Comparative Studies of Nitrogen Fixation by Soybean Root Nodules, Bacteroid Suspensions and Cell-free Extracts

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SUMMARY

Rhizobium japonicum (CC711) was used to infect soybean seeds from which plants were grown. From the root nodules, bacteroid suspensions with initial rates of nitrogen fixation as high as those calculated for bacteroids in intact nodules were prepared. Oxygen, which was required for fixation by intact nodules and bacteroid suspensions, caused the eventual loss of bacteroid nitrogen-fixing ability, accompanied by an increase in O_2 -uptake. In intact nodules and in bacteroid suspensions, increasing O_2 pressures resulted in higher values for K_m of nitrogen fixation. V_{max} also increased with increasing pO_2 and this was shown to be consistent with the characteristics of nitrogen fixation by anaerobic cell-free extracts of bacteroids which required an energy source (ATP), and a reductant (sodium dithionite).

Higher concentrations of carbon monoxide were required to inhibit nitrogen fixation by intact nodules than by bacteroid suspensions. Carbon monoxide was a competitive inhibitor of nitrogen fixation in bacteroid suspensions and K_i (CO) and K_m (N_2) values showed that the nitrogenase had about 30 times the apparent affinity for CO that it had for N_2 .

In cell-free extracts of bacteroids, the nitrogen-fixing activity remained in the supernatant fluid after centrifugation at 100,000 *g* for 30 min. The extracts were inactivated at 0°. The K_m for nitrogen fixation by cell-free extracts was variable, 62–118 (N_2 concentration in mm. Hg. pressure), compared with 50–60 for intact nodules and 20 for intact bacteroids when K_m for these was measured in the range of pO_2 in which it was only slightly affected by O_2 . The K_i for inhibition of nitrogen fixation by CO in extracts was similar to values obtained with intact bacteroids.

INTRODUCTION

Considerable understanding of the biochemistry of nitrogen fixation has developed since 1960 as a result of studies with cell-free extracts of free-living nitrogen-fixing bacteria (e.g. *Azotobacter vinelandii*, Bulen, Burns & LeComte, 1965; *Clostridium pasteurianum*, Mortenson, 1966). The symbiotic nitrogen-fixing system of legume nodules has been studied in intact detached nodules since the work of Aprison, Magee & Burris (1954), in nodule breis (Bergersen, 1966) and has been shown to be located wholly in the bacteroids (Bergersen & Turner, 1967). Koch, Evans & Russell (1967*a, b*) showed that the properties of cell-free extracts of *Rhizobium* bacteroids in many respects resemble those of extracts prepared from *Azotobacter* or *Clostridium*. The results to be given in the present paper are in substantial agreement with their results. In nature, it is the intact nodule tissue which accomplishes nitrogen fixation

and it is important to relate these new findings to the active component of the intact system, the bacteroids, which have developed the ability to perform this function only after growth within the complex structures of the nodule cells.

The effects of O_2 upon nitrogen fixation in detached intact nodules were studied by Bergersen (1962) but the inhibition of nitrogen fixation in this system by CO has only been studied in experiments with whole growing plants (Lind & Wilson, 1941). In the present paper, an account is given of experiments in which the effects of O_2 and CO upon intact nodules and *Rhizobium* bacteroid suspensions are compared and some properties of cell-free extracts of bacteroids are related to these effects.

METHODS

Nodules. Soybean plants (cv. Shelby) were grown in sand + vermiculite mixture in a glasshouse, as previously described (Bergersen, 1958), from seeds inoculated at sowing with strain CC711 of *Rhizobium japonicum*. Nodules were detached when they were aged about 35 days, and were used immediately.

Bacteroid suspensions. Breis were prepared under argon using a stainless-steel anaerobic press and washed bacteroids were prepared from the breis as previously described (Bergersen, 1966; Bergersen & Turner, 1967). In some experiments the suspending and washing medium contained 0.3 M-sucrose, 0.1 M- KH_2PO_4 (pH 7.0) and 1 mM- $MgSO_4$. Following the work of Koch *et al.* (1967a), in which polyvinyl-pyrrolidone and ascorbate were used to remove plant polyphenols, the method was modified and the initial brei was prepared in 0.1 M- KH_2PO_4 (pH 7.0) containing 1.5 % (w/v) of a soluble polyvinyl-pyrrolidone (PVP) of average molecular weight 25,000 (Kollidon 25; B.A.S.F., Australia Ltd.) and 0.2 M-Na ascorbate. The bacteroids were then centrifuged down, and washed in buffered sucrose medium with the phosphate concentration decreased to 0.025 M. This modification followed the observation of inhibition of nitrogen fixation at higher phosphate concentrations (Bergersen, unpublished). The bacteroids prepared by the modified method were more active but their properties were otherwise unaltered.

