3. As reported by Virtanen (1945), haemoglobin could not be detected in nodules produced by ineffective strains of *Rhizobium*. The amount of haematin in such nodules, estimated as pyridine haemochromogen, was much less than that in effective nodules.

4. Effective and ineffective strains of *Rhizobium*

grown in pure culture differ little in the ratios of haematin/cell nitrogen.

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EXPLANATION OF PLATE 6

Fig. 1. Section through an effective soya nodule (*Rhizobium*, strain 505) showing the bacteria-containing tissue. (Iron haematoxylin and orange G.) ($\times 800$.)

Fig. 2. Part of a section of a bean nodule treated with benzidine and hydrogen peroxide in acid solution. ($\times 100$.)

Fig. 3. The central tissue of a soya nodule before the develop-

ment of detectable amounts of haemoglobin. (Iron haematoxylin.) ($\times 800$.)

Fig. 4. The central tissue of a soya nodule after the appearance of haemoglobin, showing well-developed bacteria-containing cells. (Iron haematoxylin.) ($\times 800$.)

Fig. 5. Section through an ineffective soya nodule (*Rhizobium*, strain 507) showing the bacteria-containing tissue. (Iron haematoxylin and orange G.) ($\times 800$.)

Haemoglobin and the Oxygen Uptake of Leguminous Root Nodules

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The relation between the partial pressure of oxygen and the functioning of the leguminous root nodule is twofold, as oxygen affects both nodular development and the rate of nitrogen fixation by the mature nodule. Nodules formed in the almost complete absence of oxygen are small, white, and contain no haemoglobin (Virtanen, 1947). They are also deficient in vascular strands, but do not undergo the cellular disintegration typical of ineffective nodules. Thornton (1930) has shown that such nodules fix little nitrogen. The effect of depriving normal mature nodules of their oxygen supply has been studied by Golding (1903) and by Virtanen & von Hausen (1935, 1936), who found that uptake of gaseous nitrogen in nutrient solution cultures ceased in the absence of an oxygen supply to the nodulated roots, while uptake of combined nitrogen was independent of root

aeration. Wilson & Fred (see Wilson, 1940) have produced quantitative results demonstrating the effect of growing entire clover plants in partial pressures of oxygen (pO_2) varying between 0.012 and 0.6 atm. They found that reduction of the pO_2 down to 0.012 atm. was accompanied by a proportional decrease in the uptake of gaseous and combined nitrogen. Their experiments, however, are not directly comparable with those of Golding (1903) and Virtanen & von Hausen (1935, 1936), in which the pO_2 would probably be much lower than 0.012 atm. and the green parts of the plants were in an atmosphere containing 20 % oxygen.

Thus, apart from its effect on the development of the nodule, oxygen is concerned in the process of nitrogen fixation. It may merely be involved indirectly through the release of energy by the oxida-

tion of carbohydrates, or it may in addition enter directly into the fixation mechanism.

A fair amount of information is available concerning the respiration of nodules. Two important experimental results must be kept in mind when considering this work (Wilson, 1940); (1) Bacteria from crushed nodules, separated from the tissue debris, behave, so far as their respiration is concerned, in a manner identical with those from pure cultures with the exception that the former, in the case of the faster-growing group of *Rhizobia*, attain their maximum rate of oxygen uptake at a lower pO_2 than do bacteria grown in pure culture. (2) *Rhizobium* in pure culture will grow well at a pO_2 of less than 0.01 atm.

The relative rates of respiration of nodules and roots appear to vary within fairly wide limits. All Q_{O_2} values in this paper are in $\mu\text{l./mg. dry wt./hr.}$ Values for the ratio Q_{O_2} nodules/ Q_{O_2} roots between 0.5 and 3.1 have been reported by different workers (Allison, Ludwig, Hoover & Minor, 1940; Asprey & Bond, 1941; Wilson, 1940). Allison *et al.* found that sliced or crushed nodules had a much higher Q_{O_2} than whole nodules and that the respiratory quotient of nodules respiring in air was well in excess of 1, the value of the latter increasing with the size of nodule. Such results show that the pO_2 within the nodule must be very low and that oxygen uptake must be determined largely by the rate of diffusion of the gas through the nodule tissue. This is not a surprising fact considering that most nodules are large spheres, several mm. in diameter, containing cells packed with bacteria.

