PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Nitrogen Fixation

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Nitrogen fixation

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The International Biological Programme served as a focal point for studies on biological nitrogen fixation during the 1960s. The introduction of the acetylene reduction technique for measuring nitrogenase activity in the field led to estimates becoming available of the contribution of lichens, blue-green algae, nodulated non-legumes and bacterial-grass associations, as well as of legumes. Other studies carried out on the physiology and biochemistry of the process led to the eventual purification and characterization of the nitrogenase enzyme. These studies, collectively, provided the springboard for current work, so essential in view of the present energy crisis, on how to increase the use and efficiency of nitrogen-fixing plants, on the metabolic regulation of the nitrogenase enzyme and on the genetics of the nitrogen-fixing process, both in higher plants and in free-living micro-organisms.

Introduction

Biological fixation of nitrogen was one of the two main topics included in the I.B.P. Section, Production Processes (P.P.). The other was *Photosynthetic activity and solar energy conversion*. The decision to include nitrogen fixation showed remarkable foresight, because it was taken at a time when cheap synthetic nitrogen fertilizer was readily available in many parts of the world and most agriculturalists, environmentalists and economists were showing limited interest in this topic. Today with the escalating cost of raw materials for synthetic nitrogen fertilizer (Smallpage 1975), there is a renewed interest in biological nitrogen fixation. In this connection, the work carried out during the period of the I.B.P. and stimulated by various I.B.P. discussion meetings and symposia (Allen & Fahraeus 1970; Lie & Mulder 1971; Nutman 1975*a*; Stewart 1975*a*), is helping to provide the basis on which rational decisions on the importance and potential of biological nitrogen fixation can be taken, and furthermore is providing the springboard for much of the current research effort on this vitally important process (see, for example, Evans 1975).

2. The themes

At an early stage, it was decided that the most satisfactory way to approach the problem of nitrogen fixation on an international basis was to have various themes (listed in table 1) in which workers in different parts of the world could collaborate. Themes were developed around each of the major nitrogen-fixing groups: the free-living bacteria (themes 1 and 7), the blue-green algae (themes 1 and 2), the legume-*Rhizobium* symbiosis (themes 3, 4 and 8) and the nodulated non-leguminous nitrogen-fixing plants (theme 5). Theme 6, on leaf nodule-bearing plants was set up after reports that leaf nodules fixed nitrogen. However, as subsequent critical tests showed little evidence of this (see Becking 1974) the theme was disbanded. Five other themes were also proposed (themes 9–13), but leaders for these were never appointed and there was no international collaboration under the *aegis* of I.B.P. although these topics were considered fully at various international symposia which were organized entirely, or

partially, under the umbrella of I.B.P. (Lie & Mulder 1971; Nutman 1975*a*; Stewart 1975*a*). Of the international themes to which leaders were appointed six out of eight had theme leaders or co-leaders from the United Kingdom.

TABLE 1. THE VARIOUS I.B.P. THEMES ON NITROGEN FIXATION, AND THEIR THEME LEADERS

Theme 1. *Field experiments on the accumulation of N in the absence of legumes*
D. S. Jenkinson, Rothamsted Experimental Station, Harpenden, Herts. U.K.
Theme 2. *Nitrogen fixing blue-green algae*
W. D. P. Stewart, Department of Biological Sciences, The University, Dundee, DD1 4HN, Scotland, U.K.
Theme 3. *Experiments on nitrogen fixation by nodulated legumes*
P. S. Nutman, Rothamsted Experimental Station, Harpenden, Herts., U.K.
J. M. Vincent, Department of Microbiology, University of New South Wales, Kensington N.S.W., Australia.
Theme 4. *The relationship between symbiotic nitrogen fixation and the environment*
J. S. Pate, Department of Botany, Queen's University, Belfast, Northern Ireland, U.K.
T. A. Lie, Laboratorium voor Microbiologie der Landbouwhogeschool, Hesselink van Suchtelenweg 4, Wageningen, Netherlands.
Theme 5. *Survey of root nodule formation in non-leguminous angiosperms*
G. Bond, Department of Botany, University of Glasgow, Glasgow, Scotland, U.K.
Theme 6. *Survey of leaf nodules*
G. Bond, Department of Botany, University of Glasgow, Glasgow, Scotland, U.K.
Theme 7. *Investigations on free-living nitrogen fixers*
E. G. Mulder, Laboratorium voor Microbiologie der Landbouwhogeschool, Hesselink van Suchtelenweg 4, Wageningen, Netherlands.
Theme 8. *World catalogue of Rhizobium collections*
O. N. Allen, University of Wisconsin, College of Agriculture, Madison, Wisconsin 53706, U.S.A.,
Eva Hamatova, Institute of Microbiology, Budejovicka 1083, Prague 4, Czechoslovakia.
Theme 9. *Quality of legume inoculants*
Theme 10. *Biochemistry of symbiotic nitrogen fixation, serology of* Rhizobium
Theme 11. *Genetics of* Rhizobium
Theme 12. *Ecology of* Rhizobium
Theme 13. *Miscellaneous*

3. THE ACETYLENE REDUCTION TECHNIQUE

Towards the end of the preparatory phase of the I.B.P. (1962–7), a discovery was made, not through any I.B.P. project, which had a tremendous impact on studies on biological nitrogen fixation during the operational phase of the Programme, and subsequently. This was the acetylene reduction technique which depends on the finding made by Schöllhorn & Burris (1966) in Wisconsin, and Dilworth (1966) in Perth, Australia, that the nitrogen-fixing enzyme complex, nitrogenase, reduced acetylene, and that the product was ethylene (Dilworth 1966). Thus, by adding acetylene to the test sample and measuring ethylene production, with appropriate controls, it was possible to measure for nitrogenase activity using this simple, highly sensitive, rapid and cheap assay. The reaction was shown to work with whole nodules in 1966 (Koch & Evans 1966), and after extensive field studies in which data were obtained for legumes, nodulated non-legumes, algae, lake samples and soil samples, Stewart, Fitzgerald & Burris (1967) advocated the assay for use in field studies, writing: 'The potential of the method for field investigations on N_2 fixation generally has not been appreciated by limnologists, marine biologists, and soil scientists' and reported that 'data obtained in experiments designed to test the feasibility of employing a simple method for measuring acetylene reduction as an index of N_2 fixation in the field illustrate that the method is practical and extremely sensitive.' Typical results obtained in these early studies are presented in figure 1. This assay was soon

taken up by many workers concerned with nitrogen fixation, it was used extensively in field and laboratory experiments during the I.B.P. and by 1972 over 200 papers using this method had been published (Hardy, Burns & Holsten 1973).

