The Effects of Partial Pressure of Oxygen upon Respiration and Nitrogen Fixation by Soybean Root Nodules

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SUMMARY

Increased oxygen tension (pO₂) caused increased respiration by excised soybean nodules of all ages. The increase took place in two steps, the first maximum occurring at about 50 % O₂ and the second at 90–100 % O₂ for actively nitrogen-fixing nodules. With increasing nodule age the first maximum occurred at decreasing pO₂ until, when fixation ceased at about 6 weeks, this maximum had disappeared. This effect was more marked at 30° than at 23°. The respiration of bacteroids increased with increasing pO₂ with a maximum at 2–3 % O₂; the curve indicated a simple saturation of the terminal respiratory pathway with O₂. Increased pO₂ raised nitrogen fixation by excised nodules to a maximum which corresponded to the first maximum of the respiratory response to raised pO₂; higher pO₂ than this decreased nitrogen fixation. Sliced nodules showed the same effect but the stimulation of fixation at the lower pO₂ levels was not as great as with intact nodules. The Michaelis constant (Kₘ) for nitrogen fixation by intact excised nodules was relatively unaffected by pO₂ until this reached the pO₂ for maximum fixation when the Kₘ rose sharply. At external pO₂ of 80 %, oxygen was shown to be a competitive inhibitor of nitrogen fixation.

An explanation of these results is offered; it is suggested that the first part of the nodule O₂ consumption/pO₂ curves is due to O₂ consumption by plant tissue and the second part to O₂ consumption by bacteroids. The two components are separated by an O₂ permeability barrier. When this barrier permits a rise in the pO₂ at the bacteroids, nitrogen fixation is inhibited as oxygen competes with nitrogen for the reducing power of the bacteroids.

INTRODUCTION

Allison, Ludwig, Hoover & Minor (1940) concluded from studies of the effects of increased oxygen tension upon nodule respiration that oxygen tension was low within the tissue of soybean root nodules. Ebertova (1959) measured low redox potentials within nodules during the period in which nitrogen was fixed. In contrast to these findings Ferguson & Bond (1954) found that increased oxygen tension in the root medium favoured nitrogen fixation by whole nodulated plants; and Burris, Magee & Bach (1955) found with sliced soybean nodules that oxygen tensions up to 50 % O₂ stimulated fixation but that higher tensions were inhibitory. Bond (1961) reported similar effects with root nodules of non-leguminous plants.

A unified hypothesis which relates the various phases of symbiotic nitrogen fixation in legume nodules has been proposed (Bergersen, 1960b) in which it is suggested that reducing power generated in the bacteroids brings about the ultimate production of NH₃ from N₂. This hypothesis demands that oxygen tension at
the surface of the bacteroids be low (much less than the tension which saturates their terminal respiratory enzymes) if aerobic terminal respiration is not to compete with \( N_2 \) for their reducing power. The work to be reported here was undertaken to investigate the apparent paradox of oxygen both stimulating and inhibiting nitrogen fixation by legume nodules. In doing this the work also provided a test of one aspect of the hypothesis.

**METHODS**

**Plant material.** Lincoln variety soybeans were grown and nodule age recorded as previously described (Bergersen, 1958). Nodules were detached from the roots into beakers immersed in ice and experiments were begun within 1 hr. of picking the first nodule.

**Bacteria.** *Rhizobium japonicum* strain CC711 was used throughout and was grown on yeast-extract mannitol agar. Suspensions of bacteroids (the nodular form of the organisms) were obtained from crushed nodules as previously described (Bergersen, 1958).

**Respirometry.** Respiration was measured in double capillary Warburg manometers equipped with stopcocks between the fluid reservoirs and the capillaries to permit evacuation and flushing of the respirometers with various gas mixtures. In doing this the fluid was lowered to the hole of the stopcock and the respirometers evacuated and filled through both sides of the manometer. When filled, the fluid was raised and a positive pressure maintained until just before measurements began, when the excess pressure was released.