In view of this, the possibility that the nodule haemoglobin may have some effect on oxygen uptake by the nodular tissue at these low partial pressures of oxygen must be considered. Three ways in which haemoglobin could have such an effect suggest themselves.

(1) Haemoglobin might act as a store of oxygen. However, a simple calculation shows that haemoglobin could not act as such a store over any length of time. From the data presented in the preceding paper (Smith, 1949) it can be calculated that the haemoglobin in 1 g. (fresh wt.) of the bacteria-containing cells of soya nodules, if completely oxygenated, would contain 11.2 $\mu\text{l.}$ of oxygen. The mean Q_{O_2} of this tissue may be taken as 2. Consequently this amount of stored oxygen would be used up in 3.4 min. Small amounts of haemoglobin can serve as reservoirs of oxygen over such short periods of time in the case of organisms dependent upon an intermittent supply of oxygen (e.g. *Arenicola marina*, Barcroft & Barcroft, 1924). There is no evidence, however, that the oxygen uptake of nodules is intermittent.

(2) Were the pigment able to move about within the cells, haemoglobin might act as a carrier of

oxygen, but there is no reason to believe that any such movement could occur. It is difficult to see how stationary haemoglobin can have any direct effect upon a diffusion gradient or dissolved oxygen within the nodule after a steady state has been reached.

(3) The presence of haemoglobin might directly affect the rate of combination of oxygen with respiratory enzymes over the range of values of pO_2 at which these enzymes are not saturated with oxygen. This could bring about an increase in the overall uptake of oxygen by the nodule even where this is dependent on the rate of diffusion into the nodular tissue.

If C_o is the external concentration of oxygen, C_s is the concentration of oxygen at a respiratory site, R is the rate of oxygen uptake by enzymes at this site; then, if the enzymes are not saturated with oxygen, the rate of oxygen uptake is nearly proportional to C_s , so that if K is a constant,

$$R = KC_s. \quad (1)$$

And if r is the effective length of the diffusion path from the exterior to the respiratory site and D is the diffusion constant

$$R = D(C_o - C_s)/r. \quad (2)$$

Combining (1) and (2)

$$R = \frac{C_o}{r/D + 1/K}.$$

The possibility under consideration is that the presence of haemoglobin may increase K . This will have an effect upon R , the magnitude of which will depend on the relative magnitudes of $1/K$ and r/D .

This last possibility is apparently supported by certain experimental results. Kubo (1939) found that addition of nodule haemoglobin to *Rhizobium* cells brought about an increase in the rate of respiration at low partial pressures of oxygen. Little & Burris (1947) found a similar effect in the case of *Rhizobium* grown both in the nodule and in pure culture, and a number of other bacteria (*Escherichia coli*, etc.), using a pO_2 of 0.01 atm. Hog haemoglobin had an effect similar to that of the nodule haemoglobin.

The first part of this paper is concerned with diffusion and the oxygen uptake of nodules, the second with the relation of haemoglobin to oxygen uptake—in this section the experiments of Kubo (1939) and of Little & Burris (1947) are discussed.

EXPERIMENTAL METHODS

Nodule material. Legumes were grown in pots containing sterilized soil to which had been added a suspension of cells of the appropriate strain of *Rhizobium*. Sufficient precaution against contaminant infection was obtained by placing plants inoculated with different strains of *Rhizobium* in separate parts of the glasshouse. (Groups of forty plants of each species grown in uninoculated soil simultaneously within the same glasshouse produced no nodules.) Nodules

were normally harvested when the plants were just beginning to flower. They were detached from the plant, washed thoroughly, surface moisture was removed with filter paper, and the nodules were weighed.

Rhizobium suspensions. Strains of *Rhizobium* were grown in Roux flasks at 30° on agar medium (K_2HPO_4 , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; NaCl, 0.2 g.; $CaCl_2$, 0.2 g.; $FeCl_3$, 0.001 g.; Difco yeast, 5 g.; agar, 15 g.; distilled water to 1 l.) in which a yeast-extract preparation was the sole source of carbon and nitrogen. With this medium gum production, which causes inconvenience in the handling of the suspensions, was reduced to a minimum.

Crystalline horse haemoglobin. A solution of lyzed horse red blood corpuscles was shaken with ether and centrifuged. The lower layer of oxyhaemoglobin solution was sucked off and dialyzed. Ethanol was added to give a concentration of 20% and the solution left in the ice chest until the haemoglobin had crystallized. Before use the haemoglobin was dialyzed to remove ethanol.