Gas mixtures. These were prepared from good quality commercial gases and the composition is expressed as partial pressures measured in mm. Hg. $^{15}N_2$ was included in the gas mixtures or added to incubation vessels by syringe. Mixtures for anaerobic experiments were stored over a solution of chromous sulphate to remove traces of O_2 . Mass spectrometer analysis was used to check gas composition in the vessels before and after incubation and also to measure respiration and evolution of H_2 . In experiments with CO this inhibitor was injected into the reaction vessels by means of a gas chromatograph syringe.

Measurements of nitrogen fixation. Intact nodules (1.5 g. fresh wt) were incubated in 50 ml. Erlenmeyer flasks containing the desired gas mixture, the nodules then ground in 3 N-HCl and the soluble portion containing the non-protein nitrogen analysed for ^{15}N .

Washed bacteroid suspensions evolve NH_3 -N from endogenous sources into the medium in addition to the accumulation of fixed NH_3 -N. This is illustrated by the data from an experiment in which 3.75 $\mu g.$ NH_3 -N accumulated in the medium, while only 0.49 $\mu g.$ was fixed. These effects, while of much smaller magnitude than reported for unfractionated nodule breis (Bergersen, 1966), are still sufficient to prevent the

use of direct nitrogen analysis for the measurement of nitrogen fixation; ^{15}N methods must therefore continue to be used. Fixation by bacteroid suspensions and by cell-free extracts was measured by using sidearm flasks with a gas volume of 30 or 70 ml. and containing 3 to 6 ml. liquid. The flasks were shaken at 25° and substrates, when used, were tipped from a sidearm. The fixed nitrogen accumulated as $\text{NH}_3\text{-N}$ in the suspending medium (Bergersen & Turner, 1967). The ^{15}N content of this nitrogen was measured in the following manner in 1 ml. samples of suspension, withdrawn at intervals by hypodermic syringe from rubber-capped sidearms. The samples were centrifuged at 0 to 4° and the bacteroid pellet washed once with a further portion of the suspending medium. The combined supernatant fluids, with 50 or 100 μg . unlabelled $\text{NH}_3\text{-N}$ as carrier, were distilled from a Markham still after adding an equal volume of saturated borate buffer (pH 10.5) and the distilled NH_3 trapped in 5 ml. 1% (w/v) boric acid. Alternatively, the NH_3 in the samples was recovered by diffusion from a suspension with MgO in modified Conway dishes (Freney & Wetselaar, 1967). After titration of the $\text{NH}_3\text{-N}$, it was analysed for ^{15}N content and the μg . N fixed/vessel was calculated with regard to the initial liquid volume, the $\text{NH}_3\text{-N}$ content of the samples (including the carrier $\text{NH}_3\text{-N}$), the atoms % ^{15}N excess of the N_2 in the gas mixture and the atoms % ^{15}N excess of the samples.

Protein determination. Because of the use of intact bacteroids it was necessary to use a protein-N method which was used throughout for whole nodules, bacteroids and extracts. Samples (0.5 ml.) of suspension or extract or a sample of ground dried nodules, were extracted with 4 ml. 0.1 N- H_2SO_4 containing 0.5 ml. Na tungstate (10%, w/v). The precipitate, after overnight extraction and washing, was digested and the protein-N determined after distillation and titration. Protein (mg.) was given by $6.37 \times \text{mg. protein-N}$.

Bacteroid extracts. The bacteroids after washing were suspended in 15 ml. 0.025 M- KH_2PO_4 (pH 7.4) containing 2 mM- MgSO_4 , under argon, and transferred to a previously argon-flushed pressure-release cell designed in the Division of Plant Industry, CSIRO, Canberra. Deoxyribonuclease (about 1 μg .; Mann Research Laboratories, New York) was added and the piston inserted under a stream of argon. The bacteroids were broken by using a pressure of 8 tons/sq. in. and the extract collected and centrifuged under argon. Extracts (total volume 3 to 5 ml.) were shaken with $^{15}\text{N}_2$ + argon mixtures at 25° with various additions. The ATP-generating system consisted of 6 μmoles adenosine-5-triphosphate (Na salt; Nutritional Biochemicals Co., Cleveland, Ohio), 150 μmoles creatine phosphate and 1–2 mg. creatine phosphokinase (Sigma Chemical Co., St Louis, Mo.) all dissolved in 0.025 M- KH_2PO_4 and neutralized. The dithionite ($\text{Na}_2\text{S}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$, 24 μmoles /vessel), made up daily, was dissolved in argon-saturated phosphate buffer (0.1 M; pH 7.0) and stored under argon at 0° until used. The incubation vessels with the constituents of the ATP-generating system in the sidearms, were flushed three times with argon and then filled with gas mixture to a pre-determined pressure and the extracts added by syringe through a rubber-capped port in one sidearm. The dithionite was added last, also by syringe. NH_3 was recovered from the flask contents by distillation from saturated borate (pH 10.5), titrated and analyzed for ^{15}N , and the amount of nitrogen fixed calculated as described above.