Respiration of whole and sliced nodules was measured in Warburg vessels (volume 30 ml.) containing 20 nodules and 0.25 ml. phosphate buffer (\( \times 15, \text{pH 7-0} \)) with 0.2 ml. 40% (w/v) KOH in the centre wells. Respiration of bacteroids was measured in the same vessels using 1.0 ml. bacteroid suspension and 1.0 ml. of 0.02 m-Na succinate or 1 ml. of phosphate buffer (\( \times 15, \text{pH 7-0} \)).

Preliminary tests showed that little change in pO\(_2\) due to uptake of oxygen resulted during measurement of the respiration rate during 30 min. with bacteroids or during 1 hr. with nodules; with bacteroids the pO\(_2\) fell from 2.0 to 1.8% and from 1 to 0.9%. These small changes produced no detectable change of slope when the uptake of O\(_2\) was plotted against time. With still lower pO\(_2\) values however, a decline in rate could be detected after 20 min. In these cases only the initial rates were used.

In all cases respiration was expressed as qO\(_2\) (\( \mu \text{l. uptake of O}_2/\text{hr./mg. dry-weight tissue} \)). In the first experiment a temperature of 30\(^\circ\) was used. Later this was changed to 28\(^\circ\), the optimum for nitrogen fixation.

**Gas mixtures.** These were prepared from commercial gases through manifolds equipped with mercury manometers. The lines were evacuated by a rotary pump to 0.05 mm. Hg (McLeod gauge) and various scales graduated in percentage were used on the manometers according to the barometric pressure at the beginning of the experiment. Argon was used to bring the mixtures to one atmosphere. The following are mass spectrometric analyses of the gases used:

- **Nitrogen** \( \text{N}_2 \) (98.2%), \( \text{O}_2 \) (0.1%), A (1.7%)
- **Argon** A (98.3%), \( \text{N}_2 \) (8.2%), \( \text{O}_2 \) (0.5%)
- **Oxygen** \( \text{O}_2 \) (98.7%), \( \text{N}_2 \) (1.3%), A (trace)
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For all purposes except the determination of the Michaelis constants, gas mixtures contained 10% N₂.

Isotopic nitrogen (¹⁵N). ¹⁵N₂ gas was prepared from NH₃ (generated from NH₄⁺ labelled NH₄NO₃) by oxidation over CuO at 600–700°C. The gas was passed through the oxidant for 1 hr. after constant volume was reached and was then collected over Hg after being circulated through a liquid N₂ cooled trap where nitrogen-containing impurities condensed. Mass spectrometric analyses showed less than 1% O₂ and no trace of mass 31 (¹⁵NO), 45 or 46 (¹⁵N₂O or ¹⁵NO₂) or 47 (¹⁵NO₃).

Measurement of nitrogen fixation. Nodules were exposed at 23°C to gas mixtures containing ¹⁵N₂ in 50 ml Erlenmeyer flasks attached to a manifold. After exposure, the nodules were crushed in 3 N-HCl and the soluble material analysed for ¹⁵N after Kjeldahl digestion, distillation of NH₃ and conversion of NH₃ to N₂ by alkaline NaOBr containing 0.1% KI (Francis, Mulligan & Wornall, 1959). The atoms % excess ¹⁵N in the samples was a measure of the nitrogen fixation of the nodules during their exposure to the labelled gas mixture.

