Nitrogen fixation in legume root nodules: biochemical studies with soybean

BY F. J. BERGersen
Division of Plant Industry, C.S.I.R.O., Canberra, Australia

[Plate 66]

It is now clear from studies with soybean root nodules that the nitrogen fixing activity resides in the bacteroids which are the symbiotic form of the root nodule bacteria. These develop as a result of a complex series of changes in metabolism and structure which occur in the bacteria during the final stages of growth within membrane-enclosed vesicles in the host cytoplasm. Nitrogenase appears when these changes are complete.

The primary product of nitrogen fixation is NH₃, which in intact nodules, is rapidly transformed into α-amino compounds which are used by the host plant. In suspensions of bacteroids and in cell-free extracts prepared from them, the reaction terminates in NH₃, which is released into the medium. Free O₂, which is required for the production of energy for nitrogen fixation by nodules and by bacteroid suspensions, also causes inactivation of the nitrogen fixing system and exerts important kinetic influences upon the reaction. Reducing power and energy for the reduction of N₂ to NH₃ is provided by a photosynthetic product from the host in nodules; in bacteroid suspensions, a substrate such as succinate is required. In cell-free extracts, requirements for energy and reductant are met by ATP and dithionite. The natural reductant has not yet been identified.

A schematic representation of various factors which affect nitrogen fixation in nodules, bacteroid suspensions and cell-free extracts is presented.

1. INTRODUCTION

It is seldom disputed that fixation of atmospheric N₂ by nodulated legumes is the greatest single source for the replenishment of nitrogen in the soil-plant-animal systems of the earth. It is, therefore, somewhat of a paradox that the study of the biochemistry of this symbiotic system has lagged behind studies of the economically less-important, free-living nitrogen-fixing bacteria. There are several reasons for this; first, it is convenient for biochemists to use relatively easily grown mass-cultures of bacteria. The need for quite extensive plant-growing facilities to produce similar yields of nitrogen-fixing material from nodules, restricted work to those laboratories with access to these facilities. Secondly, there are intrinsic difficulties in working with the nodule system, which is susceptible to inactivation at many points during preparation. In addition, the fact that the products of the reaction are freely soluble and not associated with any nodule components, delayed identification of the bacteroids as the nitrogen-fixing agents and led to hypotheses which have now been abandoned (e.g. Bergersen 1960, 1966a). Following the demonstration of the bacteroid as the nitrogen fixing agent (Bergersen & Turner 1967), work with cell-free extracts has drawn heavily upon previous experience with extracts prepared from Clostridium and Azotobacter. Thus within 2 years of the first reports of nitrogen fixation by disrupted nodule preparations (Bergersen 1966b, c; Klucas & Burris 1967), many of the properties of the nodule nitrogen-
fixing enzyme complex (nitrogenase) have been elucidated (Koch, Evans & Russell 1967a, b; Klucas, Koch, Evans & Russell 1968; Bergersen & Turner 1968).

For reasons of experimental convenience, nodules of the soybean (Glycine max (L.) Merrill) have been mainly used for studies of nodule biochemistry; information from nodules of other leguminous species is limited. Soybean nodules are approximately spherical in shape and consist of a mass of central tissue surrounded by a cortex. The cortex is traversed by a number of vascular elements which fuse near the base and become linked to the central stele of the root bearing the nodule. Nitrogen fixation occurs in the cells of the central tissue. In each nodule this central tissue is composed of several thousand host cells, each of which contains about 10000 fixing units consisting of 4 to 6 bacteroids enclosed, together with a solution of the haemoprotein leghaemoglobin, in a membrane envelope of host-cell origin (Bergersen & Briggs 1958; Goodchild & Bergersen 1966).

Leghaemoglobin is invariably associated with nitrogen fixing nodules in legumes and has been used as an index of fixation potential (Virtanen 1955). This is due to the pigment being present in fairly constant cellular concentration within the nitrogen-fixing cells of nodules. Its nodular concentration is therefore an index of the amount of nitrogen-fixing tissue which is present (Bergersen 1961). This correlation between nitrogen fixed and leghaemoglobin concentration was the basis for several attempts to implicate this haemoprotein in a direct role in the nitrogen-fixing process (e.g. Winfield 1955; Bauer & Mortimer 1960). Recent work has shown that its role is an auxiliary one because bacteroids which have been washed free of leghaemoglobin are still capable of nitrogen fixation (Bergersen & Turner 1967). The role of this haemoprotein in nodules will be discussed in the section dealing with the $O_2$ requirement of nodules.

The following is an account of the present knowledge of the biochemistry of nitrogen fixation in nodules, gathered from experiments in which intact soybean nodules, bacteroid suspensions and cell-free extracts have been used. It will be seen that the symbiotic system is essentially similar to that of free-living, nitrogen-fixing bacteria such as Clostridium pasteurianum and Azotobacter vinelandii. However there are some distinguishing features, some of which may be associated with the unique environment in the plant cell within which the reactions occur.