Measurement of the oxygen uptake of detached nodules. O_2 uptake was measured in Barcroft differential manometers at 28°. The plant material was usually suspended in 3 ml. of Medium 1. CO_2 was absorbed by KOH papers in the centre wells of the flasks. All volumes are expressed as μ l. of gas at N.T.P.

Medium 1 was as follows: K_2HPO_4 , 0.8 g.; KH_2PO_4 , 0.2 g.; NaCl, 0.2 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; $CaSO_4 \cdot 2H_2O$, 0.1 g.; $Fe_2(SO_4)_3 \cdot 9H_2O$, 0.01 g.; glucose, 10 g.; distilled water to 1 l.; pH 7.3.

RESULTS

Diffusion and the oxygen uptake of nodules

The path of gas uptake in nodules. While previous authors (Allison *et al.* 1940) appear to have assumed that gases enter the nodule by diffusion in aqueous solution through the wet cell walls, there is also the possibility that small pores may exist in the outermost layer of cells communicating with the intercellular spaces within the nodule. Gases could then diffuse in directly. In nature nodules are not normally submerged in fluid, but are either more or less dry on the surface or coated with a thin film of moisture. If pores existed in the outermost layer, entry of gases would then be much more rapid under natural conditions than when the nodule was immersed in a fluid in equilibrium with the atmosphere. It is therefore necessary to know how gases do in fact enter the nodule in order to interpret data obtained from experiments on nodules immersed in fluid in manometers.

Sections of nodules of various sizes were examined. A small number of air spaces were seen within the nodule, but none of these appeared to communicate with the exterior. Surface strips of nodules did not reveal any pores or gaps in the outer layer of cells.

Manometric measurements were made of the oxygen uptake of nodules totally immersed in water, and of the same nodules when directly in contact with the gas phase. For this purpose nodules were placed in Barcroft manometers together

with a small amount of water (0.2 ml.) which prevented spurious readings being caused by changes in the vapour pressure within the flasks due to absorption of water vapour by the alkali in the centre well. These nodules were in direct contact with air in the manometer flasks. Readings were taken for 30 min., after which 3 ml. of water were added to each flask and readings were taken of the O_2 uptake of the nodules when submerged. The manometric constant was recalculated to allow for the relative change in liquid and gas volumes. It had been previously established that the O_2 uptake of nodules, when kept under constant conditions, did not vary over a period of at least 60 min. Measurements on four samples of small soya nodules gave values of 1.67, 1.60, 1.45 and 1.54 for the ratio O_2 uptake of submerged nodules/ O_2 uptake of nodules directly in air, with a mean of 1.55.

This increase in oxygen uptake, observed when nodules were immersed in a fluid shaken so as to be in equilibrium with the atmosphere, was not to be expected if gases are able to diffuse directly through pores into the nodule. The observed effect may perhaps be explained by the small surface available for exchange of gas between gas and liquid phase at the surface of nodules suspended in air, as compared with that at a constantly changing surface of shaking fluid.

It may thus be concluded that gases enter the nodule by passage in solution across the wet walls of the outer layer of cells. While rates of oxygen uptake of nodules measured when immersed in water are somewhat greater than natural rates, they correspond to a known pO_2 at the surface of the nodule.

The Q_{O_2} of nodules of different sizes and of nodule slices. From some experiments, in which the rates of oxygen uptake of various nodules and nodule slices were measured, it was possible to obtain an estimate of the extent to which gas uptake of nodules is determined by diffusion.

The oxygen uptake of soya nodules (*Rhizobium*, strain 505) was followed manometrically in air and in oxygen at 28°. After washing and drying, the nodules were graded by size into three samples, small, medium and large, and suspended in medium 1. The values obtained are given in Table 1.

Table 1. Rates of oxygen uptake of soya nodules of various sizes (*Rhizobium*, strain 505)

(The values of mean volume, radius and surface area were calculated from the weight of fresh nodule (density = 1). About 50 nodules of each size were taken.)

