The Concentration and Distribution of Haemoglobin in the Root Nodules of Leguminous Plants

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All legume nodules which are actively fixing nitrogen contain a red pigment. This pigment escaped attention until Pietz (1938) suggested that it was identical with dihydroxyphenylalanine (Dopa), the red intermediate in the enzymic oxidation of tyrosine. Kubo (1939), however, prepared a crude extract of the pigment and from its spectroscopic behaviour identified it as a haemoprotein possessing properties very similar to those of haemoglobin. Burris & Haas (1944) stated that the pigment was not a haemoglobin but a haemoprotein oxidation-reduction catalyst. However, Keilin & Wang (1945), working on an extract about 50 % pure, were able to confirm the haemoglobin-like nature of the pigment by showing that it was capable of completely reversible oxygenation and deoxygenation; they determined the absorption spectra of the oxygenated and reduced haemoglobin and those of some of its derivatives. Keilin & Wang suggested that the

Table 1. *The positions of the maxima of the absorption bands of nodule haemoglobin and some derivatives*

(Values given by Keilin & Wang, 1945)

	Maxima (in mμ.)		
	α		β
Hb		557	
HbO_2	574		540
HbCO	564		538

failure of Burris & Haas to recognize the pigment as a haemoglobin was due to the fact that in their preparations the pigment had become largely oxidized to methaemoglobin. Table 1 shows the positions of the maxima of the absorption bands of reduced, oxidized and carboxy-haemoglobin (Hb, HbO_2 and HbCO). Working at 15°, Keilin & Wang found the pO_2 giving 50 % dissociation of HbO_2 to be less than 0·1 mm. Hg, and the relative affinity for CO and O_2 $\left(K=\frac{[HbCO]\,[pO_2]}{[HbO_2]\,[pCO]}\right)$ to be 37.

A remarkable fact about nodule haemoglobin is that neither the root-nodule bacteria nor the host plant is able to produce it when grown separately. It is only found in the nodule which is produced after infection of the host legume with an appropriate strain of *Rhizobium*. A similar relationship holds in the case of the property of nitrogen fixation. Neither the host plant nor the root-nodule bacteria cultivated alone will fix nitrogen. Those micro-organisms which fix nitrogen when free living (*Nostoc*, *Azotobacter* and *Clostridium pasteurianum*) do not possess haemoglobin. In a preliminary examination of the root nodules of alder, which are also believed to fix nitrogen, the author was unable to detect the pigment. There is, however, indirect evidence that the haemoglobin in the nodule is concerned in the process of symbiotic nitrogen fixation. This may be summarized as follows: (1) Haemoglobin is present in nodules of all the leguminous plants which actively fix nitrogen. (2) Haemoglobin is absent from nodules produced by certain ineffective strains of *Rhizobium* and which fix very little nitrogen. (3) Symbiotic nitrogen fixation is inhibited by a concentration of carbon monoxide much lower than that which is required to inhibit fixation by *Nostoc* or *Azotobacter*.

From these facts alone there can be little doubt that haemoglobin plays a role in nodular nitrogen fixation and in this paper further evidence based on the distribution of the pigment in the nodule will be presented. The mechanism into which haemoglobin enters is as yet unknown. Virtanen and his co-workers (Virtanen & Laine, 1946) claimed that, in addition to haemoglobin, methaemoglobin was present in nodules, and put forward a theory of nitrogen fixation in which haemoglobin functioned as an oxidation-reduction catalyst. However, on repeating Virtanen's experiments, Keilin & Smith (1947) were unable to find any evidence for the presence of methaemoglobin in nodules and from this and other considerations rejected Virtanen's theory. The suggestion that haemoglobin may, by virtue of its oxygen-carrying property, take part in the oxygen uptake of nodules will be discussed in the following paper. In the present paper the distribution and concentration of the pigment in various types of nodule will be described.

METHODS

Cultivation of the root nodule bacteria. The *Rhizobium* strains were grown at 28 or 30° in pure culture on an agar medium (K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 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.). By using this medium, in which a yeast extract preparation is the sole source of carbon and nitrogen, one avoids the copious gum production always encountered with media containing mannitol. Nodules on soya beans were produced by the effective *Rhizobium* strain 505 and the ineffective strain 507. These two strains are identical with those used by the Wisconsin group of workers (Wilson, 1940). In some experiments the strain 2193 (National Collection of Type Cultures), which infects peas, was used.

Growth of the plants. 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 infections was obtained by placing plants in separate parts of the glasshouse (see p. 589).

Cytological methods. Nodules were fixed in Bouin's fixative or in formalin-acetic-alcohol. These were sectioned, and stained either with iron haematoxylin and orange G as counterstain, or with carbol fuchsin. The sections stained with iron haematoxylin were more satisfactory.

The estimation of haemoglobin in nodules

Direct observation of haemoglobin. Haemoglobin may easily be demonstrated in legume nodules using the microspectroscope. Whole nodules are too opaque for this observation, but on slicing or crushing the nodule the twin absorption bands of oxyhaemoglobin with maxima at 574 and 540 mμ. are seen. On standing these are slowly replaced by the single band of deoxygenated haemoglobin at 557 mμ. This is due to respiratory activity of the nodule tissue.