2. Bacteroids

In free-living, nitrogen-fixing bacteria, nitrogenase appears as an adaptive enzyme. This was shown by Pengra & Wilson (1958) with Klebsiella pneumoniae (formerly regarded as Aerobacter aerogenes) in which anaerobic growth was arrested for several hours following the exhaustion of $NH_4^+$, before it resumed after the assimilation of $N_2$ began. Similar results have been obtained with other genera and in some, shorter adaptation periods of less than 1 h have been observed. There is no available information about other changes in metabolism which may accompany adaptive production of nitrogenase in these organisms.
Electron micrographs of thin sections of growing and bacteroid forms of soybean nodule bacteria.

**Figure 33.** Growing nodule bacteria within an infection thread in a young soybean nodule. (c) Bacterial cell wall, (f) nuclear filaments, (r) ribosomes. The scale shows 1 μm.

**Figure 34.** A bacteroid isolated from a mature soybean nodule. (c) Bacteroid wall, (m) protoplast membrane, (f) nuclear filaments, (g) dense granules and (p) polar granules of polymer. Ribosomes are almost completely absent. The scale shows 1 μm.

*(Facing p. 403)*
Nitrogen fixation in legume root nodules

While soybean nodules are developing, the bacteria have the normal appearance and other properties associated with vegetative cells grown in artificial culture. During the last stages of development within the membrane envelopes in the host cell (Goodchild & Bergersen 1966) the bacteria attain their final form and develop the special properties which distinguish the so-called 'bacteroids' from the vegetative cells from which they are derived. The changes which take place are profound, involving much more than the adaptive acquisition of the enzyme complex, nitrogenase, and they coincide with the appearance of leghaemoglobin in the nodule cells.

In many legumes, gross changes in morphology distinguish the bacteroids from cultured cells, but in soybean there are only slight changes in the appearance of the nuclear material and in cell size. Ribosomes, which are prominent in vegetative cells (figure 33, plate 66), disappear from bacteroids (figure 34, plate 66) which also lose their ability to grow on ordinary culture media (Bergersen 1968; cf. Almon 1933).

Table 17. Cytochromes and co-reactive pigments of *Rhizobium japonicum* (CC705)

(From the data of Appleby 1968 a, b.)

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Cultured Cells</th>
<th>Bacteroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cytochromes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.51</td>
<td>0.94</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>0.45</td>
<td>0.42</td>
</tr>
<tr>
<td>Cytochromes aa₃</td>
<td>0.15</td>
<td>0</td>
</tr>
<tr>
<td>CO-reactive pigments:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome a₃</td>
<td>0.09</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome o</td>
<td>+ *</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c (552)</td>
<td>0</td>
<td>0.24</td>
</tr>
<tr>
<td>P-450</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td>P-428</td>
<td>+ *</td>
<td>+ *</td>
</tr>
<tr>
<td>P-420</td>
<td>0</td>
<td>+ + *</td>
</tr>
<tr>
<td>Rhizobium-haemoglobin</td>
<td>+ *</td>
<td>0</td>
</tr>
</tbody>
</table>

* +, ++ denote presence at estimated low or high concentration, of pigments whose absorption bands are partly or completely obscured by those of other pigments.

Appleby (1968 a, b) has studied the differences between cytochromes and related haemoproteins of cultured cells and of bacteroids of the soybean nodule bacteria (*Rhizobium japonicum*). His results are summarized in table 17, from which it is seen that cytochrome aa₃, cytochrome o and rhizobium-haemoglobin are exclusively present in cultured cells, whereas cytochrome c (552), P-450 and P-420 are exclusively present in bacteroids. P-428, which is present in both types of cells, and bacteroid P-420 are apparently not identical with cytochromes aa₃, cytochrome o, catalase or peroxidase; they were identified from the adsorption spectra.
of their CO-complexes. Rhizobium-haemoglobin is not an oxidase and is antigenically unrelated to leghaemoglobin. These results are sufficient to indicate the extent of changes which occur in the electron transport system, and which accompany the acquisition of nitrogen fixing ability by bacteroids.

Some of the morphological features of bacteroids can be duplicated experimentally (Jordan 1962) and some of the changes in cytochrome pattern can be approached when cultures are grown at low oxygen concentrations (Appleby & Bergersen 1958). However, duplication of other changes which occur during bacteroid development have not been attained and only natural bacteroids fix nitrogen. It is possible that the host cell may be involved in the transfer to the bacteria of genetic information, or in the alteration of the expression of information in the genome of the bacteria, which results in the initiation of the above changes. In addition, the host cell provides an environment within which the changes can be induced and in which the new enzyme can function.