	Small nodules	Medium-sized nodules	Large nodules
Mean volume of nodule (cu. mm.)	2.99	6.38	10.1
Mean radius of nodule (mm.)	0.895	1.15	1.35
Mean surface area of nodule (sq. mm.)	10.1	16.6	22.9
Q_{O_2} in air	1.26	1.05	0.99
Q_{O_2} in oxygen	3.29	2.82	2.65
			38

Similarly, the Q_{O_2} of whole soya nodules (strain 505) and that of nodule slices of various thicknesses were measured at 28° (Table 2). Nodules were sliced with a razor and the slices allowed to stand in distilled water for about 20 min. before the experiment. During the experimental period (about 20 min.) the rates remained constant showing no indication of falling off. This, together with the fact that the slices were previously thoroughly washed with water, discounts the possibility that the high Q_{O_2} values of the thin slices of tissue were due to substances liberated from the cells on cutting the tissue. Such high values of Q_{O_2} are not uncommon with plant tissues such as young roots.

Table 2. Q_{O_2} of whole nodules and nodule slices

(Soya nodules, *Rhizobium*, strain 505. The same nodule sample was used for experiments in air and in oxygen.)

	Whole nodules	Nodule slices (1 mm. thick)	Nodule slices (<0.3 mm. thick)
Q_{O_2} in air	1.32	3.59	7.01
Q_{O_2} in oxygen	3.56	8.25	7.76

The enzymes concerned in the uptake of oxygen are saturated at a pO_2 far below 0.2 atm., so that an increase brought about by increasing the pO_2 above this value shows that diffusion is to some extent determining the rate of oxygen uptake. Only in the case of nodule slices less than 0.3 mm. in thickness is there practically no such effect on increasing the pO_2 . The value for the Q_{O_2} of the nodule tissue, when independent of the rate of diffusion, may thus be taken as 7–8. Whole nodules have values very much less than this, and furthermore the value of Q_{O_2} decreases as the size of the nodule increases (and thus the ratio surface area/volume decreases). It must be concluded that the rate of uptake is largely determined by diffusion of oxygen within the nodule.

The presence of haemoglobin and oxygen uptake

Two methods of approach were used to find out whether haemoglobin takes any part in oxygen uptake of nodules by virtue of its oxygen-binding capacity. First the rates of oxygen uptake by effective nodules and ineffective nodules (which contain no haemoglobin) were compared, and secondly the effect of carbon monoxide upon oxygen uptake by effective nodules (both detached and attached to the plant) was investigated.

The oxygen uptake of effective and ineffective nodules. The rates of oxygen uptake of detached nodules produced by the effective *Rhizobium* strain 505, and of nodules produced by the ineffective *Rhizobium* strain 507, were measured manometrically at 30°.

The values of Q_{O_2} of thin slices of these two types of nodule were also compared (Table 3). The whole nodules were selected so as to be of uniform size. Plants bearing the two types of nodule were grown under identical conditions.

Table 3. *Oxygen uptake of effective and ineffective nodules*

Material	Medium	Oxygen uptake	
		Q_{O_2} in air	Q_{O_2} in oxygen
Whole effective nodules	0.05 M-phosphate buffer, pH 7.3 + 1% glucose	1.50	—
Whole ineffective nodules		1.69	—
Sliced effective nodules (<0.3 mm. thick)	Medium 1	—	7.76
Sliced ineffective nodules (<0.3 mm. thick)		—	6.84

The rates of oxygen uptake of whole effective and ineffective nodules, when expressed on a dry weight basis, are equal within the limits of error due to varying sizes of nodule. (This is also true when the results are expressed on a fresh weight basis.) The rates of oxygen uptake by thin slices of these two types of nodule in oxygen (where diffusion of gases within the tissue played no part in determining the rate) did not differ greatly, that of ineffective nodule slices being slightly lower. This result is in contrast to that reported by Asprey & Bond (1941), who found values of Q_{O_2} of whole effective soya nodules (strain 505) to vary between 2.31 and 6.25, while that of ineffective soya nodules (strain 507) was only 0.7. It may be that their ineffective nodules were older, and in a more advanced state of disorganization. In so far as effective and ineffective nodular tissues are comparable, the results (Table 3) show no indication that the haemoglobin in effective nodules affects oxygen uptake.

Carbon monoxide and the oxygen uptake of nodules. By converting all or most of the nodule haemoglobin (Hb) to carboxyhaemoglobin (HbCO) its property of reversible oxygenation is removed, and from the resulting rate of respiration it can be seen whether this property plays any part in determining the normal oxygen uptake of nodules.

The maximum partial pressure of carbon monoxide (pCO) necessary to obtain a ratio HbCO/total Hb equal to 0.5 may be approximately calculated from data given by Keilin & Wang (1945).