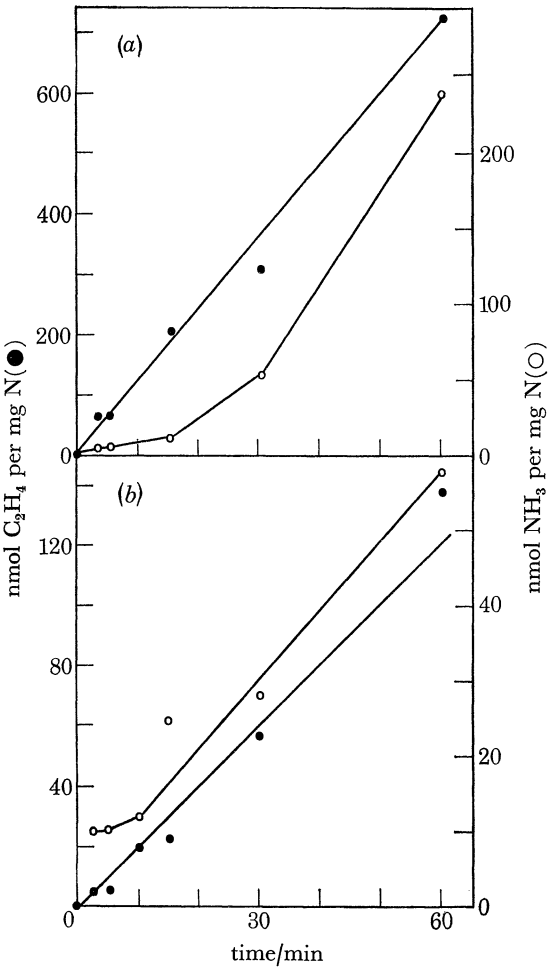

FIGURE 1. (*a*) Time course of acetylene reduction (●—●) and N_2 reduction (○—○) by *Alnus rugosa* root nodules. (*b*) Time course of acetylene reduction (●—●) and N_2 reduction (○—○) by soybean root nodules (from Stewart, Fitzgerald & Burris 1967).

4. THE NODULATED NON-LEGUMINOUS PLANTS

The nodulated non-leguminous plants considered in this theme are perennial angiosperms which bear N_2-fixing root nodules. The first evidence that such plants fixed N_2 was obtained by Hiltner (1896) who studied *Alnus glutinosa*, and since then, largely as a result of the work of Bond and his collaborators in Glasgow, and Quispel and Becking in Holland, detailed knowledge of the morphology, taxonomy, physiology and biochemistry of these N_2-fixing plants has become available (see Becking 1970*a*; Bond 1974; Quispel 1974).

The group is taxonomically and phylogenetically diverse, containing 13 N_2-fixing genera belonging to 8 families (table 2). Bond (1974) has stated: 'although some of the genera are closely related, the overall impression is one of disaffinity'. Except in the Elaeagnaceae,

nodule formation is not a family characteristic, and even within the one family, some genera, or even species, possess N_2-fixing root nodules whereas others do not. For example, in the Rosaceae *Dryas octopetala* has root nodules in North America (Lawrence 1958) whereas in Europe this species is not nodulated. Geographically, nodulated non-leguminous angiosperms are found on all five continents (table 3) and range in distribution from *Arctostaphylos uva-ursi* in the Arctic, to *Hippophaë rhamnoides* in temperate regions, to *Casuarina equisetifolia* in the tropics. Characteristically, they are pioneer plants of nitrogen-deficient habitats, ranging from the acid nutrient-poor bogland of the west of Scotland where *Myrica gale* is found, to the sand-dunes of tropical islands where *Casuarina equisetifolia* may occur.

TABLE 2. GENERA OF NON-LEGUMINOUS ANGIOSPERMS WITH NODULE-BEARING SPECIES, AND THEIR CLASSIFICATION ACCORDING TO ENGLER (1954)

genus	family	order
Casuarina Adans.	Casuarinaceae	Verticillatae
Myrica L.*	Myricaceae	Juglandales
Alnus Mill.	Betulaceae	Fagales
Dryas L.	Rosaceae (tribe Dryadeae)	Rosales
Cercocarpus Kunth.	Rosaceae (tribe Dryadeae)	Rosales
Purshia DC.	Rosaceae (tribe Dryadeae)	Rosales
Coriaria Hook.	Coriariaceae	Sapindales
Ceanothus L.	Rhamnaceae	Rhamnales
Discaria Hook.	Rhamnaceae	Rhamnales
Elaeagnus L.	Elaeagnaceae	Thymelaeales
Hippophaë L.	Elaeagnaceae	Thymelaeales
Shepherdia Nutt.	Elaeagnaceae	Thymelaeales
Arctostaphylos Adans.	Ericaceae	Ericales

* Includes *Comptonia* L'Herit. ex Ait. From Bond (1974).