3. Methods

(a) Intact nodules

Experiments with nitrogen fixation by intact detached soybean nodules began with the work of Aprison & Burris (1952). These authors used nodules which had been quickly detached from active plants and exposed to atmospheres containing $^{15}$N$_2$. After a period of incubation, the isotopic label was located in the non-protein nitrogen of the nodules. These methods are still used, together with mass-spectrometer gas analysis for studies of hydrogen evolution and exchange as these are related to nitrogen fixation. Reduction of acetylene to ethylene by nitrogen-fixing systems has also been used in studies with nodules and extracts in place of the isotopic method (Koch & Evans 1966; Koch et al. 1967a).

(b) Bacteroid suspensions

Attempts to obtain nitrogen fixation by disrupted nodule preparations were unsuccessful (Delwiche 1966) until it was shown that it was not only essential to preserve anaerobic conditions during preparation, but also that O$_2$ was required in order to detect nitrogen fixation in nodule breis (Bergersen 1966b, c). Later experiments showed that nitrogen fixing bacteroid suspensions could be prepared from the crude breis by centrifugation under argon (Bergersen & Turner 1967). Koch et al. (1967a) used an argon-filled glove bag in which to perform the anaerobic steps of the preparation of active bacteroid suspensions. Bergersen (1966c) used a stainless-steel screw-press which could be flushed with argon and all manipulations were done under streams of argon.

At least a part of the damaging effects of O$_2$ arises from the presence in the nodule sap of phenolic compounds and polyphenol oxidases. The effects of these can be minimized by removal of the phenolics by adsorption on polyvinyl-pyrrolidone (PVP) in the presence of ascorbate. Koch et al. (1967a) added a particulate
Nitrogen fixation in legume root nodules

PVP to nodule macerates in phosphate buffer containing ascorbate and Mg$^{2+}$ and after 10 min, squeezed the macerate through 100-mesh bolting cloth to produce a brei from which the bacteroids were centrifuged. Bergersen & Turner (1968) used phosphate buffer containing a soluble PVP, ascorbate and Mg$^{2+}$, as the medium in which the nodules were crushed in their press. After centrifuging, the PVP was discarded with the washings when the bacteroids were resuspended and again centrifuged in several changes of buffer containing 0·3 m sucrose. Bacteroids prepared in this way were able to fix nitrogen at rates which were approximately equivalent to rates of fixation calculated for bacteroids in intact nodules.

Following incubation in the presence of $^{15}$N$_2$ and O$_2$ the fixed nitrogen appeared in the medium as $^{15}$NH$_4^+$ and was analysed by standard methods. As is the case with other nitrogen-fixing preparations (Bulen, Burns & LeComte 1965), nitrogen fixation by bacteroids was inhibited at high phosphate concentrations (figure 28). Other features of the activities of bacteroid suspensions will be discussed under other headings.

**Figure 28.** The effect of phosphate concentration upon nitrogen fixation by a bacteroid suspension. Vessels contained, in a total volume of 6·0 ml., 21 mg of bacteroid protein, 100 μmoles of succinate, 1 mm Mg$^{2+}$ and KH$_2$PO$_4$ (pH 7·0) to final concentrations of ● 0·025 M, ○ 0·05 M, ◦ 0·10 M and + 0·15 M. Incubated at 25 °C with shaking. Gas phase contained O$_2$—42 mm Hg and $^{15}$N$_2$ (79 atoms %)—70 mm Hg with argon to 700 mm Hg. Nitrogen fixation measured as atoms % excess $^{15}$N$_2$ in NH$_4^+$ after addition of 100 μg carrier NH$_4^+$ N to 1 ml. samples withdrawn from the vessels at intervals.

(c) Cell-free extracts

Cell-free extracts of bacteroids used for nitrogen-fixation studies, have been prepared by breakage in pressure-release cells and collection of the broken bacteroids under argon. Koch *et al.* (1967a) used a French press, with a pressure of 24 tons/in.$^2$. In a later paper (Koch *et al.* 1967b) these authors described
the removal of small molecules including endogenous NH$_4^+$ (after clarification of the extracts by centrifugation at 34000 g) by chromatography on polyacrylamide gel. This treatment permitted the use of standard nitrogen analysis without the use of $^{15}$N. Bergersen & Turner (1968) continued to use $^{15}$N$_2$ for experiments with cell-free extracts prepared from bacteroids broken in a modified French press, using a pressure of 8 tons/in.$^2$. The extracts were then clarified by centrifugation for 30 min. at 100000 g and the clear, brownish-yellow supernatant contained the nitrogen-fixing activity. This activity was sensitive to storage at 0 °C and for this reason all manipulations following breakage of the bacteroids were done at 20 to 23 °C.