In the equilibrium $HbO_2 \rightleftharpoons Hb + O_2$

$$K_1 = [Hb] \cdot pO_2 / [HbO_2].$$

In the equilibrium $HbCO \rightleftharpoons Hb + CO$

$$K_2 = [Hb] \cdot pCO / [HbCO].$$

If C is the total concentration of haemoglobin in all forms

$$C = [\text{Hb}] + [\text{HbO}_2] + [\text{HbCO}].$$

When $[\text{HbCO}]/C = 0.5$

$$p\text{CO} = K_2 + \frac{K_2}{K_1} \cdot p\text{O}_2.$$

From Keilin & Wang's data

$$K_1 = 0.1 \text{ mm. Hg,} \\ K_2 = 0.0027 \text{ mm. Hg.}$$

According to Keilin & Wang K_1 may be less than 0.1 mm. but the difference does not alter the calculated value of $p\text{CO}$ to any significant extent.

Taking the maximum value of $p\text{O}_2$ which is found at the surface of the nodule in air $p\text{O}_2 = 152$ mm. mercury, the maximum value of $p\text{CO}$ is calculated to be 0.0055 atm. (0.55 % CO). With 5 % CO practically all the haemoglobin would be in the form HbCO .

The effects of these concentrations of carbon monoxide (which are below those affecting cytochrome oxidase or other respiratory enzymes) on the oxygen uptake of excised nodules and of nodules attached to the plant were studied.

The effect of carbon monoxide on the oxygen uptake of detached nodules. Whole nodules, and in some experiments sliced nodules and roots for comparative purposes, were suspended in Medium 1 in Barcroft

no inhibitory effect on oxygen uptake by detached nodules. (In fact there was often a slight accelerating effect. This will be discussed later, together with some experiments on bird red blood cells.)

Carbon monoxide and the oxygen uptake of nodules attached to the plant

A striking and at present inexplicable fact of symbiotic nitrogen fixation is that, after their separation from the rest of the plant, nodules and nodulated roots lose all but a trace of their nitrogen-fixing ability. Nodules when excised are clearly different in some way from those still attached to the plant. It was thought that this difference might be reflected in the response of their oxygen uptake to poisoning of the haemoglobin with carbon monoxide. With this end in view the experiments of the last section were repeated using intact nodulated plants.

Experimental method. While Bond (1939) has measured the CO_2 output of nodulated roots attached to the plant, similar measurements of O_2 uptake cannot be traced in the literature.

Fig. 1. Apparatus used in the measurement of oxygen uptake of nodulated roots attached to the plant.

Table 4. *Oxygen uptake of whole soya nodules (Rhizobium, strain 505)*

(q_{O_2} is the oxygen uptake expressed as $\mu\text{l./mg.}$ of fresh tissue/hr. Temp. 28° .)

q_{O_2} in air	0.134	0.155
q_{O_2} in air + CO (0.0055 atm.)	0.172	0.172

Table 5. *Oxygen uptake of Vicia faba nodules (effective Rhizobium, strain 2195) sliced nodules and roots 30°*

	Whole nodules	Whole nodules	Sliced nodules	Roots	Roots
Rate of O_2 uptake in air ($\mu\text{l./hr.}$)	80	117	150	102	144
Rate of O_2 uptake in 20 % O_2 , 5 % CO , 75 % N_2 ($\mu\text{l./hr.}$)	80	122	156	84.6	123

manometers at 28 or 30° , and their rate of oxygen uptake measured in air. The flasks were filled with gas mixtures, containing 20 % oxygen in nitrogen with varying concentrations of carbon monoxide, and readings continued. Readings in the gas mixture containing carbon monoxide were continued over 40 min. (Tables 4 and 5).

Carbon monoxide in concentrations up to 5 %, when all the Hb would be converted into HbCO , had

For this purpose the simple apparatus (Fig. 1) was devised. The plant was held tightly in the apparatus by means of the split bored rubber bung, so that the nodulated roots projected into the cylindrical glass vessel A (volume, about 20 ml.). This vessel, together with the syringe B (connected by a glass capillary and rubber tube), was filled with nutrient solution saturated with a gas mixture, and all gas bubbles were expelled through tap C which was then closed. There was then no gas phase in contact with the fluid surrounding the roots. The apparatus was attached to a holder which fitted into the shaking mechanism of the manometer bath. By this means the vessel A was given a horizontal oscillatory movement so that the fluid within was adequately stirred by a number of large rolling glass beads placed in A. Fluid in B was not in equilibrium with that in A owing to the narrow capillary connecting A and B.

