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# Hydrogenase in Pea Root Nodule Bacteroids

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*Summary.* Hydrogen uptake has been shown to occur with pea root nodule breis and this uptake has been shown to be confined to the bacteroids. The uptake of hydrogen by washed bacteroids, in the absence of any added substrates, has been shown to be accompanied by oxygen uptake and the ratio of hydrogen uptake to oxygen uptake in these preparations has been found to be 2:1. Substrates, provided to washed bacteroids, inhibit the uptake of hydrogen and it has been found that the utilisation of substrates, as measured by carbon dioxide evolution, is inhibited by hydrogen. It is suggested that hydrogen and substrates compete for electron carriers and that electrons from the hydrogen reduce components of the electron transport pathway and ATP is produced.

The action of hydrogen on nitrogen fixation in nodule breis and washed bacteroid preparations was examined and the evidence shows that some non-competitive inhibition of nitrogen fixation, by hydrogen, occurs.

While some nitrogen fixing organisms possess a hydrogenase capable of activating or evolving hydrogen, it has been shown that the hydrogen evolution of most nitrogen fixing organisms is not due to a true hydrogenase but to the ATP dependent hydrogen evolving system which appears to be responsible for the activating of electrons for nitrogen fixation (Hardy and Knight, 1968).

*Azotobacter* possesses a hydrogenase that takes up hydrogen and has a low activity with regard to hydrogen deuterium exchange (Hyndman *et al.*, 1953). This hydrogenase takes up such hydrogen as is evolved by the ATP dependent hydrogen evolving enzyme so that hydrogen evolution is not detected from this nitrogen fixing organism.

Pea root nodules similarly take up hydrogen and have no net hydrogen evolution from them (Dixon, 1967). However it has been shown by other workers (Hoch *et al.*, 1960; and Bergersen, 1963) that soya bean root nodules evolve hydrogen.

Following the preparation of root nodule breis that were capable of fixing nitrogen (Bergersen, 1967), it was decided to investigate further the uptake of hydrogen by pea root nodules. With nodule breis and separated bacteroids it is possible to supply substrates and inhibitors in order to establish the role of hydrogen uptake in nodule metabolism.

The experiments, reported here, show that hydrogen uptake is associated with oxygen uptake and there is circumstantial evidence to show that ATP is produced as a result.

In the course of this investigation it was necessary to investigate the effect of hydrogen on nitrogen fixation in different preparations. Evidence from these experiments leads to the conclusion that, in pea root nodules, some non-competitive inhibition of nitrogen fixation by hydrogen occurs.

## Methods

Seeds of *Pisum sativum* (cultivar Meteor) were surface sterilised and then inoculated with strain *311*, O.N. Allen, of *Rhizobium leguminosarum*. They were then planted in sand containing five percent John Innes Compost No. 2 and supplied with nitrogen-free mineral nutrient medium (Bond's modfied Crone medium). They were grown in a greenhouse kept at 18° C, except during warm weather when the temperature sometimes went above this. Supplementary lighting was provided, using Philips mercury-vapour lamps MBFRU 400, to give 15 h of daylight.

The plants were harvested after 4 weeks. The nodules were removed and placed in distilled water cooled in ice until the total amount of nodules, 1 to 2 g, had been collected. The nodules were then drained and washed with more ice cold distilled water and then transferred to a cooled mortar in a glove box. The glove box was sealed and then evacuated and flushed with argon several times. The nodules were then ground with the pestle and mortar in 4 ml of buffer: 0.05 M phosphate, pH 7.0, 0.0025 M $MgCl_2$ and 4.0% polyvinylpyrrolidone, PVP, average MW 40000. The resulting brei was filtered through a No. 1 porosity sintered glass filter using argon pressure. The resulting filtrate was then transferred to flasks or to centrifuge tubes.

To obtain washed bacteroids the filtrate was centrifuged at top speed on a bench centrifuge, about 4000 g, for 15 minutes, the supernatant was removed and the bacteroids resuspended in the phosphate buffer from which PVP had been omitted, and recentrifuged for 15 minutes. Finally the bacteroids were resuspended in the second buffer solution. 1 ml of the bacteroid suspension, containing from 0.8 to 1.5 mg N, was then placed in the flask by injecting it through the rubber serum cap with a syringe. The nodule preparations were kept under an argon atmosphere at all times.