Klucas et al. (1968) have recently separated the extracts into two components, neither of which alone fixed nitrogen but which regained activity following recombination of the components. These results are similar to those of Bulen & LeComte (1966) with Azotobacter vinelandii and of Mortenson, Morris & Jeng (1967) with Clostridium pasteurianum.

4. The primary product is ammonia

Study of the products of nitrogen fixation in soybean nodules followed immediately upon the success of the initial experiments with detached nodules (Aprison & Burris 1952). Aprison, Magee & Burris (1954) studied the distribution of $^{15}$N among the components of the non-protein nitrogen of nodules which had been exposed to $^{15}$N$_2$ for 1 h. It was found that glutamic acid contained the most $^{15}$N, followed by serine, threonine or asparagine and ammonia. The distribution was consistent with the entry of the fixed nitrogen by way of ammonia but there was presumed to be a comparatively large amount of ammonia in the nodules, which was not in equilibrium with the fixed ammonia. This resulted in relatively low labelling of the total nodule ammonia. With improved $^{15}$N techniques, Bergersen (1965) was able to use much shorter exposures of nodules to $^{15}$N$_2$ and demonstrated that, after 1 min, an average of 94 % of the fixed nitrogen of the soluble portion of the nodules could be recovered as ammonia by a method which gave minimal hydrolysis of amides. This percentage fell rapidly with time as ammonia was incorporated into $\alpha$-amino compounds. Equilibrium was reached after about 10 min, when fixed nitrogen in ammonia accounted for about 20 % of the total fixed nitrogen. Qualitatively similar results were obtained with nodules of Ornithopus sativa by Kennedy (1966a, b), who also identified glutamic acid and glutamine as the primary amino compounds in these nodules.

In nodule breis (Bergersen 1966c), bacteroid suspensions (Bergersen & Turner 1968) and cell-free extracts (Koch et al. 1967a, b; Bergersen & Turner 1968), the nitrogen-fixation process terminated in ammonia which was released into the medium. No other products in solution have been detected. Slight incorporation of this ammonia into $\alpha$-amino compounds within the bacteroids, has been detected during declining nitrogen fixation by bacteroids following 30 min incubation.
The extent of this incorporation was slight, amounting to only 7 to 10% of the fixed nitrogen found as soluble ammonia (F. J. Bergersen & W. B. Sylvester, unpublished). Apart from this small incorporation, none of the aminating reactions, which are so rapid in intact nodules, operate in the conditions which are imposed in experiments with breis, bacteroids or cell-free extracts.

Nitrogen fixation by bacteroid suspensions was relatively insensitive to $\text{NH}_4^+$ in the medium, being unaffected at 1 mm (table 18). Moreover, the inhibition of $\text{H}_2$ evolution which accompanied declining nitrogen fixation at higher ammonia concentrations, suggests that the inhibition which occurred was not end-product inhibition, which would be expected to result in increased $\text{H}_2$ evolution. This lack of end-product inhibition may be regarded as evidence for the lack of reversibility of the final step in nitrogen fixation.

### Table 18. The effect of $\text{NH}_4^+$ upon bacteroids

<table>
<thead>
<tr>
<th>$\text{NH}_4^+$ concentration (mm)</th>
<th>$\text{N}_2$ fixation (ng N(mg protein)$^{-1}$ h$^{-1}$)</th>
<th>$\text{H}_2$ evolution (atm × 10$^{-4}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1103</td>
<td>3.7</td>
</tr>
<tr>
<td>1</td>
<td>1152</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>1041</td>
<td>2.1</td>
</tr>
<tr>
<td>100</td>
<td>698</td>
<td>1.7</td>
</tr>
</tbody>
</table>

5. The energy and reductant requirements

(a) The oxygen requirement of intact nodules

Detached nodules require $\text{O}_2$ in order for nitrogen fixation to proceed and in soybean nodules, none occurs below a $p\text{O}_2$ of about 0.05 atm. Fixation has been found to increase with increasing $p\text{O}_2$ up to about 0.50 atm $\text{O}_2$ (Burris, Magee & Bach 1955; Bergersen 1962a). The most obvious explanation of this in the light of recent work is that substrates are oxidized and that oxidative phosphorylation provides ATP as an energy source for the reactions of nitrogen fixation. There are probably accessory roles of $\text{O}_2$, associated with the partial oxidation of some carbon substrates (Bergersen 1958) which may provide acceptors for the synthesis of amino acids from the newly fixed nitrogen. In the range of $p\text{O}_2$ from 0.05 to 0.5 atm, increasing $p\text{O}_2$ increased $V_{\text{max}}$, as well as $v$ in the Michaelis equation, indicating that $\text{O}_2$ provided something which was directly limiting in the nitrogen fixation reaction (Bergersen 1962a).

