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General Discussion

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General discussion

J. R. Postgate. I enquire why there has been so little mention of stem nodulation in *Sesbania* spp. What are the modes of initial infection in these plants?

C. Elmerich. The details of infection of stems of *Sesbania rostrata* are being studied presently. So far the signals involved in nodule initiation are unknown but the physiological steps are known. Infection of stems occurs in incipient root primordia, which are arranged in several rows vertically on each stem. It seems to involve breaks in the stem surface where these primordia emerge but do not develop into roots. Comparisons of strains of bacteria that form both stem and root nodules with those that form only root nodules suggest that the same *nod* genes are involved in both.

J. Sprent. It is interesting to observe that, in plants as different as *Arachis* spp. and *Sesbania* spp., root and stem nodules respectively are always produced in or near lateral root primordia. This form of infection may be very much more widespread than presently believed, especially in shrub and tree legumes. It is interesting also, to speculate on the possible relations with the requirements for boron, for example, in promotion of vascular development in roots and nodules, as described by Thornton, many years ago.

C. Kennedy. Does the repression by nitrate of infection of legume roots by rhizobia involve inhibition of production or action of 'nod-on' flavones and flavonones?

A. W. B. Johnston. Apparently not. Exudates from nitrate-grown plant are active.

P. S. Nutman. More than 30 years ago, we observed that certain legumes produced coloured layers in various clays placed near the hypocotyls of developing seedlings. Tests suggested that these colours were due to carotenoids and aromatic nitrogen compounds. Perhaps some of them were related to 'nod-on' substances.

A. H. Stouthamer (*Vrije Universiteit, Amsterdam, The Netherlands*). I have a suggestion about the preferred utilization of dicarboxylic acids by bacteroids. Non-equilibrium thermodynamics of microbial growth have shown that the thermodynamic efficiency of substrate consumption is dependent on the degree of reduction of the substrate. It is lower for reduced than for oxidized compounds. Malate and succinate are more oxidized than sugars. By utilizing only dicarboxylic acids during multiplication of the bacteria in nodules, a high thermodynamic efficiency of substrate utilization would be assured and the occurrence of energy-dissipating mechanisms in the bacteroids would be minimized, ensuring a high efficiency during the nitrogen fixation stage.

J. R. Postgate. I should like to try to arrest the growth of a myth that the Va-nitrogenase is less efficient than the Mo-nitrogenase. *In vivo*, the Va-enzyme seems to release more hydrogen than the Mo-enzyme but, in *A. chroococcum*, the genetic background of the test organism has three mutations (Δ*nifKDH*, *hup*$^-$, W-resistance) and we do not know that the knock-on effect of these

does not impair nitrogenase function for metabolic reasons. *In vitro*, we need to know the minimum ATP:ε ratio and minimum NH_3–H_2 stoichiometry, as well as the suitability of the potentially damaging dithionite as reductant, before we can make realistic comparisons. The Va system may be less efficient than the Mo system, but we cannot yet be sure.

R. R. Eady (*AFRC Unit of Nitrogen Fixation, the University of Sussex, Brighton, U.K.*). Although I would agree with Professor Postgate's comments on possible knock-on effects of mutations in general, I am not sure that in this instance it provides a basis for explaining enhanced H_2 evolution by the Va-nitrogenase *in vivo*.

Our chemostat studies of N_2-grown *Azotobacter vinelandii* and a derived *nifHDK* deletion strain, both grown in Mo-deficient media, revealed the following:

Firstly, under these conditions the biomass yield for both strains was similar, indicating that the parental strain which possessed both nitrogenase systems had no nutritional advantage, and also that elimination of the *nifHDK* genes had no detectable deleterious effect on the deletion strain.

Secondly, cultures of both strains evolved substantial amounts of H_2 during aerobic growth under our conditions. Because both strains were apparently using the alternative nitrogenase for N_2 fixation, extensive H_2 evolution would not appear to be a consequence of the mutation eliminating *nifHDK* genes. However, further studies will be required to resolve the question as to the relative efficiencies of both nitrogenase systems *in vivo*.

J. R. Postgate. What Dr Eady says about his experiments with *A. vinelandii* and the deletion strain is, of course, perfectly correct. But we have some evidence, mentioned in Dr Kennedy's talk, that *A. vinelandii* contains not one but two nitrogenase systems in addition to the conventional Mo-nitrogenase. Thus he cannot be certain that it is a Va-nitrogenase which was giving the seemingly ineffecient phenotype in his cultures.

J. Witty (*Welsh Plant Breeding Station, Aberystwyth, U.K.*). Could Dr Newton comment on the relative energetic efficiencies of industrial, laboratory chemical and biological nitrogen-fixing systems?