The washed bacteroid preparations were examined by light microscopy. The only detectable particles, using oil immersion, were bacteroids, which appeared to be undamaged in any way.

The flasks, in which the breis or the washed bacteroid suspensions were incubated, were modified Thunberg tubes and had a volume of about 18 ml. The flasks were filled with the appropriate gas mixture before being placed in the glove box. When a liquid was added by syringe the same quantity of gas was withdrawn. The reaction mixtures in the flasks were then incubated in a water bath at 25° C with shaking.

At the end of the incubation period, the gas was transferred to a Thunberg tube before admittance to the mass spectrometer. The accurate volumes of the flasks were known and the mass spectrometer readings were corrected for the changes in pressure which occurred.

In some experiments duplicate determinations were made but more often only single determinations were possible because of the shortage of material. Each

experiment has been repeated, some several times, and the results of these repetitions have been substantially the same.

Gas mixtures were prepared from cylinder gases obtained from the British Oxygen Company with the exception of deuterium, which was supplied by Imperial Chemical Industries from Norsk Hydro, Norway. The composition of the gas mixtures was assayed using an A. E. I. MS 3 mass spectrometer.

$(^{15}NH_4)_2SO_4$ was supplied by 20th Century Electronics Ltd. and contained 97.8% $^{15}N$. This was diluted to about 30% $^{15}N$ for use in the experiments. $^{15}N$ enrichment was determined after digesting the whole reaction mixture by the Kjeldahl method and the total nitrogen determined by distillation and titration.

The PVP buffer contained a large amount of nitrogen which would dilute the $^{15}N$ incorporated. Accordingly, for nitrogen fixation experiments carried out on nodule breis, the ascorbic acid buffer system with the use of 'Polyclar', as used by KOCH *et al.* (1967) was employed.

Because of the shortage of material, the nodules used for each experiment were collected from about 48 plants, exhaustive tests were not made to ensure that the buffer system used was that which resulted in the optimum activity of the hydrogenase or nitrogen fixing enzymes. Comparative tests with the buffer systems used by other workers, who have made active preparations of breis and bacteroids from soya bean root nodules, were however made. Very little or no activity was obtained if the buffer used by BERGERSEN (1967) was tried. This system of sucrose buffered with phosphate however gave active preparations if PVP was added. The activity differed little if the sucrose was omitted which is the buffer used in the experiments to be described.

The combination of grinding with insoluble PVP, 'Polyclar' and buffered ascorbic acid solution, used by KOCH *et al.* (1967), gave no increase in hydrogenase activity over the buffer system used in the experiments described below. It was however used in nitrogen fixation experiments with nodule breis.

## Results

Preliminary experiments were conducted with nodule breis. It was found that hydrogen was taken up in accord with the results obtained with whole nodules. It was also found that the addition of hydrogen increased oxygen uptake while the evolution of carbon dioxide was reduced.

Washed bacteroids were prepared and compared with the supernatant fraction for hydrogenase activity. No activity of any kind with regard to hydrogen could be obtained with the supernatant fraction.

The uptake of hydrogen was found to be linear for at least four hours with both preparations of nodule breis and with washed bacteroids. The results of an experiment with washed bacteroids are shown in Fig. 1.

### *Hydrogen and Oxygen Uptake*

In previous experiments it has been shown that there is no detectable isotope effect when deuterium is supplied instead of hydrogen. Deuterium was thus used in experiments on the hydrogenase so that true uptake

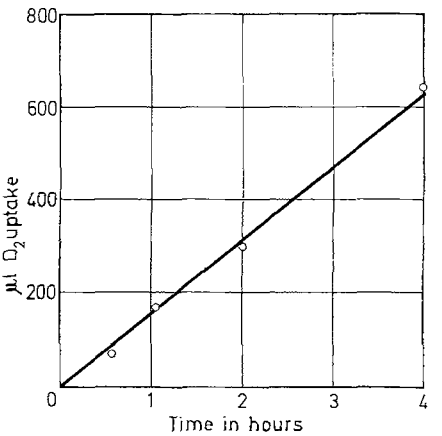


Fig. 1. Time course for the uptake of deuterium by washed bacteroids. Gas mixture (in atmospheres) $D_2$ 0.10, $O_2$ 0.05, A 0.85

Table 1. *The effect of deuterium on oxygen uptake*

| | $H_2$ | $D_2$ | $O_2$ | $CO_2$ | $D_2/O_2$ | $D_2/O_2$-E |
|---|---|---|---|---|---|---|
| $-D_2$ | — | — | 13 | 36 | — | — |
| | — | — | 17 | 36 | — | — |
| $+D_2$ | 5 | 190 | 105 | 25 | 1.81 | 2.04 |
| | 6 | 204 | 112 | 29 | 1.82 | 2.08 |

Results expressed as µl gas evolved ($H_2$ and $CO_2$) or taken up ($D_2$ and $O_2$). All per mg N/h. Gas mixtures (in atmospheres): $-D_2$; $O_2$ 0.05, A 0.95, $+D_2$ 0.1, $O_2$ 0.05, A 0.85.