The nitrogen-fixing tissue in nodules is dense and ventilation of the energy-producing sites is possibly assisted by the leghaemoglobin solution which surrounds the bacteroids. This suggestion (Yocum 1964) followed from the work of Scholander
1966) who showed that \( \text{O}_2 \) was transported, at low concentration gradients, up to eight times more rapidly through haemoglobin solutions than through water. If this is the role of leghaemoglobin, quite high fluxes of \( \text{O}_2 \) must be maintained without significant oxygenation, because the pigment is predominately in the reduced, non-oxygenated form in the intact tissue (Bergersen 1966b).

The effects of \( p\text{O}_2 \) higher than 0.50 atm will be discussed in the sections dealing with electron transport pathways and kinetic measurements.

**(b) The oxygen requirement of bacteroid suspensions**

Suspensions of bacteroids also require \( \text{O}_2 \) in order to fix nitrogen. Provided they had been carefully prepared in media of relatively high osmotic pressure, fixation proceeded with no other additions but a gas mixture containing \( \text{O}_2 \) and \( \text{N}_2 \) (Bergersen & Turner 1967). The endogenous substrates which provide the energy for oxidative phosphorylation will be discussed later in this section. With increasing \( p\text{O}_2 \) above 0.03 to 0.06 atm, doubling the \( p\text{O}_2 \) more than doubled initial nitrogen fixation rates but decreased the period for which the reaction proceeded because the system was inactivated by \( \text{O}_2 \) (Bergersen & Turner 1967). Attempts to supply exogenous \( \text{ATP} \) from a generating system at low \( p\text{O}_2 \), when endogenous energy supply would be expected to be low, produced consistent but minor stimulation of fixation in nodule breis. The use of 2,4-dinitrophenol as an uncoupling reagent suggested that oxidative phosphorylation was involved in the supply of \( \text{ATP} \), (Bergersen 1966c).

**(c) The ATP requirement of bacteroid extracts**

The addition of adenosine-5-triphosphate (\( \text{ATP} \)) was found to be an absolute requirement for nitrogen fixation by cell-free extracts of bacteroids (Koch *et al.* 1967a, b; Bergersen & Turner 1968) and the reaction was prolonged when an \( \text{ATP} \) generating system such as creatine phosphate-creatine kinase was used. In experiments using \( \text{ATP} \) without the generating system, there was a sharp inhibition of nitrogen fixation at \( \text{ATP} \) concentrations above 2 \( \mu \)moles/ml. (6 \( \mu \)moles vessel; figure 29a) at which concentration the reaction was sustained for only a few minutes (figure 29b). The initial rate of nitrogen fixation with \( \text{ATP} \) alone was similar to the more sustained rates obtained with the \( \text{ATP} \) generating system.

**(d) Intact nodules and photosynthesate**

Nodules have a requirement for a product or products of photosynthesis for nitrogen fixation. This was shown by experiments such as those of Virtanen Moisio & Burris (1955) in which it was found that nodules detached from pea plants which had been kept in the dark for 24 h fixed only about 6% of the nitrogen fixed by nodules from plants illuminated at natural day length. About two-thirds of the nitrogen fixing capacity was regained by nodules from darkened plants which were returned to normal illumination. The time-course of nitrogen
Nitrogen fixation in legume root nodules

fixation by excised soybean nodules also indicated depletion of an endogenous substrate with time. The rate was linear with time for about 1 h and declined thereafter (Aprison & Burris 1952). Bach, Magee & Burris (1958) studied the distribution of 14C-labelled photosynthate in soybean and found a high proportion in the nodules. The label was distributed among organic acids, amino acids and carbohydrates. The addition of sucrose, glucose and fructose to nodule slices enhanced nitrogen fixation.

As well as providing energy for nodule activities, photosynthetic products would supply reducing power, by way of some components of the electron-transport pathway, for the reduction of N₂ to NH₃.

---

**Figure 29.** The effect of ATP upon nitrogen fixation by cell-free extracts of bacteroids without an ATP generating system.

(a) The effect of ATP concentration upon nitrogen fixed in 30 min at 25 °C using 24 µmole of Na₃S₂O₄ in a total volume of 3 ml. of 0.025 M KH₂PO₄ (pH 7.0) containing 2 mM Mg²⁺.

(b) The time course of nitrogen fixation at 25 °C using 6 µmole of Na₃S₂O₄. Other details as in (a). In both experiments, the extract was the supernatant from centrifugation of broken bacteroids at 100000 g for 30 min.

(c) Bacteroid substrates

Bacteroids prepared anaerobically in a medium containing 0.3 M sucrose fixed N₂ without further additions but when sucrose was omitted from the medium, fixation was reduced to about 20% of the original endogenous rate. The addition of substrate amounts of sucrose did not restore the nitrogen fixing activity of bacteroids in sucrose-free medium but succinate and fumarate restored and enhanced fixation; pyruvate was much less effective (Bergersen & Turner 1967).
These observations suggested that endogenous substrates were being removed from the bacteroids during washing in sucrose-free medium. It has been found subsequently (F. J. Bergersen & W. B. Sylvester, unpublished), that succinate was a prominent constituent of organic acids leached from bacteroids which had been shaken in sucrose-free medium.