RESULTS

Comparison of bacteroid and nodule activity

Nitrogen fixation was greatest in bacteroid suspensions prepared from nodules disrupted in the presence of buffered PVP and ascorbate and washed and incubated in buffered sucrose containing 1 mM-Mg²⁺ and 16.7 mM-Na succinate. Initial fixation rates (0 to 20 min.) of 700 to 900 mμg. N/mg. protein/hr with *p*O₂ 42 mm. Hg and *p*N₂ 140 mm. Hg at 25°, was consistently obtained with this method. Bacteroids prepared from nodules disrupted in buffered sucrose (Bergersen & Turner, 1967) had initial fixation rates of 450–500 mμg. N/mg. protein/hr under the same conditions. To compare these rates of nitrogen fixation with the rates of fixation by bacteroids in intact nodule tissue, samples of nodules aged 35 days were incubated for 1 hr at 25° and the nitrogen-fixation rates and protein content of the nodules measured. These nodules contained 179 mg. protein and they fixed at a rate of 21 μg. N/hr at *p*O₂ 140 mm. Hg, and at a rate of 60 μg. N/hr at *p*O₂ 350 mm. Hg (both with *p*N₂ 140 mm. Hg). In previous work (Bergersen & Turner, 1967, Table II), bacteroid protein accounted for about 56% of the nodule brei protein. Assuming the same proportion, the above rates of nitrogen fixation by intact nodules can be expressed as 210 and 599 mμg. N/mg. bacteroid protein/hr at *p*O₂ values of 140 and 350 mm. Hg, respectively. From these results it is concluded that bacteroids prepared anaerobically in the manner described are comparable in their initial nitrogen-fixing capacity with bacteroids in intact nodule tissue, although the high rates of fixation by suspensions are transitory (Fig. 1).

Effects of O₂ upon bacteroid suspensions

The results obtained were similar to those obtained with nodule breis (Bergersen, 1966); air had to be excluded during preparation of the bacteroids, but O₂ was required for nitrogen fixation. Table 1 shows the effect of vigorously aerating a bacteroid

Table 1. *Inactivation of bacteroids by aeration*

Inactivation was by bubbling air for 15 min. at 0°. Aerated treatments contained 53 mg. bacteroid protein/vessel and non-aerated treatments 48 mg. bacteroid protein/vessel. Incubated 30 min. at 23° with ¹⁵N₂ (168 mm. Hg) and O₂ (42 mm. Hg). Respiration and H₂ evolution measured mass-spectrometrically. Non-aerated bacteroids were also kept 15 min. at 0°. Bacteroids suspended in 0.1 M-phosphate (pH 7.0) + 1 mM-Mg²⁺.

Treatment	Succinate (100 μmoles)	N ₂ fixed (mμg. N/mg. bacteroid protein)	H ₂ evolution (Δ <i>p</i> H ₂ atm.)	Respiration	
				Δ <i>p</i> O ₂ (atm.)	Δ <i>p</i> CO ₂ (atm.)
Aerated	—	0	0	−46 × 10 ^{−4}	+48 × 10 ^{−4}
Aerated	+	43	1.4 × 10 ^{−4}	−91 × 10 ^{−4}	+86 × 10 ^{−4}
Non-aerated	—	383	5.5 × 10 ^{−4}	−90 × 10 ^{−4}	+53 × 10 ^{−4}
Non-aerated	+	424	6.0 × 10 ^{−4}	−101 × 10 ^{−4}	+86 × 10 ^{−4}

suspension in sucrose-free medium for 15 min. at 0°. First, this treatment decreased the endogenous O₂ uptake, presumably because of depletion of endogenous substrate resulting from the vigorous agitation in the sucrose-free suspending medium. The O₂ uptake was restored on adding succinate. These results may be compared with those obtained previously (Bergersen & Turner, 1967) in which it was found that anaerobic

washing of bacteroids in sucrose-free medium decreased the endogenous nitrogen-fixing activity to a very low value, the activity being restored by adding succinate or other substrates. Secondly, nitrogen fixation and hydrogen evolution were reduced by the aeration, and these activities were not restored by adding succinate.

Fig. 1. The effect of O_2 concentration upon the time course of nitrogen fixation by washed bacteroids of *Rhizobium japonicum*. The dotted lines indicate the initial rates of fixation used for kinetic studies. The gas phase contained $^{15}N_2$ (70 mm. Hg) and pO_2 values (mm. Hg) of ●—● 21, ○—○ 42 and ⊙—⊙ 84; the balance to 700 mm. Hg was argon.

Fig. 2. The relationship between the time courses of nitrogen fixation, hydrogen evolution and respiration by washed bacteroids of *R. japonicum*. The distribution of the fixed N between non-protein-N (NPN) of bacteroids and suspending medium is also shown.