TABLE 3. NUMBER OF SPECIES AND DISTRIBUTION OF NODULATED NON-LEGUMINOUS ANGIOSPERMS

genus	number of species in genus	present distribution
Casuarina	45	Australia, tropical Asia, Pacific islands
Myrica	35	many tropical, subtropical, and temperate regions
Alnus	35	Europe, Siberia, North America, Japan, Andes
Dryas	4	Arctic, mountains of north temperate zone
Cercocarpus	20	North America
Purshia	2	North America
Coriaria	15	Mediterranean, Japan, New Zealand, Chile, Mexico
Ceanothus	55	North America
Discaria	10	South America, New Zealand, Australia
Elaeagnus	45	Asia, Europe, North America
Hippophaë	3	Asia, Europe
Shepherdia	3	North America
Arctostaphylos	70	Northwest and Central America, Europe, Asia

From Bond (1974).

The root nodules of these plants are all perennial structures forming distinct coralloid masses surrounded by a thick cork covering except at the white actively growing apices. Some such as *Myrica gale* produce white, negatively geotropic nodule rootlets which arise from the nodule lobes (see Bond 1952). The zone of infection in these nodules is the cortex, and the endophytes

which appear, on the basis of their filamentous habit, size, and prokaryotic cell type, to be actinomycetes (Becking, De Boer & Houwink 1964; Gardner 1965; Gatner & Gardner 1970), have never been isolated with certainty since Koch's postulates have never been satisfied. Becking (1970*b*) classifies these actinomycetes in the genus *Frankia*. There is some indirect evidence, based on their high reducing activity (Akkermans 1971) and on the correlation with the nitrogenase activity of the nodules, that the vesicles in which the hyphae terminate are the loci of nitrogenase activity. Full details of these nodules are available in a recent review (Bond 1974).

TABLE 4. ISOTOPIC TESTS FOR FIXATION IN DETACHED *DRYAS* ROOT NODULES, WITH COMPARABLE DATA FOR OTHER ROOT NODULES

nodule material	N fraction analysed	total N in fraction (mg)	atom % excess ^{15}N present after exposure of nodules
Dryas drummondii	acid-soluble	1.3	2.899
	residual	5.1	0.255
Myrica cerifera	whole N of sample	5.1	1.020
Elaeagnus angustifolia	whole N of sample	3.6	0.637

From Lawrence *et al.* (1967).

The aim of the theme on N_2-fixing non-leguminous angiosperms was simple and exemplifies a type of project which can be carried out successfully on a collaborative basis. A circular was sent to botanists and allied workers in various parts of the world telling them about the plants and the root nodules, and inviting them to seek out any species of the genera listed in table 2, and also of related genera, growing in their neighbourhood, to inspect the roots for nodules, and to report the findings to the theme leader. In all, some 50 workers from 30 countries collaborated. The collection of this information required a good deal of effort, because the plants are characteristic of inhospitable habitats, and herbarium specimens often proved to be of limited value because taxonomists and systematists in general do not collect the root systems of their plants. The contributions of the individual participants will be recorded in the survey report of this theme (Bond 1975). In summary, the project was very successful, yielding much better information on the regularity of nodulation in these species already known to be capable of nodule formation, while nodulation was recorded in 36 additional species of the genera listed in table 2. Furthermore, nodulation was reported for the first time in the genera *Colletia* (Rhamnaceae) and *Rubus* (Rosaceae).

TABLE 5. DRY MASS AND NITROGEN DATA FOR *MYRICA FAYA* PLANTS GROWN IN NITROGEN-FREE CULTURE SOLUTION FOR SIX MONTHS

material	number of plants	dry mass of nodules (mg)	dry mass per plant (mg)	nitrogen content per plant (mg)	mg N fixed per g nodule dry mass
non-nodulated control plants	5	0	299 (232–353)	2.7 (2.6–2.9)	—
nodulated plants	11	329 (167–607)	4483 (1872–7661)	79 (31–143)	242 (146–415)

Note: Figures in parentheses indicate minimum and maximum values.
From Miguel & Rodriguez-Barrueco (1974).

In certain cases, the discovery of nodules was followed up by critical tests to establish N_2 fixation. Good examples of this were the demonstrations that *Dryas drummondii* (table 4), *Myrica faya* (table 5) and certain African species of *Myrica* (Grobbelaar, Strauss & Groenwald 1971) fixed nitrogen. Studies on the contribution which such nitrogen-fixing nodules make in the field were also carried out in some instances. For example, Stewart & Pearson (1967) showed that on the sand dune system at Gibraltar Point, Lincolnshire, 3–180 kg nitrogen ha^{-1} a^{-1} accumulated under N_2-fixing *Hippophaë rhamnoides* plants. Information of this type is not only of intrinsic scientific interest, but provides the rationale for growing such plants in nitrogen-deficient soils to improve soil fertility (Tarrant & Trappe 1971; Uemura 1971).

5. The blue-green algae

Blue-green algae were first shown to fix N_2 almost 50 years ago (Drewes 1928) and are unique among N_2-fixing micro-organisms in having a higher plant type of photosynthesis with water acting as a source of reductant and O_2 being evolved. Work during the period of I.B.P. was concerned with three main aspects: (1) identifying which blue-green algae fixed N_2; (2) studying the morphological, physiological and metabolic factors associated with N_2 fixation; (3) determining the ecological distribution of such N_2-fixing algae. Studies towards these ends were carried out within and outside I.B.P. in various laboratories throughout the world. In the United Kingdom workers in Dundee, Durham, Liverpool, London and Rothamsted all contributed.