From time to time samples of the fluid in *A* were removed through a short piece of sealed rubber tubing *E* into a syringe, devised by Roughton & Scholander (1943), the capillary end of which had been cemented to a no. 20 hypodermic needle (Fig. 2). As the sample was removed from *A* it was replaced by an equal volume of fluid, containing the initial amount of dissolved gas, from the syringe *B*. (The plunger of this syringe was kept under a slight positive pressure by a rubber band.) Allowance was made for this replacement in the calculation of the results. (Transpiration by the small

Fig. 2. Modified Roughton-Scholander syringe.

clover plants used was negligible over the period of the experiment.) The dissolved gases in the sample (about 0.4 ml. fluid) were extracted with CO_2 in the main part of the syringe, the CO_2 absorbed with KOH , and the bubble of extracted O_2 and N_2 pushed into the capillary of the syringe where its volume was measured (after immersion of the syringe in a bath of water at room temperature). The O_2 was then absorbed by alkaline $\text{Na}_2\text{S}_2\text{O}_4$ and the bubble of N_2 measured. This analytical procedure is described by Roughton & Scholander (1943). During the shaking operations the needle was closed with a small piece of spongy rubber.

During the course of these experiments the $p\text{O}_2$ was constantly changing, as no provision was made for the replacement of the O_2 used. It was found experimentally that, above a certain value, changes in $p\text{O}_2$ did not affect the rate of O_2 uptake by nodulated roots of small clover plants. (This would not be true of thick roots owing to diffusion

Fig. 3. Oxygen uptake of nodulated clover roots attached to the plant, with and without CO ($p\text{CO} = 0.02$ atm.)

effects.) It was found convenient to fill the apparatus at the start of an experiment with Medium 1 (without glucose) approximately saturated with a gas mixture containing 80% O_2 and 20% N_2 . In filling the apparatus this was first washed with the gas mixture, passed through via the sampling tube *E*, and then the medium saturated with the gas mixture was passed through. The tube *E* was then closed by the piece of sealed rubber tubing and any gas bubbles expelled through tap *C*.

Values of the rate of oxygen uptake of pieces of plant tissue measured in the apparatus and in Barcroft manometers agreed to within 2%.

Experiments. Those roots bearing few or no nodules were removed from a well-nodulated clover plant (inoculated with *Rhizobium*, strain 2192). The latter was then washed thoroughly and placed in the apparatus filled with Medium 1 equilibrated with a mixture containing 20% N_2 and 80% O_2 . The apparatus was shaken and readings of the O_2 content of the fluid taken. After about 90 min. tap *C* was opened, and 0.4 ml. of medium 1 saturated with CO was introduced through *E* by means of a syringe with a needle. This displaced an equal volume of medium through tap *C*. By this means a $p\text{CO}$ of 0.02 atm. was obtained. Tap *C* was then closed and readings were continued.

Several experiments of this kind were carried out; typical results are shown in Fig. 3. No effect of carbon monoxide at this partial pressure on the oxygen-uptake rate could be detected. Any change in rate of oxygen uptake of about 5% or more would easily have been observed.

Haemoglobin and the oxygen uptake of the root nodule bacteria

Mention has already been made of the experiments of Little & Burris (1947), who found that addition of mammalian or nodule haemoglobin to resting suspensions of *Rhizobium* brought about an increase in the rate of oxygen uptake at low partial pressures of oxygen (0.01 atm.). Their interpretation of this result as a direct effect of haemoglobin on the oxygen uptake of the nodule bacteria, the pigment acting through its capacity for reversible oxygenation, made it likely that the same effect might be present in the nodule. From the experiments with effective and ineffective nodules, and with carbon monoxide, described above, it is apparent that this is not so. In order to learn if their results could be interpreted in another way the experiments of Little & Burris (1947) were repeated at values of $p\text{O}_2$ of 0.01 and 0.2 atm. with carboxyhaemoglobin as a control in addition to denatured haemoglobin.