In Fig. 1, the effect of O_2 concentration upon the initial rates of nitrogen fixation and upon the time course are illustrated. Doubling the pO_2 value more than doubled the initial fixation rates but the course was much shortened. The inactivation of the nitrogen-fixing activity of bacteroids during incubation in the presence of O_2 may be due to the oxidation of labile-SH groups. Attempts to preserve the activity by the use of β -mercaptoethanol or thioglycollate (Fraenkel-Conrat, 1957) were unsuccessful; neither compound prolonged the activity and both were inhibitory to nitrogen fixation. The decline in fixation, which occurred after about 40 min. with pO_2 42 mm. Hg was accompanied by a decline in H_2 evolution and by an increase in respiration rate. This is illustrated in the data of Fig. 2, which presents the results from an experiment in which 6 vessels were incubated for the times shown, before the gas phase was sampled and analysed and the distribution of the newly fixed N in the bacteroid suspension determined. During nitrogen fixation, only about 3–5 % of the fixed N was found in the bacteroids after one washing, but after 60 min. when fixation had ceased, this increased slightly to 7 %. In other experiments, the soluble NH_3 -N, which accounted for most of the fixed N in this experiment, declined after fixation ceased. These observations suggested that there may have been some assimilation of newly fixed soluble NH_3 -N at this stage. This was been confirmed in experiments which showed slight labelling of α -amino-N of the bacteroids after 40 min.

Effects of O_2 upon reaction kinetic measurements with nodules and bacteroids

The characteristics of an enzymic reaction are most conveniently described in terms of the Michaelis equation:

$$v = V_{max}s/K_m + s$$

in which v is the reaction velocity, V_{max} the velocity with non-limiting substrate concentrations, s the substrate concentration and K_m the equilibrium constant of the reversible combination of enzyme and substrate (the Michaelis constant). In the work to be described, N_2 was the substrate and the values obtained were for the reduction of N_2 to NH_3 .

In previous work with intact detached nodules (Bergersen, 1962) the apparent K_m for nitrogen fixation increased sharply at high values of pO_2 and was accompanied by inhibition of nitrogen fixation. Although V_{max} also increased in this range of pO_2 values, the increase was not significant and it was concluded that O_2 was a competitive inhibitor of nitrogen fixation in this system.

The effects of O_2 upon fixation by bacteroid suspensions appeared to be different: no inhibition was obtained at the highest concentration which permitted measurement of initial rates of nitrogen fixation. K_m and V_{max} for isolated bacteroids were therefore measured by using the reciprocal method of Dixon (1953). Six experiments were done, in each of which a pair of pO_2 values was compared. The data are shown in Fig. 3. In this work v was taken as the average rate of fixation over the first 10 or 20 min. (see Fig. 1) and s was measured in portions of an atmosphere of 700 mm. Hg. Statistical treatment was necessary in order to give confidence limits for the values obtained. A preliminary fit of $1/v$ upon $1/s$ was made, ignoring the increasing array dispersion with $1/s$. Deviates from the line were calculated and the absolute values of these plotted against estimated $1/v$. A quadratic trend was indicated and it was found that the array standard deviation was proportional to $(1/v)^2$. The values of $1/v$ and $1/s$

were then fitted with weights inversely proportional to the fourth power of the estimated $1/v$ of the first fitting, and the confidence limits of the intercepts giving $1/K_m$ and $1/V_{max}$ were calculated.

Fig. 3. Dixon-Lineweaver-Burk plots for nitrogen fixation by washed bacteroids of *R. japonicum* at pO_2 values of 21, 42, 53 and 84 mm. Hg. v measured as initial fixation rates in $\mu\text{g. N/mg. protein/hr}$ and s as portions of an atmosphere of 700 mm. Hg. The regression lines are fitted according to the weighted method described in the text. In each case, data for 3 experiments are shown.

Both K_m and V_{max} increased with increasing pO_2 values over the range 52 to 84 mm. Hg, while from 21 to 42 mm. Hg there was a significant increase in V_{max} but no change in K_m . In Fig. 4 these results are compared with data for intact nodules, used previously (Bergersen, 1962). Although no inhibition of nitrogen fixation by O_2 was obtained with bacteroids, in both intact nodules and bacteroids the kinetic effects of O_2 are comparable. A fourfold increase in pO_2 changed K_m and V_{max} by similar factors in each system, although with intact nodules the increase in V_{max} at high pO_2 was not significant, because of large experimental error. It is clear that increasing O_2 pressures decreased the apparent affinity of the nitrogenase for N_2 as shown by the increased values of K_m at higher pO_2 values. At the same time, increasing pO_2 values increased the velocity of the reaction. In these experiments it was observed that O_2 -uptake was greatest at the lowest N_2 concentrations. This is in general agreement with results of

experiments such as that illustrated in Fig. 2, which suggests competition between O_2 and N_2 for reducing power.

The inhibition of nitrogen fixation by carbon monoxide

The effect of carbon monoxide upon nitrogen fixation by intact nodules is shown in Fig. 5, which presents data from a series of experiments, in which samples of 1.5 g. fresh weight of nodules were incubated for 1 hr at 25°, with pCO values of 0 to 8 mm.