$D_2/O_2$-E the endogenous oxygen uptake has been substracted from the total oxygen uptake. Washed bacteroids were suspended in 0.05 M phosphate buffer, pH 7.0, 0.0025 M $MgCl_2$.

could be determined without complications of concurrent hydrogen evolution. It was also necessary in some experiments to determine such hydrogen evolution.

The effect of supplying deuterium on the oxygen uptake of washed bacteroids was investigated. The results of one such experiment are given in Table 1. Deuterium considerably stimulated oxygen consumption and, if the endogenous oxygen uptake is taken into account, the amount of extra oxygen taken up in the presence of deuterium is found to be about half of the volume of deuterium taken up. 14 results, obtained

with conditions as pertaining to Table 1, gave $D_2/O_2$ ratios varying from 1.8 to 2.1.

The result of an experiment to determine the optimal partial pressure of oxygen for hydrogen uptake is shown in Fig. 2. The uptake of deuterium increased with increasing partial pressures of oxygen up to 0.04 atm and remained the same for 0.1 atm. Higher oxygen tensions were not

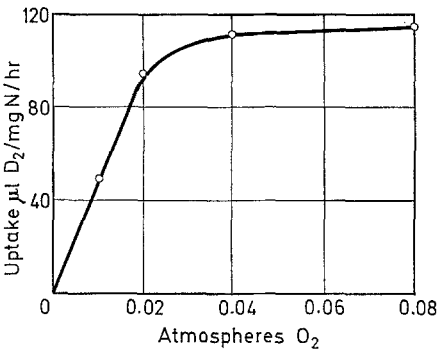


Fig. 2. Effect of the partial pressure of oxygen on deuterium uptake. Gas mixtures $D_2$ 0.10, oxygen as indicated A to make up to 1 atmosphere

tried. Hydrogen evolution and exchange decreased with increasing partial pressures of oxygen. It is assumed that these decreases are due to the increased amount of uptake of deuterium thus decreasing any back reaction and also taking up some of the hydrogen or HD that may have been evolved from the nitrogen fixing system.

### *The Effect of Substrates*

With the very low endogenous rate of respiration found with washed bacteroids, it has been shown above that hydrogen is taken up and oxidised either directly or indirectly by oxygen. The results obtained with nodule breis, which had a high endogenous rate of respiration, were not so clear. The extra oxygen taken up compared with the volume of deuterium taken up showed different ratios in different preparations. While it is clear that the hydrogen, taken up by washed bacteroids in the absence of added substrate, cannot take part in any major metabolic reactions apart from the oxidation reactions connected with the uptake of oxygen, this is not clear from the results obtained from nodule breis. The effect of substrates on hydrogen uptake by the washed bacteroids was therefore examined. The results of an experiment, in which three substrates, pyruvate, succinate and $\beta$ hydroxybutyrate, were supplied, are presented in Table 2. $\beta$ hydroxybutyrate was tried as poly $\beta$ hydroxy-

Table 2. *The effect of substrates on deuterium uptake*

| Substrate added 20 μM | $H_2$ | HD | $D_2$ | $O_2$ | $CO_2$ | % inhibition |
|---|---|---|---|---|---|---|
| 0 | 0.0 | 0.0 | 148 | 80 | 11 | — |
| β-hydroxybutyrate | 0.0 | 0.0 | 138 | 76 | 9 | 0 |
| pyruvate | 5.0 | 3.0 | 77 | 80 | 74 | 48 |
| succinate | 12.0 | 4.0 | 46 | 77 | 45 | 69 |

Results expressed as μl gas evolved ($H_2$, HD and $CO_2$) or taken up ($D_2$ and $O_2$). All per mg N/h. Gas mixture (in atmospheres): $D_2$ 0.1, $O_2$ 0.05, A 0.85. Washed bacteroids were suspended in 0.05 M phosphate buffer, pH 7.0, 0.0025 M $MgCl_2$.