Soybean bacteroids contain prominent inclusions of a polymer (Goodchild & Bergersen 1966) which may be identical with the large amounts of lipoidal material having the melting point of poly-β-hydroxy butyric acid which can be extracted from bacteroids (C. A. Appleby, private communication). It is possible that this material serves as a reservoir of substrate which allows nitrogen fixation to proceed for some time following darkening of the host plants, removal of the nodules from the plants, or the preparation of bacteroid suspensions. If this is so, the rate at which the polymer could be degraded into utilizable form may govern the rate of nitrogen fixation, following depletion of utilizable substrates. When rates of nitrogen fixation by intact nodulated plants and by detached nodules are compared using similar conditions, it is seen that detached nodules fix at a much reduced rate (table 19). It has been shown that initial rates of fixation by bacteroid suspensions can be the same as rates of fixation in the intact detached nodules (Bergersen & Turner 1968). That is, fixation rates in these two types of material can be attributed to the bacteroid endogenous substrate. The loss of activity which follows detachment of the nodules would thus seem to be a consequence of the removal of the supply of a readily available photosynthetic product from the host.

### Table 19. Comparison of nitrogen fixation by nodulated soybean plants and detached nodules

**Intact plants (from data of Bergersen 1958)**

<table>
<thead>
<tr>
<th>Nodule age (days)</th>
<th>Total N/plant (mg)</th>
<th>Nodule fresh wt./plant (g)</th>
<th>N₂ fixed h⁻¹ (g fr. wt. nodules)⁻¹*</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>43</td>
<td>0.55</td>
<td>211</td>
</tr>
<tr>
<td>42</td>
<td>83</td>
<td>0.58</td>
<td></td>
</tr>
</tbody>
</table>

Detached nodules: Plant grown as above; nodules incubated 1 h at 23 to 25 °C with 0.2 atm O₂ and 0.2 atm ¹⁵N₂. Soybean varieties Lincoln and Shelby.

<table>
<thead>
<tr>
<th>Nodule age (days)</th>
<th>Non-protein-N (g fr. wt. nodules)⁻¹ (mg)</th>
<th>Atoms ¹⁵N excess†</th>
<th>N₂ fixed (g fr. wt. nodules)⁻¹ h⁻¹ (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cv. Lincoln</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>1.11</td>
<td>0.616</td>
<td>6.8</td>
</tr>
<tr>
<td>32</td>
<td>1.48</td>
<td>0.453</td>
<td>6.7</td>
</tr>
<tr>
<td>24</td>
<td>2.05</td>
<td>0.383</td>
<td>7.9</td>
</tr>
<tr>
<td>cv. Shelby</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3.71</td>
<td>0.372</td>
<td>13.8</td>
</tr>
</tbody>
</table>

* Assuming fixation 24 h/day.
† ¹⁵N excess corrected to 100% in gas phase.
Nitrogen fixation in legume root nodules

(f) The reductant requirement of bacteroid extracts

Cell-free extracts of bacteroids have an absolute requirement for dithionite as well as for ATP, in nitrogen fixation. This is true for crude (Bergersen & Turner 1968) and for partially purified extracts (Koch et al. 1967b; Klucas et al. 1968). It has been reported that pyruvate, α-keto-glutarate, β-hydroxy-butyrate, reduced NAD and reduced NADP were not able to replace dithionite (Koch et al. 1967b). In our laboratory, attempts to replace dithionite with succinate, fumarate, reduced NAD and reduced NADP, in centrifugally clarified bacteroid extracts have also been unsuccessful. However, in some experiments using differentially centrifuged extracts, it has been found that a proportion of the N₂-fixing activity present in the supernatant from centrifugation of broken bacteroids at 20000 g for 15 min, was removed by centrifugation at 100000 g for 15 to 30 min. This particle-bound portion of the nitrogenase fixed N₂ when supplied with ATP and reduced NAD. Also present in the 20000 g supernatant was a particle bound hydrogenase which evolved H₂ from reduced NAD in the absence of an ATP generating system (F. J. Bergersen & G. L. Turner, in preparation).

It is possible that the natural reductant which in vitro is replaced by dithionite, is very labile. The isolation from bacteroids of a brown, non-haem-iron-protein which lost 90 % of its labile sulphide in 5 h under an argon atmosphere, has been reported (Koch et al. 1967b). This protein may be a very labile ferredoxin and if so, it could be the natural electron donor for nitrogen fixation in bacteroids.