Fig. 4. Changes of K_m and V_{max} as a function of pO_2 . Data for intact nodules from Bergersen (1962) ○—○, and for bacteroid suspensions from the data of Fig. 3 ●—●. The values given are for s measured in mm. Hg: v for nodules was atoms % excess/hr and for bacteroids, v was $\mu g.$ N/mg. protein/hr. The vertical lines give the magnitude of the 95 % confidence limits.

Hg, a pO_2 of 140 mm. Hg, and pN_2 values of 56 to 140 mm. Hg. Although inhibition by CO was greater at lower pN_2 values, suggesting that inhibition was competitive, it was not possible to apply kinetic treatment because of the slightly stimulatory effect of low pressures of CO. Inhibition by 50 % occurred in the pCO range of 4 to 8 mm. Hg with a pN_2 range of 56 to 140 mm. Hg. There was no effect of CO upon respiration or H_2 evolution in these experiments.

Fig. 5. Inhibition of nitrogen fixation in intact nodules by CO. Fixation measured as atoms % ^{15}N in nodule non-protein-N. $p\text{O}_2$ 140 mm. Hg; $p\text{N}_2$ values (mm. Hg): ■—■ 55, ○—○ 99, ●—● 97 and ⊙—⊙ 140.

Fig. 6. Inhibition by CO of nitrogen fixation by *R. japonicum* bacteroid suspensions with a $p\text{O}_2$ 42 mm. Hg. v = initial fixation rates in $\mu\text{g. N/mg. protein/hr.}$ (a) $p\text{N}_2$ = 70 mm. Hg; data for two experiments. (b) Competitive inhibition shown by the method of Dixon (1953). $p\text{N}_2$ (mm. Hg): ●—● 70 and ⊙—⊙ 140. Data for one experiment.

The inhibition of nitrogen fixation by CO in thrice-washed bacteroid suspensions was studied in experiments at pO_2 42 mm. Hg. The results are illustrated in Fig. 6, which shows that the inhibition was of standard form and that it was competitive (Dixon, 1953). The mean inhibitor constant (K_i , the equilibrium constant of the reversible combination of the nitrogenase with CO) was in these experiments 0.43 ± 0.11 with s and i measured in mm. Hg pressure, and the mean K_m was the same as that obtained in previous experiments with pO_2 42 mm. Hg (Fig. 4). There was no effect of CO upon respiration by bacteroids at concentrations which produced almost complete inhibition of nitrogen fixation. At higher CO concentrations there was partial inhibition of O_2 -uptake which showed some evidence of light reversibility (Table 2).

Table 2. *Effects of CO upon nitrogen fixation and respiration of bacteroid suspensions*

Suspensions incubated for 30 min. at 25°. Gas phase contained O_2 (35 mm. Hg) and $^{15}N_2$ (140 mm. Hg). Nitrogen fixed measured as NH_3 -N after addition of carrier and respiration measured mass-spectrometrically as changes in partial pressure. One atm. = 760 mm. Hg. Results of two experiments. Darkened flasks wrapped in foil; lighted flasks illuminated by Phillips Altrilux lamp (500 W) at a distance of 1 foot above the vessels.

pCO (mm. Hg)	N fixed ($\mu g.$)	Respiration	
		ΔpO_2 (atm.)	ΔpCO_2 (atm.)
0 (dark)	4.71	-81.4×10^{-4}	$+73.2 \times 10^{-4}$
0.06 (dark)	2.88	-80.7×10^{-4}	$+77.7 \times 10^{-4}$
6.0 (dark)	0.18	-81.8×10^{-4}	$+77.8 \times 10^{-4}$
0 (light)	4.87	-111.9×10^{-4}	$+62.0 \times 10^{-4}$
14 (dark)	0	-69.3×10^{-4}	$+54.4 \times 10^{-4}$
14 (light)	0	-84.8×10^{-4}	$+59.7 \times 10^{-4}$
300 (dark)	0	-69.0×10^{-4}	$+57.3 \times 10^{-4}$
300 (light)	0	-92.0×10^{-4}	$+54.3 \times 10^{-4}$

Experiments with cell-free extracts of bacteroids

Bacteroids broken under argon fixed N_2 into NH_3 under strictly anaerobic conditions when supplied with dithionite and an ATP-generating system; unbroken bacteroids did not fix N_2 under these conditions (Table 3). Omission of the dithionite from the broken-cell preparation decreased the activity by more than 90%. In other experiments, omission of the creatine phosphate and creatine phosphokinase decreased nitrogen fixation by 30 to 60% and omission of ATP decreased it to zero.