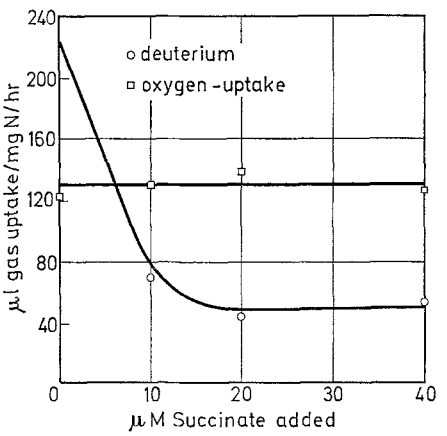


Fig. 3. Effect of substrate concentration on deuterium uptake by washed bacteroids. Gas mixture (in atmospheres) $D_2$ 0.01, $O_2$ 0.05, A 0.85

butyrate is a reserve food substance of the bacteroids. β hydroxybutyrate had no effect on hydrogen metabolism but there was no evidence that it was itself metabolised. Both pyruvate and succinate inhibited the uptake of hydrogen though to different extents. That both of these substrates were metabolised can be seen from the fact that carbon dioxide evolution was considerably increased when they were added. The oxygen uptake did not differ appreciably whether a substrate or hydrogen alone was added.

The effects of different concentrations of succinate are shown in Fig. 3. When no substrate was added no hydrogen or HD could be detected above the control values. As deuterium uptake was inhibited,

hydrogen and HD could be detected. This may reflect the back reaction or it may be that the hydrogen evolved by the ATP dependent hydrogen evolving enzyme can be detected as less of the hydrogen so evolved is being taken up by the hydrogenase. However the HD is likely to come from the hydrogenase itself as the ATP dependent hydrogen evolving enzyme does not catalyse hydrogen deuterium exchange and the nitrogenase is completely inhibited under the conditions of this experiment as is shown below.

The fact that oxygen uptake remained the same with different substrates and with different concentrations of the same substrate suggested that oxygen consumption was limited by some other mechanism than lack of substrate to be oxidised. Consumption of deuterium would then be limited by the same factor. In order to test whether ADP was limiting, an attempt was made to increase ADP concentration by uncoupling oxidative phosphorylation. 2.4. dinitrophenol was therefore added at a concentration of $2 \times 10^{-4}$ M. This concentration of dinitrophenol had no effect on oxygen uptake or carbon dioxide though hydrogen uptake was increased by about 20%.

It has been shown that substrates inhibit hydrogen uptake. The effect of hydrogen on the metabolism of the substrates can be inferred from the fact that the volume of carbon dioxide evolved in the presence of 10% hydrogen decreased, on average, by about 15%, compared with controls in which no hydrogen was supplied.

### *The Effect of Nitrogen*

The effect of nitrogen differed between nodule breis and washed bacteroid preparations. With washed bacteroids no effect of nitrogen could be detected on hydrogen uptake or exchange or on respiration, measured as oxygen uptake or as carbon dioxide evolved. When nodule breis were investigated, nitrogen affected all gases measured except for HD. Hydrogen evolution was inhibited and deuterium uptake enhanced when nitrogen was present and respiration was increased both measured as oxygen uptake and as carbon dioxide evolved. The amount of the increase of oxygen uptake was always more than the increase in carbon dioxide evolution, but the extra increase in oxygen uptake could be accounted for if it is associated with the increase in deuterium uptake. In the experiment quoted in Table 3, the extra increased amount of oxygen uptake over the increased amount of carbon dioxide evolution, due to the presence of nitrogen, can be calculated to be 43% of the increased volume of deuterium taken up at the same time. This is in reasonable agreement with the extra oxygen uptake being associated with deuterium uptake, taking into account the amount of variation of the duplicates in this experiment.