Mass-spectrometric determinations. All isotope measurements and gas analyses were done with an M86 (Atlas-Werke, Bremen) mass spectrometer. Atoms % ¹⁵N were determined from the mass 29:28 ratios of the samples and the excess calculated from alternative readings of a sample of air N₂ contained in the other half of the double inlet system. All samples and standards were measured at the same inlet pressure (5 mm. Hg). Corrections were made for air leakage occurring during preparation of the samples by measuring the magnitude of the mass 28 and 32 peaks and applying corrections to the atoms % excess ¹⁵N (Francis et al. 1959). In these experiments the maximum analytical error of repeated determinations on the same sample of gas was ± 2% of the atoms % excess. The maximum analytical error of a complete determination, including digestion, distillation, conversion to N₂, measurement and correction was ± 6.5% of the atoms % excess. In any one experiment all values of ¹⁵N excess of samples were adjusted by multiplying by c/x where c was the chosen atoms % excess of the incubation gas mixture and x was the actual value analysed. This step was necessary because contaminating N₂ in the other gases used in the mixture caused small variations in the percentage of ¹⁵N in the mixtures of different composition which were used in an experiment; the step thus put all measurements on a common basis. Gas analyses were prepared from mass-spectra, the relative peak heights of which gave the partial pressures of the components of the mixture.

RESULTS

The relationship between pO₂ and qO₂ for nodules and bacteroids

The effect of nodule size and slicing. The general form of the effects of pO₂ upon nodule respiration is seen in Fig. 1, which illustrates the results obtained with 28-day nodules. When qO₂ was plotted against pO₂ (the qO₂/pO₂ curve) the result was a distinct two-step curve with the first maximum at about 50% O₂ and the second at about 90% O₂. This type of curve was repeatedly obtained throughout this work as long as nodules of a single age were used. The plants from which the nodules used in this experiment were taken fell into two well-defined groups: those with few large (8–4 mm. diam.) nodules and those with many small (1.5–3 mm. diam.) ones. These nodules were of the same age to within one day and each plant bore the same...
Fig. 1. The effects of nodule size and slicing on qO₂/pO₂ curves for nodules. Respiration measured at 30°. ×, 23-day-old nodules: large sliced; ○, 23-day-old nodules: small, whole; ●, 23-day-old nodules: large, whole.

Fig. 2. The effects of nodule age on the qO₂/pO₂ curves for intact nodules, measured at 30°.
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weight of nodules. The effects of this difference in size are seen in Fig. 1. The small nodules had a slightly higher $qO_2$ at each $pO_2$ value and the maximum occurred at slightly lower $pO_2$ than was the case for the large nodules. In all subsequent experiments only the large nodules were used because they were more easily handled.

When the large nodules were sliced (about 1 mm thick) the general form of the $qO_2/pO_2$ curve was still apparent, but the first maximum was not as marked as with the intact nodules and the $qO_2$ at each $pO_2$ value was raised. The magnitude of the first step of the curve was increased by a little more than a third and that of the second step was slightly reduced.

The effects of nodule age on the $qO_2/pO_2$ curve. The $qO_2/pO_2$ curves were determined for nodules of different ages. The results are illustrated in Fig. 2. The two-step curve was found at all ages except 46 days. With young nodules (7–14 days) the two maxima occurred at lower $pO_2$ values than with actively nitrogen-fixing (21–23 days) nodules. As the nodules aged, both maxima occurred at progressively lower $pO_2$. At 46 days the first maximum, when present at all, occurred at less than 5% $O_2$ and nodule respiration was completely saturated with respect to $O_2$ at 10%. From previous work (Bergersen, 1958, 1960a) it is known that fixation ceases when the nodules are about 42 days old. Concurrently with this experiment measurements were made of the respiration of isolated bacteroids and the relative proportions of the various nodule fractions were determined.

The effects of nodule age on the $qO_2/pO_2$ curves for bacteroids. Bacteroids were prepared from nodules aged 8, 15, 22 and 47 days and the respiration measured at a range of $pO_2$ (Fig. 3). The maximum $qO_2$ was attained at 2–3% $O_2$, and the curves are similar to those given by Burris & Wilson (1939). The $qO_2/pO_2$ curves are typical of the saturation of an enzyme with increasing concentrations of its substrate and there was no evidence of a two-step curve as seen with intact nodules. The main
effect of nodule age was that which has already been reported, as variations occurred in the maximum \( q_{O_2} \) which were in close agreement with the results of Bergersen (1958). At all ages the endogenous respiration was one-third of the respiration with succinate as substrate and was very stable, the \( q_{O_2} \) of 22-day-old bacteroids being unaltered after they had been standing in buffer at room temperature for 30 hr.