Experiment 1. A 4-day culture of *Rhizobium* (strain 2193) grown at 30° was washed and suspended in 0.1 M-phosphate buffer pH 7.3. Crystalline haemoglobin, prepared from horse red blood cells as described (p. 593), was dissolved in 0.1 M-phosphate buffer pH 7.3. Denatured haemoglobin was obtained by heating this solution to 100° for 1 min. (following the procedure of Little & Burris, 1947). O_2 uptake was measured manometrically at 37.2° in atmospheres of O_2 of partial pressure 0.01 atm. or 0.2 atm. in N_2 . In each flask was placed 1 ml. of the *Rhizobium* suspension, 1 ml. of 3% glucose, together with either 1 ml. of buffer solution, 1 ml. of the haemoglobin solution in buffer, or 1 ml. of denatured haemoglobin.

Table 6 shows that the effect of haemoglobin is large at both values of $p\text{O}_2$. The haemoglobin denatured by heating inhibited oxygen uptake to some

extent and was unsatisfactory as a control, consequently in the next experiment it was replaced by carboxyhaemoglobin.

Table 6. *Haemoglobin and oxygen uptake of Rhizobium*

Contents of manometer flask ...	<i>Rhizobium</i> (suspension in glucose buffer + haemoglobin)	<i>Rhizobium</i> (suspension in glucose buffer + buffer)	<i>Rhizobium</i> (suspension in glucose buffer + de-natured haemoglobin)
Rate of O ₂ uptake (μl./hr.) at pO ₂ =0.01 atm.	49.5	23.4	15
Rate of O ₂ uptake (μl./hr.) at pO ₂ =0.2 atm.	53.5	39.6	21

The oxygen capacity of the haemoglobin solution added, determined manometrically by the ferri-cyanide method, was 29.2 μl. O₂/ml. solution.

Increasing the rate of shaking of the flasks did not affect the rates of oxygen uptake.

Experiment 2. The first experiment was repeated with some flasks filled with air and others with a gas mixture containing 20% O₂, 5% CO and 75% N₂. Haemoglobin in flasks filled with the CO mixture was entirely converted to HbCO.

The oxygen-uptake curves (Fig. 4) showed that, in addition to haemoglobin, carboxyhaemoglobin brought about equally large increase in the oxygen

Fig. 4. Oxygen uptake of *Rhizobium* suspensions: A, in air; B, in air containing CO; C, in air, with haemoglobin; D, in air containing CO, with haemoglobin.

uptake of the bacteria. The effect of haemoglobin in this and probably in the other experiments, including those at low partial pressures of oxygen, is not connected with its ability to undergo oxygenation. A probable explanation is that *Rhizobium* and many other bacteria are able to break down haemoglobin and use it as a nitrogen source, so that in the presence of haemoglobin, the bacteria change over from a rate of oxygen uptake corresponding to a resting metabolism to a higher rate characteristic of a proliferating metabolism. A similar effect of haemoglobin on bacterial respiration at low values of pO₂, noted

by Baumberger (1939), may possibly be interpreted in the same way.

Haemoglobin and oxygen uptake in bird red blood cells. The nucleated red blood corpuscle of birds provides a model cell containing a large quantity of haemoglobin and having a reasonably high respiratory activity. Warburg (1929) has used these cells in an experiment intended to find out whether stationary haemoglobin within cells can have any effect on their oxygen uptake. He measured the change in oxygen uptake of bird red blood cells on converting all their haemoglobin to carboxyhaemoglobin, and found the unexpected result that treatment of these cells with carbon monoxide caused an increase in their oxygen uptake. Warburg does not explain this odd effect satisfactorily and gives no details of his experiments.

His experiment has been repeated here using fresh washed chick red blood corpuscles suspended at 37° in bird Ringer solution containing 0.2% glucose. (Bird Ringer solution: NaCl, 9 g.; KCl, 0.4 g.; CaCl₂, 0.25 g.; water to 1 l.). Into each of a number of Warburg manometers were placed 2 ml. of this suspension. Oxygen uptake was measured in air and in a gas mixture containing 5% carbon monoxide, 20% oxygen and 75% nitrogen.

Immediately after replacing the air in the flasks with the gas mixture containing carbon monoxide the effect noted by Warburg was observed, the oxygen uptake increasing by more than 50%. However, after about 20 min the rate fell quickly back to the original value obtained in air (Table 7).

Table 7. *Oxygen uptake of bird red blood corpuscles in air and in air containing 5% carbon monoxide*

(Each flask contained 2 ml. corpuscles. O₂ capacity of the haemoglobin in 2 ml. corpuscles = 133 μl. Temp., 37°.)