Table 3. *The effect of nitrogen on gas exchanges in a nodule brei*

| | $H_2$ | HD | $D_2$ | $O_2$ | $CO_2$ |
|---|---|---|---|---|---|
| $-N_2$ | 8.5 | 3.5 | 47 | 87 | 74 |
| | 7.0 | 4.0 | 55 | 93 | 76 |
| $+N_2$ | 4.5 | 3.5 | 100 | 120 | 80 |
| | 5.5 | 4.0 | 112 | 122 | 82 |

Results expressed as μl gas evolved ($H_2$, HD and $CO_2$) or taken up ($D_2$ and $O_2$). All per mg N/h. Gas mixtures (in atmospheres): $-N_2$; $D_2$ 0.10, $O_2$ 0.05, A 0.85, $+N_2$; $D_2$ 0.10, $O_2$ 0.05, $N_2$ 0.30, A 0.55. Nodule brei in 4.0% PVP, 0.05 M phosphate buffer pH 7.0, 0.0025 $MgCl_2$.

Table 4. *The effect of hydrogen on nitrogen fixation in a nodule brei*

| | Atoms % excess | μg N fixed/mg N/h |
|---|---|---|
| $-H_2$ | 0.068 | 1.22 |
| | 0.630 | 1.13 |
| $+H_2$ | 0.019 | 0.29 |
| | 0.022 | 0.34 |

Gas mixtures (in atmospheres): $-H_2$; $N_2$ 0.10 (28.0% $^{15}N$), $O_2$ 0.05, A 0.85, $+H_2$; $N_2$ 0.10 (32.0% $^{15}N$), $H_2$ 0.10, $O_2$ 0.05, A 0.75. Nodule brei in 0.3 M ascorbic acid, 0.05 M phosphate buffer, pH 7.0, 0.0025 M $MgCl_2$. 20 μM succinate was added to each flask.

Table 5. *The effect of substrate and hydrogen on nitrogen fixation in washed bacteroids*

| | Addition | Atoms % excess | μg N fixed/mg N/h |
|---|---|---|---|
| $-H_2$ | 0 | 0.050 | 1.26 |
| | succinate | 0.087 | 2.64 |
| $+H_2$ | 0 | 0.0 | 0.0 |
| | succinate | 0.0 | 0.0 |

Gas mixtures (in atmospheres): $-H_2$; $N_2$ 0.10 (26.6% $^{15}N$) $O_2$ 0.05, A 0.85, $+H_2$; $N_2$ 0.10 (35.4% $^{15}N$), $H_2$ 0.10, $O_2$ 0.05, A 0.75. Washed bacteroids were suspended in 0.05 M phosphate buffer, pH 7.0, 0.0025 M $MgCl_2$. 20 μM of succinate was added where indicated.

The reduction in the amount of hydrogen evolved, as a result of the presence of nitrogen, is real and cannot be due to errors in the mass spectrometric measurement of hydrogen caused by nitrogen. If such

errors, as have been reported by Bradbeer and Wilson (1963), were occurring then a decrease in hydrogen evolution should be measured in the experiments when washed bacteroids were used and also it would be expected to affect the HD measurements. In neither case was there any decrease in gas measured when nitrogen was present.

The lack of any effect of nitrogen on hydrogen-deuterium exchange is contrary to the observations of other workers who have found an increased evolution of HD in the presence of nitrogen in soya bean root nodules (Hoch *et al.*, 1960) and in *Azotobacter* preparations (Hardy and Knight, 1967). It is however in accord with previous results obtained with whole pea root nodules (Dixon, 1967).

### *Nitrogen Fixation*

The lack of effect of nitrogen on the gas exchanges of washed bacteroid preparations indicated that they lacked the capability to fix nitrogen. Experiments were therefore conducted to investigate this.

Table 4 contains the results of an experiment where $^{15}N_2$ was supplied to a nodule brei together with succinate. The presence of $10\%$ hydrogen inhibited nitrogen fixation by about $70\%$.

Table 5 contains the results of an experiment with washed bacteroids. Succinate doubles the amount of nitrogen fixed by these preparations. The inhibition of nitrogen fixation by $10\%$ hydrogen is however complete. This explains why no effect of nitrogen could be detected on gas exchanges in preparations of washed bacteroids.

## Discussion

The evidence presented previously, as a result of studies on whole pea root nodules, and that presented here, shows that a hydrogenase is present in pea root nodule bacteroids as well as the ATP dependent hydrogen evolution. That the latter is active can be inferred from the reduction of hydrogen evolution in the presence of nitrogen. Such a hydrogenase has been demonstrated in close connection with the nitrogen fixing system in *Azotobacter* (Hyndman *et al.*, 1953), but has not been shown to be present in soya bean root nodule bacteroids.