The effects of age on the composition of nodules. The composition of nodules is given in Table 1. For these determinations the nodules were crushed and the dry weights of the various fractions obtained after centrifugal separation. The nature of the fractions was checked microscopically. The composition became fairly stable from 15 days with the bacteroids making up about 25% of the dry weight and the cell walls and large particles of the host accounting for about 40%.

Table 1. The composition of soybean root nodules of various ages determined on a dry-weight basis

<table>
<thead>
<tr>
<th>Nodule age (days)</th>
<th>Fraction</th>
<th>5</th>
<th>8</th>
<th>15</th>
<th>22</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large particles and cell walls (1)</td>
<td>80</td>
<td>56</td>
<td>43</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Bacteroids (2)</td>
<td>3</td>
<td>19</td>
<td>28</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Small particles (3)</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Soluble (4)</td>
<td>15</td>
<td>23</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
</tbody>
</table>

(1) Sedimented from nodules crushed in buffer at 300 g for 4 min.
(2) Sedimented from the supernatant of (1) by 4000 g for 6–8 min.
(3) Sedimented from the supernatant of (2) by 6000 g for 20 min.
(4) Supernatant from (3).

The effects of \( pO_2 \) upon nitrogen fixation (\( ^{15}N_2 \) uptake)

It has already been shown by Burris et al. (1955) that \( pO_2 \) affects nitrogen fixation by excised soybean nodules, and it was necessary to ascertain that these effects were not due to an increase or a decrease in the time during which N-fixing capacity was retained by the excised nodules. Figure 4 shows that this was not the case, since at 20, 37 and 81% \( O_2 \), fixation was linear with time for at least 90 min. No fixation at all was detected at 5% \( O_2 \); this is in agreement with all the present experiments, in which \( N_2 \) fixation could be extrapolated to zero at about 5% \( O_2 \) for intact nodules. In this experiment, exposure to \( ^{15}N_2 \) commenced 55 min. after the first nodule was detached.

Nitrogen fixation increased with increasing \( pO_2 \) up to 80°–50%, above which it was inhibited. With intact nodules, the rise in fixation with increasing \( pO_2 \) from 10 to 50% was greater than the decrease which occurred from 50 to 80%, but with sliced nodules the inhibition was as great as the stimulation over a similar increment of \( pO_2 \) (Table 2; Fig. 5).

The effects of nodule age upon \( ^{15}N \) uptake with different \( pO_2 \) values. The amount of nitrogen fixed at the optimum \( pO_2 \) varied with nodule age, being greatest at 28–32 days (Table 2), and the optimum \( pO_2 \) decreased from 50% at 32 days to 30% at 40 days.

The relationship between the \( qO_2/pO_2 \) curve and \( ^{15}N_2 \) uptake with varying \( pO_2 \). The
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data in Table 2 clearly show that the \( pO_2 \) range of the first maximum of the \( qO_2/pO_2 \) curve corresponded with the \( pO_2 \) range for maximum nitrogen fixation. Figure 5 illustrates the form of the curves for nodules aged 32 days. This relationship was not found in older nodules (86 and 40 days) when respiration was measured at 30°. In these cases the first maximum of the \( qO_2/pO_2 \) curve occurred at a lower \( pO_2 \) than at 23° (compare Fig. 2 and Table 2).

![Fig. 4](image1.png)

**Fig. 4.** The time course of \( ^{15}N_2 \) uptake by 26 day old intact nodules. ●, 37% \( O_2 \), 61% \( O_2 \); ×, 20% \( O_2 \), 10%; +, 5% \( O_2 \), all gas mixtures contain 10% \( ^{16}N_2 \) (46 atoms %).