6. Nitrogen-fixation and hydrogen metabolism

Intact, detached soybean nodules evolve H₂ and this is inhibited in the present of N₂. They also catalyse an exchange between D₂ and endogenous H-donors to produce HD; this reaction occurs only in the presence of N₂ (Hoch, Schneider & Burris 1960; Bergersen 1963). Similar effects are observed during nitrogen fixation by bacteroids and cell-free extracts.

Many treatments which reduce nitrogen fixation also enhance H₂ evolution. For example, in experiments with bacteroid suspensions and extracts, in which two concentrations of N₂ were used with increasing CO concentrations, more N₂ was fixed and less H₂ evolved at the higher N₂ concentration. Less N₂ was fixed and more H₂ evolved at the higher CO concentrations (figure 30). When these results were calculated in terms of μ-equivalents of reductant, it was seen that the decrease in fixation caused by CO was matched by an almost equivalent increase in H₂ evolution by the bacteroids. In the extracts, H₂ evolution was increased rather more than expected from the reduction in fixation of N₂ produced by CO. Differences between the two N₂ concentrations in terms of N₂ fixed and H₂ evolved were approximately equivalent in terms of reductant, for both bacteroids and extracts.

Hydrogen evolution in nodules and bacteroid suspensions requires O₂ and in extracts requires both ATP and dithionite. It has been shown that H₂ competitively
inhibited nitrogen fixation in cell-free extracts (Koch et al. 1967b). Conversely, when two different external O₂ concentrations were used to produce two levels of ATP dependent reductant in detached nodules, it was shown that N₂ competitively inhibited H₂ evolution (Bergersen 1963). These results show that in the nodule system, NH₃-formation from N₂ plus H⁺ and H₂-formation from H⁺ are both the expression of the same ATP-dependent reduction.

(a) Washed bacteroids (40 mg protein/vessel) plus 100 µmoles of succinate, incubated for 30 min at 25 °C. Gas phase contained O₂—42 mmHg and ^12N₂—70 mmHg (● ▲), or 140 mmHg (○ △), with argon to 700 mmHg. Suspending medium 0·025 M KH₂PO₄ (pH 7·0), 1 mM Mg²⁺.

(b) Centrifuged cell-free extract (supernatant from 100 000 g for 30 min; 8·6 mg of protein/vessel) incubated 30 min at 25 °C with 24 µmoles of Na₂S₂O₄, 5 µmoles of ATP, 164 µmoles of creatine phosphate and 1 mg of creatine kinase in a total volume of 3·0 ml. Gas phase contained ^18N₂—35 mmHg (● ▲) or 140 mmHg (○ △), with argon to 700 mmHg.

There are a number of possibilities for the explanation of the D₂ exchange reaction in the presence of N₂. In the opinion of this author, it results from exchange between D₂ and the H of intermediates such as the equivalent of di-imide and/or hydrazine as these remain bound on the nitrogenase. The fairly constant ratio of 2 to 3 moles exchanged/mole of N₂ fixed over quite wide rates of fixation (Bergersen 1963) results from the interaction of the rate of D₂ exchange with the intermediate(s) and the time for which these remain bound and exchanging H before the final reduction and release of NH₃.
7. The relationship between bacteroid electron transport pathways

In the functioning soybean nodule, the bacteroids are not growing, and from the comparative absence of ribosomes, are not engaged in much protein synthesis. It is, therefore, likely that oxidation of substrates to produce reducing power is fulfilling two main functions. First, it is producing ATP from oxidative phosphorylation in a coupled terminal respiratory pathway with O₂ as the terminal acceptor. Secondly, it is providing reducing power for the reduction of N₂ to NH₃. In the event that reducing power becomes limiting due to the demands of one of these functions, the other reaction may be diminished. This was the interpretation of experiments which showed that at pO₂ above 0.5 atm, nitrogen fixation by detached

![Graph showing the effects of N₂ upon reductant used in nitrogen fixation, hydrogen evolution and oxygen uptake by washed bacteroids](image_url)