W. E. Newton. Laboratory-scale chemical systems usually use more energy than biological ones. *In vitro* test tube assays of nitrogenase are similar to the Haber reaction in the energy requirement. In fact, if the energetically wasteful evolution of H_2 could be eliminated, the energy demands would be the same.

C. Pickett (*AFRC Unit of Nitrogen Fixation, The University of Sussex, Brighton U.K.*) I disagree with Dr Newton. I think that chemical systems are capable of being much more efficient than they seem at present.

R. N. F. Thorneley (*AFRC Unit of Nitrogen Fixation, The University of Sussex, Brighton, U.K.*). The electrochemical synthesis of ammonia developed by Dr Pickett is most efficient when protonation and electronation of coordinated N_2 are temporally separated. Electronation in the presence of excess tosylic acid causes increased H_2 evolution and a decreased yield of NH_3. The key to efficient enzymic reduction of N_2 seems to me to be the mechanism by which

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wasteful H_2 evolution is suppressed. Protons cannot be excluded from the active site, because they are probably required to generate a metallodihydride intermediate to which N_2 binds by H_2 displacements and they are certainly required for the stepwise protonation of coordinated N_2 to yield $2NH_3$. How are the postulated metallodihydride intermediates constrained to react with N_2 rather than with excess protons to yield H_2 and an oxidized metal centre? Nature with nitrogenase has achieved something that Dr Pickett has so far not achieved with his chemical system, that is a tightly coupled electron transfer–proton transfer system. Could this be the reason that electron transfer between the component proteins is tightly coupled to ATP hydrolysis? Computer simulations based on the Lowe–Thorneley mechanism for nitgrogenase action show that high concentrations of the nitrogenase component proteins (up to 40% of the total cytoplasmic protein), a slow rate-limiting protein dissociation reaction ($k = 6.4\ s^{-1}$) and a ratio of Fe:Mo protein of 2.5:1 are optimal for N_2 fixation rather than H_2 evolution. Bacteria pay a high price to fix N_2 rather than evolve H_2.

J. G. Leigh (*AFRC Unit of Nitrogen Fixation, The University of Sussex, Brighton, U.K.*). Chemistry is showing already that there is no unique route for conversion of dinitrogen to ammonia. We know that in the protonation of dinitrogen coordinated to molybdenum as far as hydrazide^{2-}, the precise mechanistic route depends on the acid strength, the solvent, and the other ligands bound to molybdenum. For the conversion from hydrazide^{2-} to ammonia, at least two routes are available. Consequently, it is not justifiable to assume that the mechanism for conversion of dinitrogen to ammonia mediated by nitrogenase is the same whatever the source of the nitrogenase. Subtle differences between otherwise rather similar proteins could well induce changes in detailed reaction mechanism.

Similarly, considerable discussion has taken place concerning the relative efficiencies of the vanadium and molybdenum nitrogenases, based on the proportions of electrons apparently used by dihydrogen production. Such discussions can only be valid if the mechanisms involving vanadium and molybdenum are precisely the same. This we simply do not know. It could be that the different proportions used by vanadium systems are inherent in vanadium chemistry and would persist even were such a system giving a maximum stoichiometric yield of ammonia. Until this question is settled, discussions of relative efficiencies are not very meaningful.

S. Hill (*AFRC Unit of Nitrogen Fixation, The University of Sussex, Brighton, U.K.*). I should like to ask Dr Sprent whether there is any other N_2-fixing symbiosis besides *Azolla* in which the symbiont is retained during the sexual reproduction of the host, as this host presumably does not have to establish a new symbiosis for each generation.

Janet Sprent. I'm not sure that this happens, even in *Azolla*.

J. Becking (*Research Institute ITAL, Wageningen, The Netherlands*). During vegetative reproduction, which accounts for most of the biomass production in nature, the cyanobacteria are located near the stem apex containing the apical meristem. During the formation of new leaves from leaf primordia of the apical meristem, the cyanobacteria invade the developing leaf cavities of the young leaves.

During sexual reproduction, which is rather complicated in a fern like *Azolla*, the cyanobacteria are only transmitted by means of the macrosporocarps (female organs) and not by

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the microsporocarps (male organs). Macrosporocarps are urn-shaped and harbour the cyanobacteria, i.e. the *Anabaena* endophyte, near the tip just below the indusium cap. These cyanobacteria are extracellular and embedded in polysaccharide-like material. They are probably in a resting stage, so-called akinetes. When, in a mature macrosporocarp, the macrospore located at the bottom of the macrosporocarpic structure is fertilized, it germinates (embryo) forming a new sporophyte. The developing sprout growing to the tip of the macrosporocarpic structure comes in direct contact with the *Anabaena* cells present there and the newly developing leaves become infected just as in vegetative reproduction. (See *Plant and Soil* **100** (1987).)

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