**Fig. 5.** The relationship between the \( qO_2/pO_2 \) curve and the nitrogen fixation/\( pO_2 \) curve for intact 32 day-old nodules. Both fixation and respiration measured concurrently at 23°.

*The effects of \( pO_2 \) on the kinetics of nitrogen fixation.* The kinetics of nitrogen fixation by intact nodules were examined by measuring the effect of \( N_2 \) concentration upon the nitrogen fixed in 1 hr. with different \( pO_2 \) values. The \( ^{15}N \) excess of the nodules after 1 hr. was a valid measurement of the velocity \( (v) \) at any particular \( N_2 \) concentration \( (s) \) since fixation was linear with time for at least 90 min. The Dixon (1953) modification of the Lineweaver & Burk (1934) graphical method was then used to determine the Michaelis constant \( (K_m) \) and the maximum velocity \( (V_{max}) \) for nitrogen fixation in the Michaelis-Menten equation

\[
\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max} \cdot pN_2}
\]

by plotting \( 1/v \) against \( 1/s \) at each \( pO_2 \), for nodules aged from 25 to 27 days. The oxygen tension in this work as in the respiratory work was taken as the initial value. The \( N_2 \) concentration was taken as the final \( pN_2 \) measured in a gas sample taken at the end of the incubation. At each \( pN_2 \) for any \( pO_2 \), duplicate determinations of \( ^{15}N \) excess were made on each of two separate samples of nodules.

Statistical methods were necessary in order to exploit the data fully. The following is an outline of the methods of analysis used as a result of examination of the form of the data. The variance of \( 1/v \) increased with \( 1/v \) so that a weighted regression procedure was necessary. To determine the weight function the regression of the
difference between (or range of) the sample means of two duplicates on the average of the means was determined in a relationship of the form: range = \( b_1 (1/v) + b_2 (1/v)^2 \).

This relationship itself was fitted using weights inversely proportional to \((1/\text{range})^2\). The coefficient of the linear term, \( b_1 \), was significant while \( b_2 \) was not, although there is little doubt of the reality of the curvature. However, these data do not conform to a relationship: range = \( b(1/v) \), as suggested by Wilkinson (1961). The weights were taken as the inverse of the squares of the range estimated from the regression relation of any average \( 1/v \). The weighted regression of \( 1/v \) on \( 1/pN_2 \) was fitted using standard least squares procedure. The confidence limits of the intercepts on the \( x \) axis \((-1/K_m)\) and on the \( y \) axis \((1/V_{max})\) were calculated using standard procedures.

### Table 2. The influence of the partial pressure of \( O_2 \) upon respiration and nitrogen fixation of excised soybean nodules of various ages

<table>
<thead>
<tr>
<th>Nodule age (days)</th>
<th>Measurement</th>
<th>Partial pressure of ( O_2 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>23</td>
<td>( qO_2^* )</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>( ^{15}N ) excess†</td>
<td>—</td>
</tr>
<tr>
<td>(atoms %)</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>23 (sliced)</td>
<td>( qO_2^* )</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>( ^{15}N ) excess†</td>
<td>—</td>
</tr>
<tr>
<td>(atoms %)</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>28</td>
<td>( qO_2 )</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>( ^{15}N ) excess†</td>
<td>—</td>
</tr>
<tr>
<td>(atoms %)</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>32</td>
<td>( qO_2 )</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>( ^{15}N ) excess†</td>
<td>—</td>
</tr>
<tr>
<td>(atoms %)</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>36</td>
<td>( qO_2 )</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>( ^{15}N ) excess†</td>
<td>—</td>
</tr>
<tr>
<td>(atoms %)</td>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

\( qO_2 = \mu l/hr/\text{mg. dry wt. nodule. Respiration was measured on 20 nodules in each Warburg vessel; 0.5 ml. m/15 phosphate buffer (pH 7-0), was added to prevent drying out of the tissue; centre wells contained 0-2 ml 20 % KOH.} \)

\(^{15}N\) uptake was measured by analysing the portion of the nodules which was soluble in 3N-HCl after 1\(\frac{1}{2}\) hr exposure at 23° to gas mixtures enriched in \(^{15}N\). Figures for duplicate nodule samples are shown.