**Figure 31.** The effects of N₂ upon reductant used in nitrogen fixation, hydrogen evolution and oxygen uptake by washed bacteroids (23·2 mg protein/vessel) suspended in 0·025 M KH₂PO₄ (pH 7·0), 2 mM Mg²⁺ containing 100 μmoles succinate/vessel. Gas phases contained 0 (○) or 70 (●) mmHg of ^1⁵N₂ and 35 mmHg of O₂ with argon to 700 mmHg. Hydrogen evolution and oxygen uptake measured mass-spectrometrically and nitrogen fixation measured from the ^1⁵N content of NH₄⁺-N after the addition of 100 μg carrier NH₄⁺-N.
soybean nodules was reduced with some of the characteristics of competitive inhibition (Bergersen 1962a). This interpretation was strengthened by the observation that in this range of O$_2$ concentration, oxygenation of leghaemoglobin in the intact tissue increased, indicating increased availability of free O$_2$ (Bergersen 1962b). This resulted in reducing power being used in respiration rather than in nitrogen fixation. Because nitrogen fixation by bacteroid suspensions is rapidly inactivated in the presence of free O$_2$ it has not been possible to test this interpretation of results obtained with intact nodules. However, other observations lend support to it. First, when nitrogen fixation by bacteroid suspensions declines due to inactivation by O$_2$, a concurrent rise in O$_2$ uptake has been observed (Bergersen & Turner 1968). Secondly, experiments in which bacteroids were incubated for various times with and without N$_2$ and in which the amount of reductant used in nitrogen fixation, H$_2$ evolution and O$_2$ uptake has been calculated, have shown the results illustrated in figure 31. Nitrogen depressed the amount of reductant used in both H$_2$ evolution and respiration but the effects were not equivalent. After 30 min, nitrogen fixation consumed 1.5 μ-equivalents of reductant, whereas H$_2$ evolution was reduced by 3 μ-equiv. and O$_2$ uptake was reduced by 6 μ-equiv. The relatively greater effect upon respiration may be a reflection of the relative efficiency of ATP-use in the processes associated with nitrogen fixation.

8. Kinetic measurements

Measurements of the Michaelis constant ($K_m$) in the soybean nodule system has been made for N$_2$ in nitrogen fixation by nodule slices (Burris et al. 1955), intact detached nodules (Bergersen 1962a), bacteroid suspensions (Bergersen & Turner 1968) and cell-free extracts of bacteroids (Koch et al. 1967b; Bergersen & Turner 1968). Values for intact nodules, slices and bacteroids ranged from 0.025 to 0.066 atm (17 to 50 mm Hg) at the lower O$_2$ concentrations. Increased pO$_2$ above 0.3 atm for nodules and above 0.06 atm for bacteroids, increased the apparent $K_m$ values (Bergersen 1962a; Bergersen & Turner 1968). Values of $K_m$ for N$_2$ in cell-free extracts of 0.056 atm (Koch et al. 1967b) and 0.08 to 0.156 atm (Bergersen & Turner 1968), have been reported. These values are higher than values for intact bacteroids. The increase in $K_m$ following breakage of nitrogen fixing cells has been reported for other bacteria (e.g. Mortenson 1964). Bergersen & Turner (1968) discussed the possibility that this effect may be due to altered rates of product formation and not to a real difference in the (nitrogenase:N$_2$) dissociation constant.

Other kinetic measurements which have been made with the nodule system are of the inhibitor constants of H$_2$ and CO, both of which behave as competitive inhibitors of nitrogen fixation. $K_i$ for H$_2$ was 0.016 to 0.058 atm in cell-free extracts (Koch et al. 1967b) and $K_i$ for CO was 0.00057 atm (0.4 mm Hg) for bacteroids and 0.00030 atm (0.2 mm Hg) for cell-free extracts (Bergersen & Turner 1968). With the exception of $K_i$ for H$_2$ in extracts, all of the values obtained in kinetic experiments are similar to values obtained for corresponding intact or cell-free systems of other nitrogen-fixing bacteria.
Nitrogen fixation in legume root nodules

9. Conclusion

Nitrogen fixation in soybean nodules is a property of the bacteroids, which develop under specific host-cell influences in a specialized environment. During this development, a number of associated changes in metabolism can be observed. Some of these changes are related to electron transport pathways which provide reducing power for both oxidative phosphorylation and the reduction of \( \text{N}_2 \). Figure 32 summarizes the matters which have been discussed in a diagram in which the relationships between the known properties of the nodule system are shown. It is clear that there are many features common to the symbiotic system in legumes and the systems of free-living, nitrogen-fixing bacteria (cf. Burris in this Discussion).

The author wishes to acknowledge the assistance of Mr G. L. Turner and the technical help of Mrs A. Marcina and Mrs L. Hush in the experimental programme from which some of the results presented have come. Thanks are due to Dr C. A. Appleby for permission to quote unpublished material and to Professor H. J. Evans for making available results of work in progress in his laboratory. Dr D. J. Goodchild and Miss M. Paulin kindly provided the electron micrographs.
REFERENCES (Bergersen)


Electron micrographs of thin sections of growing and bacteroid forms of soybean nodule bacteria.

Figure 33. Growing nodule bacteria within an infection thread in a young soybean nodule. (c) Bacterial cell wall, (f) nuclear filaments, (r) ribosomes. The scale shows 1 μm.

Figure 34. A bacteroid isolated from a mature soybean nodule. (c) Bacteroid wall, (m) protoplast membrane, (f) nuclear filaments, (g) dense granules and (p) polar granules of polymer. Ribosomes are almost completely absent. The scale shows 1 μm.