At the start of I.B.P. 30 strains of blue-green algae were known to fix N_2 and all were characterized by the presence of peculiar thick-walled differentiated cells called heterocysts. Their function was unknown and Fritsch (1951) in his presidential address to the Linnean Society had aptly called such cells a 'botanical enigma'. Despite the lack of knowledge on their function, the fact that they were characteristic of N_2-fixing algae provided a simple method by which scientists, even in the remotest parts of the world, with no facilities other than a microscope and slide, could determine whether blue-green algae potentially capable of fixing N_2 were present in their study area. This, coupled with a literature survey of the occurrence of heterocystous algae, allowed the construction of distribution maps of potential N_2-fixing algae. The results showed that N_2-fixing algae were of world-wide distribution, but in terrestrial habitats were more important in extreme than in temperate ecosystems, and reached their maximum development relative to other plant species in polar and sub-polar regions (see Alexander 1975), and in some moist tropical areas (see Venkataraman 1973; Henriksson, Henriksson & DaSilva 1975). In aquatic systems they were common in mesotrophic and eutrophic freshwaters (see Fogg 1971) but were rare in marine habitats, apart from intertidal regions (Stewart 1975*b*) and in some oceanic waters where *Trichodesmium* predominates (see Bunt *et al.* 1973).

Although terrestrial blue-green algae are of worldwide distribution they are characteristic of certain habitats. Information accumulated in Scotland from a study of 12 types of habitat (table 6) sampled in various parts of the country showed, in general, that blue-green algae were usually present and often abundant in moist, non-acidic habitats where there was little competition with eukaryotic species. Indeed from a knowledge of soil type and climatic conditions, fairly accurate predictions can be made as to whether or not blue-green algae are likely to be important components of particular ecosystems.

During the period of I.B.P., attention was also focused on the possible role of the heterocyst

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in N_2-fixing organisms and, on the basis of indirect data, Fay, Stewart, Walsby & Fogg (1968) hypothesized that the heterocysts were the sites of N_2 fixation in the blue-green algae. Direct evidence that heterocysts fix nitrogen has since been obtained and there is now detailed physiological and biochemical knowledge available on the ways in which they perform this function (see Stewart 1973; Stewart, Rowell & Tel-Or 1975). Such studies led ultimately to the discovery of a whole new group of N_2-fixing algae, the non-heterocystous filamentous forms. The rationale which led to this discovery was that if heterocysts protected the nitrogenase from O_2-inactivation in aerobic cultures, it may be that under anaerobic or microaerobic conditions the vegetative cells of some algae may also fix N_2. This hypothesis was shown to be correct

TABLE 6. ABUNDANCE OF NITROGEN-FIXING BLUE-GREEN ALGAE IN SCOTTISH SOILS

habitat	no. of sites tested	% with N_2-fixing blue-green algae
arable soil	48	75
acid bogland	7	43
coniferous woodland	39	8
deciduous woodland	32	62
freshwater marshes	30	68
river banks	48	72
pasture	54	50
heath and moorland	33	3
rock outcrops	12	76
marine rocky shores	4	25
sand dunes	12	50
salt marshes	5	80

TABLE 7. BLUE-GREEN ALGAE KNOWN TO FIX NITROGEN AT START AND END OF I.B.P

	species and number†	
genus	start	end
Anabaena	10	12
Anabaenopsis	1	2
Aphanizomenon	0	2
Aulosira	1	1
Calothrix	4	4
Chlorogloea	1	1
Cylindrospermum	4	4
Fischerella	0	2
Hapalosiphon	0	1
Mastigocladus	1	1
Microchaete	0	2
Nostoc	6	10
Scytonema	0	2
Stigonema	1	1
Tolypothrix	1	1
Westiellopsis	0	1
Gloeocapsa	0	2
Lyngbya	0	2
Oscillatoria	0	5
Phormidium	0	1
Plectonema	0	6
total	30	63

† In addition field populations of *Trichodesmium* fix N_2.

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(Stewart & Lex 1970) and now about half of the non-heterocystous filamentous forms examined are known to fix nitrogen (Stewart 1971, unpublished; Kenyon, Rippka & Stanier 1972). Thus the species of blue-green algae known to fix N_2 increased markedly during I.B.P. as shown by the information given in table 7.

The amounts of N_2 fixed by blue-green algae range from a few kilograms per hectare per year in temperate regions (Stewart 1967, 1975*a*; Day, Harris, Dart & Van Berkum 1975) to over 100 kg ha^{-1} a^{-1} in some polar regions (see Alexander 1975) and in tropical rice paddy soils (Singh 1961; Venkataraman 1973). Henriksson *et al.* (1975) have stressed, however, that the blue-green algae fix N_2 as efficiently in temperate regions as in tropical regions, but in the tropics they are more plentiful. Details of the ecological importance of N_2-fixing blue-green algae have been presented at the I.B.P. symposia in Wageningen (see Lie & Mulder 1971) and Edinburgh (see Nutman 1975*a*; Stewart 1975*a*).

6. Free living heterotrophic bacteria

Since the discovery that *Clostridium* fixed nitrogen (Winogradsky 1893) there have been conflicting reports on the range of heterotrophic bacteria which fix nitrogen and on their contribution to soil fertility. An up-to-date list of known N_2-fixing bacteria is given by Mulder & Brotonegoro (1974) and during I.B.P. substantial knowledge has been accumulated on their role in natural ecosystems.

Table 8. Organic carbon, nitrogen, sulphur and phosphorus gained by the top 22.9 cm (9 in) of soil during reversion to wilderness at Rothamsted

		Broadbalk stubbed	Broadbalk wooded	Geescroft wooded
gained by soil since 1883‡†(kg ha^{-1})	org. C	45500	43100	20900
	org. N	3930	3670	1090
	org. S	560	560	300
	org. P	480	450	190
mean annual gain(kg ha^{-1} a^{-1})	org. C	560	530	250
	org. N	49	45	13
	org. S	6.9	6.9	3.7
	org. P	5.9	5.6	2.3
initial soil ratios‡	C/N	9.0	9.0	9.0
	S/N	0.16	0.16	0.13
	P/N	0.25	0.25	0.22
ratios in gain	C/N	11.6	11.7	19.2
	S/N	0.14	0.15	0.28
	P/N	0.12	0.12	0.17

† All gains calculated on an equivalent depth basis.
‡ For Broadbalk the initial status is taken to be that of Broadbalk plot 3, as sampled in 1881: for Geescroft that of Geescroft plots 3 + 4, as sampled in 1883.
From Jenkinson (1971).