* The respiration of the 23-day-old nodules was measured at 30°. All other \(qO_2\) values shown were measured at 23°.

† Corrected \(^{15}N\) excess figures; all results are expressed as if the incubation gas contained 55-8 atoms % excess \(^{15}N\).

The results are given in Table 3. It will be seen that \( V_{max} \) increased with \( pO_2 \). \( K_m \) was only slightly affected by \( pO_2 \) at the lower levels but above 40 % increased very sharply. The intercepts on the \( y \) axis for 60 and 80 % \( O_2 \) were not significantly different but the slope was greater for 80 % \( O_2 \). These results meet the criteria for competitive inhibition of nitrogen fixation by oxygen when comparing 60 and 80 % \( O_2 \). The intercepts on the \( y \) axis for 50 and 60 % \( O_2 \) were just significantly different, but the slope was greater for 60 % \( O_2 \). Thus, comparing these two partial pressures
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of O₂, the results approach competitive inhibition. The Dixon–Lineweaver–Burk plots for these three partial pressures of O₂ are presented in Fig. 6.

Figure 7 shows the change in Kₘ with pO₂ and the change in nitrogen fixation at a constant pN₂ (10 %). These latter figures were determined from the regression diagrams of 1/v on 1/pN₂ at each pO₂. Figure 7 thus shows the change in the kinetics of the nitrogen-fixation reaction in relation to the earlier experiments, in which the effects of pO₂ upon fixation were determined with pN₂ equal to 10 % (Table 2 and Fig. 5).

Fig. 6. The Dixon-Lineweaver-Burk plot for nitrogen fixation by intact 26 day-old nodules at 50, 60 and 80 % O₂. O—O, 80 % O₂; ●—●, 60 % O₂; ×—×, 50 % O₂.

Fig. 7. The relationship between the change in Kₘ with pO₂ and the change in fixation (v) with pO₂ when the gas phase contained 10 % ^15N₂.

Table 3. Data from the Lineweaver–Burk plots of nitrogen fixation at different pO₂ values. The intercepts on the x axis gave −1/Kₘ and on the y axis 1/Vₘₙₐₓ.

<table>
<thead>
<tr>
<th>Initial pO₂ (%)</th>
<th>1/Vₘₐₓ Estimate</th>
<th>95 % limits</th>
<th>Vₘₐₓ Estimate</th>
<th>95 % limits</th>
<th>−1/Kₘ Estimate</th>
<th>95 % limits</th>
<th>Kₘ Estimate</th>
<th>95 % limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>3.4157</td>
<td>3.9365</td>
<td>2.5058</td>
<td>1.1769</td>
<td>0.7245</td>
<td>0.5660</td>
<td>0.4433</td>
<td>0.2927</td>
</tr>
<tr>
<td>30</td>
<td>2.5058</td>
<td>3.1868</td>
<td>0.3991</td>
<td>0.3384</td>
<td>1.8712</td>
<td>18-2242</td>
<td>0.0842</td>
<td>0.0549</td>
</tr>
<tr>
<td>40</td>
<td>1.7690</td>
<td>1.3145</td>
<td>0.8393</td>
<td>1.1915</td>
<td>7.8666</td>
<td>12-7561</td>
<td>0.1269</td>
<td>0.0784</td>
</tr>
<tr>
<td>50</td>
<td>0.7245</td>
<td>0.9440</td>
<td>1.3002</td>
<td>1.0593</td>
<td>3.7775</td>
<td>5.4518</td>
<td>0.2647</td>
<td>0.1834</td>
</tr>
<tr>
<td>60</td>
<td>0.5660</td>
<td>0.7168</td>
<td>1.7667</td>
<td>1.3951</td>
<td>2.1954</td>
<td>8-0111</td>
<td>0.4554</td>
<td>0.3321</td>
</tr>
<tr>
<td>80</td>
<td>0.4036</td>
<td>0.6247</td>
<td>2.4777</td>
<td>1.6008</td>
<td>0.9402</td>
<td>1.5798</td>
<td>1.0636</td>
<td>0.6380</td>
</tr>
</tbody>
</table>
DISCUSSION

There are intriguing effects of oxygen tension both on nodule respiration and on nitrogen fixation. In discussing these results it is convenient to divide this part of the paper into sections, but it should be realized that the results are composed of inter-related observations.