In some experiments (e.g. Table 3), centrifugation in the chilled rotor of the Spinco, under argon, to remove coarse debris and intact bacteroids, resulted in considerable loss of activity. In a later experiment (Table 4) storage of the broken bacteroids in ice, under argon, resulted in loss of half the activity in 30 min., as compared with the same preparation kept under argon at room temperature (20°). Centrifugation for 30 min. at 100,000 g in an unchilled rotor with the chamber at 20–25° and measurement of fixation by the pellet ($P_{100,000}$) and the supernatant fluid ($S_{100,000}$) showed that the highest specific activity resided in the latter fraction. This amount of fixation corresponded to 70% of the activity of the unfractionated broken bacteroids, kept under argon at 20° for the same time (Table 4).

The time course of fixation was linear for the first 30 min. (Fig. 7a), and these initial rates were used for the calculation of K_m in experiments in which a range of N_2

pressures was used (Fig. 7*b*). Values of K_m with any one extract could be obtained within quite narrow confidence limits (Fig. 7*b*). However, there was considerable fluctuation between values obtained with extracts prepared on different days, even when rigidly standardized methods were used. In four experiments K_m was measured

Table 3. Nitrogen fixation by cell-free extracts of bacteroids

Vessels contained the preparations as listed in a total volume of 3.0 ml. Succinate, 100 μ moles/vessel. ATP-generating (ATP-gen.) system: ATP (Na salt) 6 μ moles; creatine phosphate 150 μ moles; creatine-creatine-phosphokinase 2 mg./vessel, $\text{Na}_2\text{S}_2\text{O}_4$, 24 μ moles/vessel. One atmosphere (700 mm. Hg) was made up of the gases shown and the balance with argon. Preparations were incubated for 1 hr at 25° with shaking.

Preparation	Additions	Atmosphere (mm. Hg)		$\text{NH}_3\text{-N}^*$ vessel (μ g.)	Protein vessel (mg.)	Atoms ^{15}N % excess	N fixed (μ g. N/ mg. protein)
		$p\text{O}_2$	$p^{15}\text{N}_2$				
Whole bacteroids	Succinate	42	70	75	12.9	2.603	151.3
Whole bacteroids	ATP gen. + $\text{Na}_2\text{S}_2\text{O}_4$	0	140	73	12.9	0.006	0.3
Broken bacteroids	ATP gen.	0	140	71	18.2	0.031	1.2
Broken bacteroids	ATP gen. + $\text{Na}_2\text{S}_2\text{O}_4$	0	140	68	20.2	3.786	127.9
5000 g supernatant fluid	ATP gen.	0	140	75	21.0	0.039	1.4
5000 g supernatant fluid	ATP gen. + $\text{Na}_2\text{S}_2\text{O}_4$	0	140	73	21.0	0.801	27.8

* Includes unlabelled carrier $\text{NH}_3\text{-N}$.

Table 4. The distribution of activity in cell-free extracts of bacteroids and the effects of storage at 0°

Vessels contained the extracts shown and, in a total volume of 3.0 ml., ATP (6 μ moles), creatine phosphate (150 μ moles), creatine phosphokinase (2 mg.), $\text{Na}_2\text{S}_2\text{O}_4$ (24 μ moles). Vessels were incubated with shaking at 25° for 1 hr with an atmosphere containing $p^{15}\text{N}_2$ 140 mm. Hg (balance was argon).

Treatment	Protein/ vessel (mg.)	$\text{NH}_3\text{-N}^*$ vessel (μ g.)	^{15}N atoms % excess	Nitrogen fixed (m μ g.)		
				per vessel	per mg. protein	Total prepara- tion
Broken bacteroids 20°; 45 min.	13.8	65	1.435	933	67.8	12,126
Broken bacteroids 0°; 45 min.	13.8	61	0.721	440	32.0	5,718
$P_{100,000} \cdot \frac{1}{2}$	7.7	38	0.192	73	9.5	711
$S_{100,000} \cdot \frac{1}{2}$	7.1	69	1.156	798	113.1	8,425

* Includes carrier $\text{NH}_3\text{-N}$.

as 61.7, 64.5, 95.0 and 118.0 (N_2 pressure in mm. Hg). V_{max} varied from 1 to 2 m μ moles NH_3 /min. mg. protein, higher values being obtained with experiments in which K_m values were low. The variation was too great to permit pooling of data to obtain confidence limits, as was done for bacteroid suspensions. These results show a three- to five-fold increase in K_m for extracts, as compared with the value for intact bacteroids measured in the range of $p\text{O}_2$ in which K_m was unaffected.

The effects of CO were measured in a manner similar to measurements made with

Fig. 7. (a) Nitrogen fixation by a cell-free extract of bacteroids of *R. japonicum*. Vessels contained 11.4 mg. $S_{100,000} \cdot \frac{1}{2}$ protein, ATP (6 μmoles), creatine phosphate (150 μmoles), creatine phosphokinase (0.8 mg.), $Na_2S_2O_4$ (24 μmoles), in a total volume of 4 ml. Incubated with $^{15}N_2$ (119 mm. Hg) with shaking at 25°. (b) Determination of K_m for nitrogen fixation by $S_{100,000} \cdot \frac{1}{2}$ protein. Details as in (a). s measured in mm. Hg and v measured as the initial velocity (0 to 30 min.) in mμg. N/mg. protein/hr. Data for one experiment.