There is now good evidence that in temperate regions nitrogen fixation by heterotrophic N_2-fixing bacteria is unimportant compared with other N_2-fixing groups. For example, Jenkinson (1971) presented data on the quantities of organic nitrogen which had accumulated in the 'wilderness' areas at Rothamsted, mainly in the absence of legumes since 1881 and

found, as shown in table 8, that the organic nitrogen gained per annum varied between 13 and 49 kg N ha^{-1}. Subsequently Frogatt *et al.* (1973) and Day *et al.* (1975) carried out acetylene reduction assays in various parts of these areas and concluded that free-living bacteria contributed only 2–3 kg N ha^{-1} a^{-1}, and that N_2-fixation by blue-green algae was relatively more important, contributing 7–26 kg ha^{-1} a^{-1} with as much as 1–2 kg N ha^{-1} d^{-1} accumulating on occasions after heavy rainfall. Their most consistent finding, however, was that nitrogenase activity was usually higher in the rhizosphere of higher plants than in soil cores, due presumably to the angiosperms providing the necessary energy substrate for heterotrophic growth. For example substantial activity was associated with *Nepeta glechoma* (ground ivy), *Stachys sylvatica* (hedge woundwort), *Heracleum sphondylium* (hogweed) and *Mercurialis perennis* (dog's mercury). Mean fixation rates for *Heracleum*, *Nepeta* and *Stachys* averaged just over 400 g N ha^{-1} d^{-1} (Day *et al.* 1975). The general conclusion from these and other data (see, for example, Brouzes, Mayfield & Knowles 1971) is that in temperate regions N_2 fixation by heterotrophic bacteria, either free-living or in associative symbioses is usually low.

TABLE 9. NITROGENASE ACTIVITY ON ROOTS AND IN RHIZOSPHERE SOIL OF TROPICAL FORAGE GRASSES IN BRAZIL†

plant species	no. of sites‡	nmol C_2H_4 per g dry roots per h	nmol C_2H_4 per g dry soil per h
Brachiaria mutica	6	156–730§	0
B. rugulosa	3	5–148	—
Hyparrhenia rufa	6	17–29	0–0.148
Digitaria decumbens	5	21–404§	0–0.349
Pennisetum purpureum	5	5–954§	0–0.085
Panicum maximum	5	20–299	0–0.148
Melinis minutiflora	3	13–41	0–0.187
Cynodon dactylon	2	17–269	0–0.068
Paspalum notatum	6	2–283	0–0.330

† Minimum and maximum mean values (6 root or 3 soil samples).
‡ Areas of approximately 10 m^2 were designated 'sites'.
§ These maximum values were obtained in experiments with high phosphorus fertilization.
From Dobereiner & Day (1975).

In the tropics, however, there is increasing evidence that N_2 fixation by bacteria, particularly those growing in associative symbioses may be very important. Over the past 20 years there have been reports of high rates of nitrogen accumulation in the absence of legumes in many tropical soils (Parker 1957; Jaiyebo & Moore 1963; Moore 1966; Dobereiner & Campelo 1971) but it has only been since the introduction of the acetylene reduction assay that it has been possible to investigate in any detail the contribution of biological N_2 fixation towards these gains. The latter studies have formed an important part of the I.B.P. (Rinaudo, Balandreau & Dommergues 1971; Dobereiner & Day 1975; Balandreau, Rinaudo, Fares-Hamad & Dommergues 1975). Since Dr Nutman's laboratory at Rothamsted has been, and still is, collaborating in the work which Dr Johanna Dobereiner is carrying out in Brazil it is appropriate to mention it at this symposium on the U.K. contribution to I.B.P. There have been various reports of N_2-fixing bacteria, usually species of *Azotobacter*, *Beijerinckia*, *Derxia*, *Klebsiella* and *Spirillum*, occurring in tropical soils. Since the introduction of the acetylene reduction technique, it has been shown that, as in temperate regions, highest activities are associated with the

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roots of higher plants. Tropical grasses with which high N_2 fixation rates are associated include *Panicum*, *Pennisetum*, *Paspalum*, as well as sugar cane. Typical results, obtained with Brazilian material, are shown in table 9.

The most studied association during I.B.P. was that between the N_2-fixing bacterium *Azotobacter paspali* and tetraploid cultivars of the tropical forage grass *Paspalum notatum*. This grass is very abundant in Brazil and its nitrogen nutrition is thus of considerable importance. In early studies it was shown that nitrogenase activity was intimately associated with the higher plant, there was little activity in the surrounding soil, and that the activity associated with the roots did not diminish after the roots were washed (Dobereiner, Day & Dart 1972). *Azotobacter paspali* was isolated consistently from such roots and typical data are presented in table 10. The results suggest that this association fixes 90 kg N ha^{-1} a^{-1}. More recently Dobereiner and co-workers have isolated from tropical Brazilian soils a very efficient N_2-fixing *Spirillum* which, in association with maize, may fix several hundred kg N ha^{-1} a^{-1} (Dobereiner, personal communication).

TABLE 10. NITROGENASE ACTIVITY OF THE *PASPALUM NOTATUM–AZOTOBACTER PASPALI* ASSOCIATION

	nmol C_2H_4 per g per h†	100 × no. *A. paspali* per g root	100 × no. *A. paspali* per g soil
roots + surface soil	6.44	204	298
rhizomes + surface soil	2.44	130	504
roots + rhizomes + surface soil	8.53	—	—
roots + rhizomes (washed)	7.60	136	—
root surface soil	0.180	115	226
rhizosphere soil	0.072	—	26

† Maximum rates obtained; mean of four replicate vials; assay P_{O_2}, 0.04 atm (4 kPa).
From Dobereiner, Day & Dart (1972).