	Rate of O ₂ uptake (μl./hr.)
In air	68.4
Initial rates in air containing 5% CO	99.6, 91.2*
Final rate in air containing 5% CO	67.2, 66.6*

* Duplicate determinations.

DISCUSSION

While in most cases the occurrence of haemoglobin can be directly related to its function as an oxygen carrier, some organisms (including some invertebrates and certain protozoa) possess a stationary intracellular haemoglobin, which cannot possibly act as an oxygen carrier in the normal sense and yet which has the same property of reversible oxygenation. This is also true of the bacteria-containing cells of the legume nodule. Here, in addition, there is good evidence that the presence of the pigment is in some way connected with the process of symbiotic nitrogen fixation.

The view that nodule haemoglobin acts in nitrogen fixation as an oxidation-reduction catalyst, the valency of the iron in the molecule undergoing reversible changes, must be rejected both on experi-

mental and theoretical grounds (Keilin & Smith, 1947). It has often been assumed that such stationary haemoglobin might bring about an increase in the oxygen uptake of cells, especially when these are in an environment deficient in oxygen. In face of the known interrelation between oxygen supply and the functioning of the legume nodule such an explanation of the presence of haemoglobin in the nodule seemed not unlikely. This was more especially so in view of the low pO_2 in the central nodular tissue.

It was therefore necessary to compare the oxygen uptake of nodules with and without haemoglobin capable of reversible oxygenation. This was done in two ways and in each case no significant difference in oxygen uptake was detected. The first comparison, that between effective and ineffective nodules is open to the objection that ineffective nodules differ from effective nodules not only because of their lack of haemoglobin, but also because of the breakdown of the bacteria-containing cells. Such criticisms do not apply, however, to the comparison between normal nodules and nodules in which the haemoglobin has been converted to carboxyhaemoglobin. Within the accuracy of the methods used in measuring oxygen uptake (approx. $\pm 2\%$) the presence of haemoglobin has no effect on the oxygen uptake of nodules whether excised or attached to the rest of the plant. Such a conclusion is compatible with the results found on examining the effect of solutions of haemoglobin upon the oxygen uptake of *Rhizobium* suspensions. The increase in oxygen uptake of *Rhizobium* suspensions on addition of haemoglobin solutions was found not to be connected with the reversible oxygenation property of haemoglobin, but to be probably due to the ability of the bacteria to use haemoglobin as a nitrogen source.

Because of its comparatively high respiratory activity and its high haemoglobin content, the nucleated bird red blood cell appeared to be an ideal model on which to investigate the effect of haemoglobin contained within the cell on its oxygen uptake. However, low concentrations of carbon monoxide bring about a temporary increase of about 50% in the oxygen uptake of these cells. It is possible that this increase in gas uptake is in reality due to the

combustion of carbon monoxide to carbon dioxide which, as has been shown by Fenn & Cobb (1932), may take place in frog muscles. It was thus not possible in these cells to find the effect on oxygen uptake of converting the haemoglobin to carboxyhaemoglobin.

SUMMARY

1. It is shown that oxygen enters the nodules of leguminous roots by diffusion in solution across the wet cell walls.

2. Values of the Q_{O_2} of whole nodules decrease with increasing size of nodule and are greater in oxygen than in air. The Q_{O_2} of thin slices of nodules, of a thickness such that diffusion does not determine the rate of oxygen uptake, is about 7–8 $\mu\text{l./mg. dry wt./hr.}$, while that for whole nodules is about 1–2 $\mu\text{l./mg. dry wt./hr.}$

3. The Q_{O_2} values of whole and sliced ineffective nodules differed very little from those of whole and sliced effective nodules.

4. Carbon monoxide in a concentration sufficient to convert practically all the nodule haemoglobin to carboxyhaemoglobin had no effect on oxygen uptake by detached effective nodules. An apparatus is described by means of which the oxygen uptake of nodulated roots may be measured when these are attached to the plant. No effect of low concentrations of carbon monoxide on the oxygen uptake of attached nodules could be detected.

5. The increase in oxygen uptake brought about by addition of haemoglobin solutions to *Rhizobium* cells is shown to be unconnected with the ability of haemoglobin to undergo reversible oxygenation.

6. Warburg's (1929) observation that low concentrations of carbon monoxide apparently stimulated oxygen uptake by bird red blood cells was confirmed. It was found that this stimulation was temporary.

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