Fig. 8. Inhibition by CO of nitrogen fixation by cell-free extracts of bacteroids of *R. japonicum*. (a) Determination of K_i ; data for one experiment. pN_2 (mm. Hg): ○—○, 140 and ●—●, 35. (b) Plot of data confirming competitive inhibition. i and s measured in mg. Hg pressure. v measured in mμg. N/mg. protein/hr. Other details as described in the text. pCO (mm. Hg): ○—○, 0 and ●—●, 1.3. The intercepts at $1/s = 0$ are not significantly different.

intact bacteroids. Carbon monoxide was a competitive inhibitor of nitrogen fixation in cell-free extracts with a K_i of 0.23 ± 0.02 (mm. Hg), but the lines for the two substrate concentrations intercepted at a low value of $1/v$ (Fig. 8*a*). A double reciprocal plot (Dixon, 1953) was therefore used to confirm the competitive nature of the inhibition (Fig. 8*b*). In all of these experiments, K_m varied between 62 and 77 mm. Hg.

DISCUSSION

The main findings of this work are summarized in Table 5 in which the effects of O_2 and CO upon the kinetics and other properties of the nitrogen-fixing system are compared for nodules, bacteroid suspensions and cell-free extracts of bacteroids. The results, which showed that the activity of cell-free extracts was decreased when stored at 0° , were similar to observations made with extracts of *Clostridium pasteurianum* and *Azotobacter vinelandii* (Dua & Burris 1965; R. C. Burns, personal communication). We have found no evidence of this effect before the disruption of the bacteroids.

Table 5. *Comparison of nitrogen fixation by intact nodules, bacteroid suspensions and cell-free extracts of bacteroids as influenced by oxygen and carbon monoxide*

	Intact nodules	Bacteroid suspensions	Extracts
Effects of O_2	Required; fixation linear with time at all pO_2 values. No fixation below pO_2 3.5 mm. Hg; maximum at 300 to 350 mm. Hg; inhibited at higher pO_2 values	Required; fixation linear with time below pO_2 14 mm. Hg; higher values increase initial rates up to 85 mm. Hg but the time course is shortened, due to inactivation	Causes inactivation; requirement replaced by use of ATP generating system + $Na_2S_2O_4$
K_m (N_2) (mm. Hg)	$50.3 \left\{ \begin{smallmatrix} 37.0^* \\ 7.8 \end{smallmatrix} \right\}$ in air increases at pO_2 above 300 mm. Hg	$20.4 \left\{ \begin{smallmatrix} 10.7^* \\ 33.4 \end{smallmatrix} \right\}$ at low pO_2 increases at pO_2 above 42 mm. Hg	Variable; 61 to 118 (compare 42.5 obtained by Koch <i>et al.</i> 1967 <i>b</i>)
Effects of CO	Inhibitory; 50 % inhibited at pCO 4 to 8 mm. Hg. Less inhibition at higher pN_2 but data not suitable for kinetic analysis	Competitive inhibition: $K_i = 0.43 \pm 0.11 \uparrow$ mm. Hg	Competitive inhibition: $K_i = 0.23 \pm 0.02 \uparrow$ mm. Hg

* 95 % confidence limits. † Range of variation from mean value.

The effects of oxygen upon nitrogen fixation by bacteroids in suspension or in host tissue can be summarized as follows. (1) Oxygen is required, for nitrogen fixation by nodules and bacteroid suspensions, to provide available energy, as shown by the increases in v and V_{max} when the pO_2 value was increased. (2) The nitrogen-fixing system is inactivated by free oxygen. This is seen from the shortened time-course of fixation by isolated bacteroids with increasing pO_2 values and by deliberate aeration during preparation. (3) The apparent affinity of nitrogenase for N_2 is decreased in the presence of oxygen, as shown by the increased K_m with increasing pO_2 . (4) The effects 2 and 3 must be separate, because the time-course of fixation by intact nodules is not shortened at high pO_2 values where K_m is sharply increased (Bergersen, 1962). This implies that the bacteroids are protected in the intact tissue from the inactivating effects of

high pO_2 but are exposed to its kinetic effects (see discussion in Bergersen & Turner 1967). (5) O_2 and N_2 may be regarded as being in competition for reducing power. This competition may not occur at the N_2 -binding site but may be an expression of the presence of two divergent electron-transport pathways, one of which terminates with O_2 and the other with N_2 . The increase in O_2 -uptake which accompanied cessation of fixation by bacteroids (Fig. 2), and the kinetic experiments with bacteroids in which it was found that O_2 -uptake was greatest at the lowest N_2 pressures, illustrate this. Because of the rapid inactivation of bacteroids at high pO_2 values it was not possible to reach concentrations of O_2 equivalent to those which produced inhibition in intact nodules. The nature of the apparent competition, therefore, remains in doubt because the cell-free system with dithionite is incompatible with the use of oxygen as an inhibitor.