The above results are particularly important, because synthetic nitrogen fertilizer is not readily available in most tropical areas, and tropical legume 'technology' is not as far advanced as that for temperate regions. It is interesting to consider the physiological and structural features which make such associative symbioses so successful. First, it is significant that all the tropical grasses (except rice) with which high rates of N_2 fixation are associated, have a C_4-dicarboxylic acid photosynthetic pathway (Hatch & Slack 1970). Thus, they do not waste CO_2, make very efficient use of high light intensities and temperatures and, unlike temperate C_3 plants, exude appreciable quantities of carbon from the roots which provides substrate for the heterotrophic bacteria. Some of these monocotyledons, e.g. *Digitaria decumbens* cultivar *transvala*, are also characterized by the presence of lacunae in their roots and it is in these spaces and associated cells that the bacteria appear to aggregate, receiving fixed carbon from the shoots and providing fixed nitrogen to them (J. Dobereiner, personal communication).

6. THE LEGUME-*RHIZOBIUM* ASSOCIATION

I.B.P. work on the legume-*Rhizobium* association was concerned with three main facets: (1) factorial field studies to measure the amounts of nitrogen fixed by legumes and to determine the nutritional and microbiological factors which promote nitrogen fixation; (2) studies on the

effects of the environment on symbiotic N_2 fixation; (3) the organization of rhizobial culture collections.

Workers in various countries and including developing as well as developed countries participated in the factorial experiments designed by Dr Nutman and Professor Vincent. The results obtained have been considered in detail by Nutman (1975*b*). The basic design of the experiment is shown in figure 2, although for various reasons not all workers in all countries included all 32 treatments. Such treatments involved rhizobial inoculation of the legume, using effective and ineffective strains, 4 levels of synthetic nitrogen fertilizer, and the inclusion of a non-nodulated grass in the series to evaluate uptake of nitrogen from the soil. The treatments were replicated with and without the addition of nutrients such as P, K, S, and sometimes lime, as required.

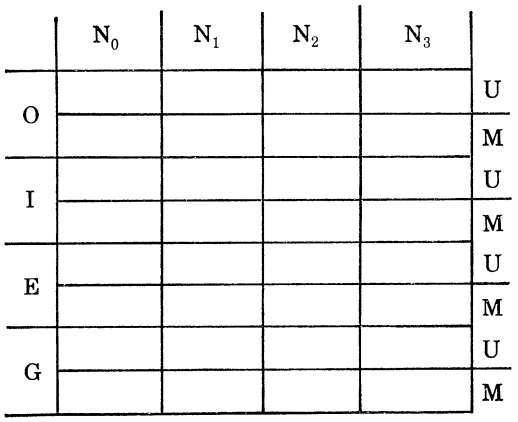

FIGURE 2. I.B.P. field experiment on legume nitrogen fixation. Treatments were as follows: O, uninoculated legume; I, legume inoculated with an ineffective (non-N_2-fixing) strain; E, legume inoculated with an effective strain; G, non-legume (grass or cereal); N_0–N_3, levels of nitrogenous fertilizer; M, non-nitrogenous fertilizer; U, no non-nitrogenous fertilizer.

Results obtained for lucerne and rye-grass grown under two extremes of soil type in the United Kingdom, the chalky loam of Stopsley near Rothamsted and the silty clayloam of Aberystwyth are presented in table 11. The Stopsley experiments were carried out by Dr Nutman and his colleagues; the Aberystwyth experiments by Mr W. Ellis Davies of the Welsh Plant Breeding Station, Aberystwyth. Many interesting points emerge from such studies. Among these, it can be seen that the crop yield obtained by using untreated lucerne is as good as that obtained with untreated rye-grass, that effectively nodulated lucerne can give a crop yield which is twice that obtained with untreated grass, and that if legumes are to be inoculated with rhizobia, it is important to ensure that effective rhizobial strains are used.

Other major findings are shown by the data in table 12 which were obtained for sites in Wales, France, England, Tunisia, Bulgaria and India. These show that uninoculated lucerne responds to the addition of synthetic nitrogen fertilizer whereas inoculated lucerne often does not, and that the nitrogen yield from inoculated plants without added nitrogen is usually as good as that obtained from uninoculated plants supplied with fertilizer nitrogen. That is, nodulated leguminous plants can often provide as much crop nitrogen as that supplied by synthetic nitrogen fertilizer to non-nodulated crops. Such experiments provide a most useful fund of basic and applied information on the role which N_2-fixing legumes can play in increasing crop yield and protein production in various parts of the world.

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It is obvious from field studies of the type just described that the environment has a large effect on the host physiology and on the crop yields obtained. Detailed studies on environmental effects were carried out in Theme 11, organized by Professor Pate and Dr Lie. The results of such findings have been presented at the Wageningen and Edinburgh symposia (see Lie & Mulder 1971; Nutman 1975*a*) and elsewhere (see, for example, Minchin & Pate 1973, 1974). As I could not do justice to the results obtained in this broadly based theme in a paper of the present type, I shall not attempt to do so and the reader should refer to the original publications.