Hyndman *et al.* showed that in *Azotobacter* the uptake of hydrogen was associated with oxygen uptake, the Knallgas reaction, and that ATP was produced as a result of this reaction. They also found that this hydrogenase had a low capability to produce HD.

Cell free preparations have not as yet been prepared from pea root nodule bacteroids so that a direct comparison of the hydrogenase with that of *Azotobacter* has not been possible. The indications are however that they are very similar. The association of hydrogen uptake

with oxygen has been shown and the bacteroid hydrogenase produces very low amounts of HD in the presence of deuterium. The effect of substrates suggests that ATP might be formed as a result of hydrogen uptake. This suggestion arises from the fact that hydrogenase and substrates appear to compete for oxygen. This could arise by competition of electrons flowing from metabolism of the substrates and electrons obtained from hydrogen, by the hydrogenase, for carriers in the electron transport pathway. In the case of substrates it is reasonable to assume that the passage of electrons along this pathway gives rise to ATP. If the competition is for a carrier at an early stage of the electron transport pathway then ATP should be produced by the passage of electrons from hydrogen. The fact that succinate inhibits hydrogen uptake more strongly than pyruvate or malate suggests that the competition is not for the reduction of pyridine nucleotide but at a later stage in the electron transport pathway.

Evidence that ATP is produced is also provided by the effect of nitrogen on respiration. The amounts of hydrogen and substrates supplied appear to be saturating, as far as respiration is concerned, as succinate does not increase the amount of oxygen uptake when added to bacteroids which are oxidising hydrogen and vice versa. The only occasions in which it has been observed that respiration was increased above these levels was when nitrogen was supplied. If the amount of ADP were limiting respiration then nitrogen could increase respiration because of the increased hydrolysis of ATP necessary to provide energy for nitrogen fixation.

Hydrogen has been shown to be a competitive inhibitor of nitrogen fixation in a number of nitrogen fixing organisms, though PARKER and DILWORTH (1963) indicate a non-competitive type of inhibition in whole cells of *Azotobacter vinelandii*. The evidence presented here shows that if the inhibition of nitrogen fixation by hydrogen is competitive there must at least be a non-competitive element in the inhibition of nitrogen fixation in pea root nodule bacteroids.

When washed bacteroids were used with $10\%$ nitrogen and hydrogen no nitrogen fixation was detectable though with the same concentrations of gases the nitrogen fixation in nodule breis was inhibited by only $70\%$. This suggests that there is an interaction of hydrogen with some part of the nitrogenase system in the washed bacteroid fraction that is not occurring, or happening to a lesser extent, in the bacteroids in the nodule breis. As the bacteroids in each case are suspended in the same quantity of fluid at the same pH it cannot be due to any difference in the diffusion of gas to the nitrogen fixing site. It seems possible that hydrogen reduces a group that is essential for the proper functioning of the nitrogenase enzyme and that in the nodule breis there are substances capable of

reoxidising any such groups reduced, while in the washed bacteroid preparations such compounds are not present. This subject is at present under further investigation.

Both *Azotobacter* sp. and some *Rhizobium* species have a hydrogenase which rapidly takes up hydrogen and both give off very low amounts of HD which suggests that they are not as reversible as the more conventional hydrogenase such as that possessed by *Clostridium* in connection with the phosphoroclastic system. Both *Azotobacter* and *Rhizobium* are aerobic aorganisms so that it is possible to assign more than one possible role for the hydrogenase activity in connection with nitrogen fixation.

Oxygen is inhibitory to the nitrogen fixing system so that even in aerobic organisms the nitrogen fixing sites must be assumed to be in anaerobic conditions. The role of a hydrogenase, such as that examined here, might be to scavenge for oxygen at the nitrogen fixing site and thus add protection to the site from inhibition by oxygen. It would appear that, if the above explanation is correct, that it is not essential for nitrogen fixation by aerobic organisms as the hydrogenase has not been reported to be present in bacteroids from soya bean root nodules.

The circumstantial evidence presented to show that the oxidation of hydrogen by means of the hydrogenase can produce ATP leads to the suggestion that this hydrogenase may conserve ATP. A large amount of ATP appears to be needlessly lost by the action of the ATP dependent hydrogen evolving enzyme. The increase in survival value of such a saving of energy by *Rhizobium* is not at the moment apparent as this activity is confined to the bacteroids which no longer divide. With regard to *Azotobacter* it seems more plausible that the hydrogenase should have arisen and then been retained for this function.

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