Lind & Wilson (1941) showed that carbon monoxide was a specific but non-competitive inhibitor of nitrogen fixation by growing nodulated redclover plants. Except for some studies on the effects of CO upon the leghaemoglobin of nodules (Smith, 1949) and upon hydrogen metabolism (Hoch, Schneider & Burris, 1960; Bergersen, 1963), little work has been done with the legume system. Inhibition of nitrogen fixation by CO has been shown to be competitive with N_2 in cell-free extracts of *Clostridium pasteurianum*, with a K_i of about 0.3 mm. Hg (Lockshin & Burris, 1965). The form of the data for intact nodules shows why the competitive nature of inhibition of nitrogen fixation by CO was not shown earlier. The slightly stimulatory effect of low concentrations (2 to 6 mm. Hg) confounds kinetic treatment of the data. No explanation of this effect is offered; it may be related to binding of CO by the leghaemoglobin of the intact tissue. These effects were avoided when well-washed bacteroids were used and it was clearly shown that CO was a competitive inhibitor. The K_i value obtained (0.43 ± 0.11) is very near to the value obtained by Lockshin & Burris (1965) with *C. pasteurianum* extracts; the value obtained with cell-free Rhizobium bacteroid extracts was slightly lower. When the solubilities in water at 25° of CO and N_2 are compared at the pressures corresponding to the K_i and K_m values for bacteroids (at pO_2 42 mm. Hg), concentrations of $0.54 \mu M$ for CO and $16.8 \mu M$ for N_2 are obtained. That is, the nitrogenase of intact bacteroids has about 30 times the apparent affinity for CO that it has for N_2 .

Studies with intact growing *Azotobacter vinelandii* (Wilson, Burris & Lind, 1942) and *Clostridium pasteurianum* (Westlake & Wilson, 1959) have indicated a K_m for nitrogen fixation of 15 to 23 mm. Hg (0.02 to 0.03 atmosphere). These values are comparable with the value of 20 (Fig. 4) obtained for non-growing Rhizobium bacteroids in the range of pO_2 in which K_m was not affected. The higher value for K_m of 50 to 60, obtained at the lower pO_2 values with intact nodules, is presumed to arise as a consequence of a N_2 concentration gradient extending from the external atmosphere to the bacteroids within the host tissue.

After the work reported here was completed Koch *et al.* (1967*b*) reported a K_m of 0.056 atmosphere (42.6 mm. Hg) for nitrogen fixation by cell-free extracts of Rhizobium bacteroids. This value is less than that obtained by us, but it is substantially higher than the K_m for nitrogen fixation by intact bacteroids. The reported K_m values for cell-free extracts of *Clostridium* and *Azotobacter* are 4 to 10 times higher than values for intact nitrogen-fixing bacteria and in general have been found to be 0.16 to 0.20 atmosphere, i.e. 122–152 mm. Hg (e.g. Mortenson, 1964). Dilworth,

Subramanian, Munson & Burris (1965) showed that this increase in K_m might be due in part to the effects of H_2 when substantial evolution of H_2 occurs or when fixation is measured in the presence of H_2 and acetylphosphate. H_2 is a competitive inhibitor of nitrogen fixation and therefore tends to increase the apparent K_m . In the work with cell-free extracts of *Rhizobium* bacteroids described here, H_2 was always present in the gas phase but only to the extent of 0.1 to 0.2 mm. Hg., concentrations too small to affect K_m .

The nitrogen-fixation reaction may be expressed as follows:

where E is the nitrogenase complex and k_1 , k_2 and k_3 are the respective rate constants. Strictly, $K_m = k_2/k_1$, but in the method used to measure K_m , the relationship between N_2 concentration and rate of NH_3 production is measured and it is assumed that k_3 is not rate-limiting. The following considerations suggest that k_3 cannot be ignored, especially when using cell-free extracts. Carbon monoxide is not reduced by nitrogenase to any significant extent; we have been unsuccessful in a search for any of the likely products of such a reaction. Therefore, in measuring K_i (CO), we have been dealing only with the affinity of the binding-site. This is also the site at which N_2 is bound, because CO is a competitive inhibitor of nitrogen fixation; it is also of the same mass and is iso-electronic with N_2 . We have shown that the binding of CO to nitrogenase is relatively unaffected by disruption of the *Rhizobium* bacteroids because the K_i (CO) was 0.4 for bacteroids and 0.2 for extracts. It is therefore unlikely that the binding-site of the enzyme was affected by disruption of the bacteroids. It is thus possible that the apparent three- to five-fold increase in K_m (N_2) which occurs when the bacteroids are disrupted is due to a decrease in k_3 . That is, the substitution of dithionite and ATP for the natural reducing system leads to less rapid production of NH_3 .

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