The effects of $pO_2$ on nodule respiration. In the widest sense the results presented are in agreement with the work of others (e.g. Allison et al. 1940; Ebertova, 1959) who have concluded that the oxygen tension in legume root nodules in air is low. This is shown by the 2–3-fold increase in $qO_2$ when the external $pO_2$ is increased from air tension (20%) to 90%. The additional information which has come from the present work is that this increase in respiration is not simple. The failure of others to observe the two-step nature of the $qO_2/pO_2$ curve can be attributed largely to the fact that no precautions were taken about nodule age. Nodules of a range of ages would have the first maximum at different $pO_2$ values and hence the whole curve would tend to be smoothed out. An explanation of the $qO_2/pO_2$ curve is offered.

Interpretation of the nodule respiration data. From studies of the anatomy and cytology of soybean nodules the bacteroids are seen to be the innermost component since in the central tissue in which they occur they are enclosed within membrane envelopes in the cytoplasm of the host cells (Bergersen & Briggs, 1958). It may also be noted again that the tissues of soybean nodules are uniform in cell age since these nodules have no growing point (Bergersen, 1958). From these considerations and assuming (i) that bacteroids within the nodules have a $qO_2$ similar to their endogenous $qO_2$ as measured in the Warburg; and (ii) that root tissue has a similar $qO_2$ to the plant tissue component of nodules, it is possible to offer an explanation of the unusual pattern of nodule respiration with increasing $pO_2$. Because the bacteroids are the innermost respiring component it is logical to suggest that their respiration is the last to be saturated with respect to $O_2$ as the external $pO_2$ increases; that is to say, the second step of the $qO_2/pO_2$ curve for the nodules represents the bacteroid respiration increasing to a maximum with increased penetration of $O_2$. Table 1 shows that for nodules aged 22 days the bacteroids composed 25% of their dry weight. Figure 3 shows that the maximum respiration of bacteroids isolated from these nodules occurs at 2–3 % $O_2$. If we now consider the data for nodules aged 21 days (Fig. 2), the bacteroids (22-day sample, Fig. 3) respiring at the maximum endogenous rate would account for 2.5 $\mu l./hr.$ of $O_2$ uptake. This is the magnitude of the second step of the $qO_2/pO_2$ curve (Fig. 2, 21 days).

Turning to the plant tissue component of the nodule respiration, it is seen that this is made up of the respiration of the cortical tissue and the central (bacteroid-containing) cells, the peripheries of which contain mitochondria (unpublished electron microscope observations). It was found that 23-day soybean root segments had a $qO_2$ of 2.52 at 20 % $O_2$ and 3.86 in 100 % $O_2$. These values are close to those of Allison et al. (1940), namely, 2.12 and 2.88, respectively. They support the suggestion that the first part of the $qO_2/pO_2$ curve represents the saturation with $O_2$ of the plant tissue respiration of the nodule, because 75 % of the nodule dry weight is plant tissue and 75 % of 3.86 (the maximum $qO_2$ of root tissue) is close to the magnitude of the first maximum of the curve for these nodules (Fig. 2). The suggested explanation is summarized in Fig. 8, which shows how the nodule $qO_2/pO_2$ curve
Nitrogen fixation by soybean root nodules could be the sum of the respiration of the plant tissue and of the bacteroids as the respirations of these components are successively saturated with O₂.

