 Influence of partial pressures of acetylene and nitrogen upon nitrogenase activity by species of *Beijerinckia*. *Aust. J. biol. Sci.* **30**, 593–596.

Minchin, F. R., Sheehy, J. E. & Witty, J. 1985 Factors limiting N_2 fixation by the legume–*Rhizobium* symbiosis. In: *Nitrogen fixation research progress* (ed. H. J. Evans, P. J. Bottomley & W. E. Newton), pp. 285–291. Dordrecht: Martinus Nijhoff.

Muthukumur, G., Arunakumari, A. & Mahedevan, A. 1982 Degradation of aromatic compounds by *Rhizobium* spp. *Pl. Soil* **69**, 163–169.

Parke, D. & Ornston, L. N. 1984 Nutritional diversity of Rhizobiaceae revealed by auxanography. *J. gen. Microbiol.* **130**, 1743–1750.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Parke, D. & Ornston, N. 1986 Enzymes of the β-ketoadipate pathway are inducible in *Rhizobium* and *Agrobacterium* and constitutive in *Bradyrhizobium*. *J. Bact.* **165**, 288–292.
Parker, C. A., Trinick, M. J. & Chatel, D. L. 1977 Rhizobia as soil and rhizosphere inhabitants. In *A treatise on dinitrogen fixation* (ed. A. H. Gibson), section 4, pp. 311–352. New York: Wiley Publishing Co.
Pickett, C. J. & Talarmin, J. 1985 Electrosynthesis of ammonia. *Nature, Lond.* **317**, 652–653.
Peters, N. K., Frost, J. W. & Long, S. R. 1986 A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science, Wash.* **233**, 977–980.
Redmond, J. W., Batley, M., Djordevic, M. A., Innes, R. W., Kuempel, P. L. & Rolfe, B. G. 1986 Flavones induce expression of nodulation genes in *Rhizobium*. *Nature, Lond.* **323**, 632–635.
Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. & Postgate, J. R. 1986 The alternative nitrogenase of *Azotobacter chroococcum* is a vanadium enzyme. *Nature, Lond.* **322**, 388–390.
Ronson, C. W., Lyttleton, P. & Robertson, J. G. 1981 C_4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4284–4288.
Sahay, M. N. & Sankaram, A. 1968 Effect of trace element and growth promoting substances on *Anabaena circinalis* RABH. *Indian J. Microbiol.* **8**, 47–48.
Saroso, S., Glenn, A. R. & Dilworth, M. J. 1984 Carbon utilization by free-living and bacteroid forms of cowpea *Rhizobium* NGR234. *J. gen. Microbiol.* **130**, 1809–1814.
Saroso, S., Dilworth, M. J. & Glenn, A. R. 1986 The use of carbon catabolic-enzymes as a probe for the carbon nutrition of snakebean nodule bacteroids. *J. gen. Microbiol.* **131**, 243–249.
Smart, J., Robson, A. D. & Dilworth, M. J. 1984 A continuous culture study of the phosphorus nutrition of *Rhizobium trifolii* WV95, *Rhizobium* NGR234 and *Bradyrhizobium* CB756. *Arch. Microbiol.* **140**, 276–280.
Spiff, E. D. & Odu, C. T. I. 1973 Acetylene reduction by *Beijerinckia* under various partial pressures of oxygen and acetylene. *J. gen. Microbiol.* **78**, 207–209.

Discussion

J. M. Lynch (*Glasshouse Crops Research Institute, Littlehampton, West Sussex, U.K.*). Chemotactic responses of *Rhizobium* to root hairs or infection sites may not be specific. In the soil, there may also be competition for substrates exuded from root surfaces.

M. J. Dilworth. There may be both positive and negative chemostactic responses and combinations of these would be selective.

J. W. Drozd (*Shell Research Ltd, Sittingbourne, Kent, U.K.*). Why does the sucrose, present in such abundance in nodule tissues, fail to reach the bacteroids? What is so special about dicarboxylic acids that they are the preferred energy source?

M. J. Dilworth. This is an important point. It may be that it is a consequence of a fundamental property of peribacteroid membranes that dicarboxylates are the major substrates for bacteroids in all legume nodules examined so far.

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