TABLE 11. DATA ON THE NITROGEN YIELDS FOR LUCERNE AND RYE-GRASS OBTAINED AT STOPSLEY AND ABERYSTWYTH AT THE END OF THE FIRST YEAR OF THE I.B.P. EXPERIMENT

place, year				Stopsley, 1969		Aberystwyth, 1969
climate†				203 mm, 17.7 °C		409 mm, 13.8 °C
previous cropping				pasture/arable		(+100 kg K ha^{-1})
soil type, pH				chalky loam, 7.5		silt clay loam, *ca.* 5.0
soil N (%)				0.64		—
fertilizers(kg N ha^{-1})				99–297 (+P, K)		168–673 (+Ca, P, K)
R. meliloti per g soil				10–10^2		none
				effects	N yield kg N ha^{-1}	effects	N yield kg N ha^{-1}
O	.			.	125	.	105
	N			+		0	
	M			+ +	164	+ +	229
	N × M			−		−	
I	.			0	124	−	70
	N			+		+ +	
	M			0		+ +	
	N × M			0		−	
E	.			+	144	+ +	248
	N			+		−	
	M			+ +	179	+ +	316
	N × M			0		− −	
G	.			.	85	.	113
	N			+		+ +	
	M			+ +		0	
	N × M			0		0	

N, nitrogenous fertilizer; M, other fertilizers (lime, P, K, etc.); ., no treatment; +, increase with respect to control > standard error; + +, increase with respect to control > twice standard error; −, decrease with respect to control > standard error; − −, decrease with respect to control > twice standard error; 0, no effect. For O, I, E, G, see legend to figure 2.

† Rainfall and mean air temperature for May–August.

From Nutman (1975*b*).

Finally, in relation to studies on the legume-*Rhizobium* association, the I.B.P. was involved in a project on Rhizobial Culture Collections. There is now good evidence that in the legume-*Rhizobium* association, the nitrogenase is coded for by the prokaryotic genome and that the bacterium also synthesizes the enzyme (Pagan, Child, Scowcroft & Gibson 1975). Despite the tremendous role which rhizobia thus play, there was, until the I.B.P., little information readily available on the rhizobial culture collections dispersed throughout the world and there was no unified effort to ensure the perpetuity of particular collections when their curators retired or

moved occupations. The objective of Theme 13 was to bring together information on various culture collections throughout the world. The theme leaders, Dr O. N. Allen of the University of Wisconsin and Dr E. Hamatova of Prague, collaborated with Dr F. Skinner of Rothamsted, in compiling the I.B.P. Catalogue of *Rhizobium* culture collections (Skinner 1973). This is providing a focal point for the international effort now being directed towards ensuring that valuable cultures of rhizobia are not lost, and that due attention is given to the maintenance

TABLE 12. FIRST YEAR RESPONSE OF LUCERNE TO NITROGENOUS FERTILIZER, EITHER WHEN UNINOCULATED AND WITHOUT LIME, P, K, ETC., OR WHEN INOCULATED WITH AN EFFECTIVE STRAIN OF *RHIZOBIUM* AND GIVEN LIME, P, K, ETC.

treatment	site	rates of application of fertilizer, kg N ha^{-1}			uptake in lucerne, kg N ha^{-1}			
		N_1	N_2	N_3	N_0	N_1	N_2	N_3
uninoculated and without lime, P, K, etc.	Aberystwyth	168	336	673	105	115	119	191
	Dijon	.	.	.	.	.	.	.
	Shardlow	33	66	99	207	251	241	236
	Rothamsted B	66	132	198	73	134	203	245
	Rothamsted A	66	132	198	106	81	152	156
	Stopsley	99	198	297	125	140	177	196
	Woburn D	99	198	297	58	119	111	172
	Woburn C	66	132	198	104	124	89	119
	Trustenik	36	72	144	117	106	134	170
	Sekirovo	36	72	144	50	54	108	128
	Bozhourishte	33	66	99	95	100	111	101
	New Dehli	10	20	30	44	40	49	48
	Ksar Gheris	99	.	.	9	105	.	.
effective inoculation and with lime, P, K, etc.	Aberystwyth	168	336	673	316	344	351	309
	Dijon	.	.	.	227	265	320	284
	Shardlow	33	66	99	228	251	284	268
	Rothamsted B	66	132	198	237	251	274	266
	Rothamsted A	66	132	198	260	225	252	231
	Stopsley	99	198	297	178	193	192	228
	Woburn D	99	198	297	145	207	177	191
	Woburn C	66	132	198	187	205	176	187
	Trustenik	36	772	144	119	107	108	132
	Sekirova	36	72	144	51	51	114	140
	Bozhourishte	33	66	99	96	96	88	82
	New Delhi	10	20	30	58	63	65	69
	Ksar Gheris	99	.	.	10	10	.	.

From Nutman (1975*b*).

of such important organisms. Indeed almost six months ago, to the day, in the rooms of the Linnean Society at Burlington House, a meeting of international experts was convened, under the auspices of the United Nations Environmental Programme, to plan a course of action to safeguard the world's rhizobial strains. Among the conclusions was one that Dr Skerman in Australia and Dr Skinner in Rothamsted draw up a plan for a continually updated *Rhizobium* catalogue and for the transfer of responsibility for this from the I.B.P. to the World Data Center (Anonymous 1975). This emphasizes how important a role the I.B.P. rhizobium work has been and how the role of the British contribution in this connection has been recognized.

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Conclusion

Work on N_2 fixation during the I.B.P. proceeded on a very broad base, and thus it has been impossible, in a paper of this type, to give anything other than a resumé of some of the activities. In so doing, I have inevitably had to omit many major and minor findings, and perhaps have done scant justice even to those which I have mentioned. However the work on N_2 fixation carried out during the I.B.P. and other recent N_2 fixation studies have been presented in two volumes which report the proceedings of the final I.B.P. synthesis meeting on N_2 fixation, held in Edinburgh, in 1973 (Nutman 1975*a*; Stewart 1975*a*). Studies on N_2 fixation have benefited, in my opinion, from the inclusion of the topic as a main project in the Production Processes section. It is to be hoped, likewise, that those responsible for its inclusion feel in retrospect that their choice was a good one. Certainly, for the first time there is evidence, obtained by international collaboration, in which United Kingdom participants played a full role, on the quantities of nitrogen which may be fixed biologically in various parts of the world, and by which organisms. These findings are summarized in figure 3. It is up to post-I.B.P. workers to exploit these well-established N_2-fixing systems to advantage, because it is likely that in the foreseeable future they will continue to supply the bulk of the combined nitrogen added each year to the surface of the Earth.