Assuming that this interpretation is correct, it is also possible to deduce the internal pO₂ at various external oxygen tensions. These deduced values are shown on the lower scale of Fig. 8.

![Graph of Fig. 8](image)

**Fig. 8.** The explanation of the qO₂/pO₂ curve for nodules. The observed curve is considered to be the sum of the curves for the plant tissue and the bacteroids as these are successively saturated with respect to O₂. ——, Observed respiration 1 mg. nodules aged 21 days; ---, observed respiration 0.75 mg. root aged 21 days; •••••, calculated respiration 0.25 mg. bacteroids.

This explanation of the nodule respiration data implies that there is an O₂ diffusion barrier between the host respiration and that of the bacteroids. The latter, with their high respiratory rate (about five times higher than that of the root tissue cells in terms of the dry weight) would respire any O₂ passing the outer respiring regions of the nodule as, with increasing external pO₂, these regions approached saturation with respect to O₂. There would be no break in the curve unless there was a further barrier which did not permit the passage of appreciable amounts of O₂ until a certain partial pressure had been exceeded. This diffusion barrier may be the membrane envelopes within which the bacteroids are enclosed (Bergersen & Briggs, 1958).

**The effects of pO₂ upon nitrogen fixation.** The data of Table 2 fully agree with those of Burrus et al. (1955) who found similar responses to increased pO₂ with sliced nodules. However, in my work there was considerably less variation in ¹⁵N₂ uptake than was the experience of these authors or of Aprison, Magee & Burrus (1954), who found it necessary to use sliced nodules to decrease variation. This decreased variability of nitrogen fixation in the present work is attributable once again to the use
of nodules of a single age in any one experiment. The use of nodules of mixed ages and of successive nodule samples from ageing plants will give rise to errors because the shape of the fixation/pO$_2$ curve changes with age.

The nature of the stimulation of nitrogen fixation with increased pO$_2$ up to about 50% in any one nodule sample remains obscure. The increased respiration of the host tissues in this pO$_2$ range may provide more substrates to the bacteroids or more acceptors for the fixed nitrogen. There is a significant correspondence between the first maximum of the qO$_2$/pO$_2$ curve and the optimum pO$_2$ for nitrogen fixation (Fig. 5). If the interpretation of the respiratory data which has been offered is correct, it would therefore seem that the penetration of O$_2$ into the vicinity of the bacteroids results in inhibition of nitrogen fixation. This has been confirmed by the kinetic data.

Parker & Scutt (1960) showed that, within certain limits, O$_2$ and N$_2$ competed as terminal hydrogen acceptors in nitrogen fixation by *Azotobacter vinelandii* and thus fixation may be regarded as a form of respiration. That a similar phenomenon might account for the inhibition of nodule nitrogen fixation at high pO$_2$, described by Burris *et al.* (1955), has been suggested by Bergersen (1960b). The kinetic studies reported here show that there was in fact a competitive inhibition of nitrogen fixation by O$_2$ at high external pO$_2$. The value for $K_m$ of 0.07 atmosphere N$_2$ for 20% O$_2$ is higher than the 0.025 obtained by Burris *et al.* (1955), probably because they used sliced rather than intact nodules. The Michaelis constant ($K_m$) also rose sharply in a range at which an internal O$_2$ diffusion barrier became permeable. The competitive nature of the inhibition is not necessarily due to a direct competition for the active site of the nitrogen-reducing enzyme. Any diversion of reducing power from any part of an electron transport chain could have a competitive effect. Thus, diversion of the bacteroid-reducing power to aerobic respiration could have a competitive effect even if the actual reduction of nitrogen or nitrogen containing intermediate compounds took place at a site remote from the bacteroids but linked with them by an electron transport chain. The results reported in the present work therefore support the hypothesis proposed elsewhere (Bergersen 1960b), that one of the main functions of the bacteroids in nodule nitrogen fixation is that they are a source of reducing power which is used for the ultimate reduction of N$_2$ to NH$_3$.

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