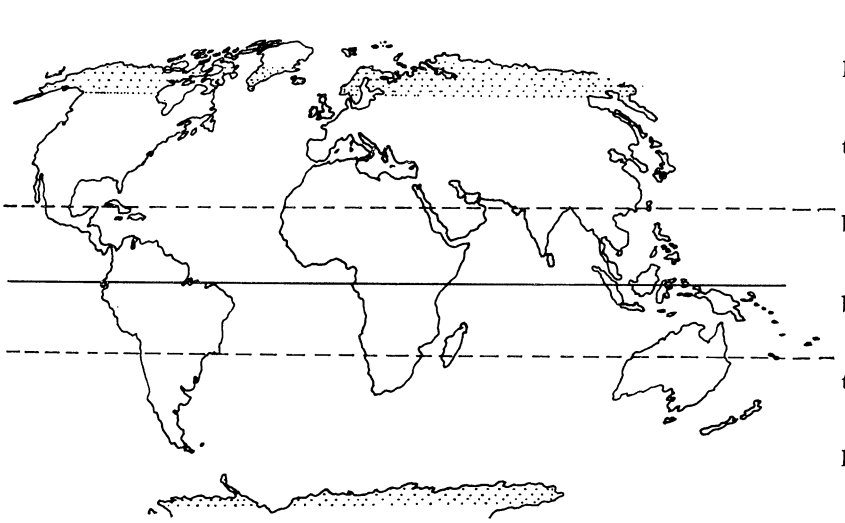

Figure 3. Quantitative significance of various biological N_2-fixing systems and the areas of the world where they appear to be most important.

In preparing this paper, I have had most useful discussions with Professor G. Bond, F.R.S., and Dr P. S. Nutman, F.R.S. I also thank Professor G. E. Fogg, F.R.S., for his help in connection with my own studies of N_2 fixation during I.B.P. The help of Mr B. J. Harbott and Miss Gail Alexander with some of these studies is also acknowledged, and I thank the various workers who have allowed me to use figures, tables, etc., from their original publications.

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Discussion

P. S. Nutman, F.R.S. (*Soil microbiology Department, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ*)

I should first like to underline the point made by Professor Stewart in his summary that the large I.B.P. effort in N-fixation coincided with important technical advances in assaying nitrogenase and a growing awareness of the world population/food/energy problem. I think one could also make the point that at the same time considerable advances were being made in the peripheral biochemistry of N-fixation, in general microbial genetics and later in the genetics of N-fixing systems; some of this research continues actively in many centres and at all levels.

Concomitantly with the I.B.P. studies but owing nothing directly to them has also been the recent painstaking unravelling of the physical chemistry of the reduction of dinitrogen by metal complexes which will in its turn interact with research over a wide area. The point I wish to make is that the I.B.P. N-fixation programmes are notable strands in a network of effort.

Now that the surveys and experiments are done and everyone (or nearly everyone) is busy computing, collating and writing, one should perhaps also point out where findings may fall short of expectations. I do not refer to programmes mentioned by Professor Stewart that have not 'taken off' but those that will appear in the final reports. It is the hope of the I.B.P. hierarchy and relevant committees that each of the 35-odd volumes of results shall be a *synthesis* of its own field. I cannot speak for the other sections, but for N-fixation this requirement will not be met in full and much of the material will be interim or even tentative. This is a consequence of the almost explosively rapid progress that is being made. Letters and articles on N-fixation appear in almost every number of *Nature*, so that it is impossible to pause and take stock without being out of date. One can illustrate this by reference to the acetylene reduction work. In spite of its enormous use and value, it is unfortunate that those working on N-fixation have not all been prepared to do what was originally recommended (Stewart, Fitzgerald & Burris 1967), i.e. 'to refer routinely to a primary standard ($^{15}N_2$-test), rather than to rely entirely upon an indirect secondary standard, attractive though it may be' and thus some of the data obtained using it are of doubtful validity. One final point on the energy/protein equation; I would like to assert, perhaps somewhat provocatively, that *there is no N problem*. The element is superabundant and a plethora of systems are at hand for its incorporation into living material using either energy inputs from fossil sources or from current photosynthesis. The question is only one of ways and means; what strategies to employ in particular situations in relation to man and the biosphere and matters of land use and agricultural development, economics, etc.

For this we will need to know very much more about the functioning of the processes of fixation in natural ecosystems and in agriculture, especially in the tropics, not neglecting those systems already known to make the most substantial contributions of biologically fixed N. These are certainly widely underexploited and also still amenable to large improvement.

G. E. Fogg, F.R.S. (*The Marine Science Laboratories, Menai Bridge, Anglesey, Gwynedd*)

It was fortunate that the I.B.P. coincided with a phase of active development in the biochemistry and physiology of nitrogen fixation. In particular, the introduction of the acetylene-

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reduction technique for the estimation of nitrogen fixation in the field resulted in a wealth of results far beyond anything that one envisaged at the outset of the I.B.P. We were also particularly fortunate in having several exceptionally vigorous workers in this field as team leaders. There can be no doubt that the U.K. has made a most valuable contribution to the I.B.P. programme on nitrogen fixation. In criticism it can only be said that it is a pity that there was not more contact between P.P. and P.M. The extent of our knowledge of nitrogen fixation in marine habitats is still meagre although it is becoming increasingly evident that it is combined nitrogen which most often limits production in the sea.

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