General method of estimation. For this purpose use was made of the microspectroscope and wedge trough as described by Keilin (1933) and by Keilin & Wang (1946). In this instrument the spectra produced by light from two different sources are compared. Light from one source passes through a double-wedge trough, one half of which contains a standard solution, the other water. The optical depth of the standard solution through which light is passing is varied by movement of the wedge trough and is proportional to the distance through which this is moved. Light from the other source passes through a cylindrical vessel containing the solution of unknown density. In matching, two adjustments are made simultaneously: the intensities of the absorption bands in the two spectra are matched by movement of the wedge trough, and the intensities of the spectral backgrounds adjacent to the bands are matched by varying the strengths of the two light sources. If the matching is carried out in this manner it can be shown that opaqueness of the unknown solution does not affect the result, and measurements may in fact be made of concentrations of pigments in slices of tissue. In this way the haemoglobin was estimated as pyridine haemochromogen, both in aqueous extracts and directly in nodule slices.

Determination of nodule haemoglobin in aqueous extracts. Nodules freshly removed from the plant were washed, and the surface moisture was removed by filter paper; the nodules were then weighed and the total volume of the nodule sample measured by placing in water and measuring the volume of water displaced. The nodules were then ground in water and the extract centrifuged to remove cellular debris, which was again extracted with water. The second extract was found to contain less than 2% of the total haemoglobin and was consequently discarded. The first extract was made up to a volume of 5 ml. and a measured volume (v) of the solution placed in a cylindrical upright tube of cross-sectional area (a) on the stage of the microspectroscope. The haemoglobin was then converted to pyridine haemochromogen by the addition of a little NaOH (to denature the proteins), pyridine and $Na_2S_2O_4$. The comparison was made against a standard pyridine haemochromogen solution (of concentration C) contained in the wedge trough. The concentration of the unknown solution (c) is given by the relation $c = H.C/h$, where H is the optical depth of the standard solution (this is obtained from the distance through which the wedge trough has been moved and from constants dependent on its dimensions), and h is the optical depth of the unknown solution ($= v/a$).

It will be shown that the haemoglobin is limited to the bacteria-containing cells in the central part of the nodule, so, in order to calculate the approximate concentration of haemoglobin in these cells, correction must be made for the volume of the outer cortical parenchyma of the nodule which contains no haemoglobin. This volume was estimated from measurements of the relative areas of internal nodular tissue and cortical parenchyma in thin slices of nodules, assuming these to be spherical. These measurements were made using a microscope with a micrometer eyepiece.

Spectroscopic examination of the aqueous extract and the cellular debris showed that practically all the haemoglobin passed into the first extract. Only haemoglobin could be identified in this extract. The solid cellular debris showed only the absorption bands of components of the cytochrome system.

As the central nodular tissue consists both of cells containing bacteria and haemoglobin, and small interstitial cells devoid of both, the value obtained for the concentration of haemoglobin in the former cells is slightly less than the true value. This error, however, should not be greater than about 10%.

Determination of haemoglobin directly in nodule slices. In this method all the haematin in a small slice of nodule was converted into pyridine haemochromogen and this determined directly by comparison with a standard haemochromogen solution. For this purpose each nodule was cut into slices 1–2 mm. thick. A slice was placed on a glass slide in a few drops of pyridine and reducer ($Na_2S_2O_4$). A piece of plane glass was placed on top of the slices and supported at the corners by small pieces of plasticine. The whole was then compressed slightly so that all surfaces of the slices were in contact with the glass, and the two pieces of glass were adjusted to make their surfaces accurately parallel. This system was then placed on the stage of the microspectroscope, to which a low power objective had been added, so that the central part of the nodule was under the objective. The slices were left in contact with pyridine and reducer until the maximum amount of haemochromogen had developed (usually after 20 min.). The mean of several readings over different parts of the nodule slice was taken. Then, as before, $c = H.C/h$, where h is the thickness of the nodule slice. This was obtained by measuring with a micrometer the distance apart of the two pieces of glass with and without the slice in position.

Accuracy in this method requires: (1) careful measurement of the thickness of the nodule slice, (2) evenness in the thickness of the slice, (3) that the light passing through the slice should be parallel.

The concentration measured in this way gives an upper limit to the amount of haemoglobin in the nodule, as haematin from the cytochromes and other haemoproteins is included in the estimation.

It might be thought that, since the haemoglobin is not distributed uniformly throughout the tissue, but is present in a number of small spherical cells, a false estimate of concentration would be obtained. However, it was found that the concentration of haemoglobin, when measured in a suspension of horse red blood cells under conditions when these were ellipsoidal in shape, was identical with that found after lysing the cells. The behaviour of this model suggests that the localization of the haemoglobin does not affect the estimation described above.

RESULTS

Localization of haemoglobin within the nodule

After the invasion of the root by the bacterial infection thread a meristem is set up in the root cortex. This meristem produces two kinds of cells; cells which later enlarge and are packed with bacteria, and smaller interstitial parenchymatous cells devoid of bacteria. The mature nodule then consists of an outer bacteria-free cortex and an inner tissue made up of the large bacteria-containing cells together with the interstitial cells (Fig. 1). In certain types of nodule these bacteria-containing cells undergo a rapid disorganization which is easily seen on cytological examination. Thornton (1939) has shown from measurements on such nodules that the rate of nitrogen fixation is proportional to the volume of bacteria-containing cells which have not undergone disorganization. It is reasonable to suppose that, since the nitrogen gas is actually taken up by the nodule, in these cells lies the seat of nitrogen fixation. It is interesting therefore to know whether the haemoglobin is present in these cells.

In order to answer this question soya nodules (*Rhizobium*, strain 505) were examined. From observation of the distribution of the red colour in cut nodules, and by careful examination of thin slices of nodules under the microspectroscope, it was apparent that there was no haemoglobin in the cortex. Use was then made of the peroxidase action of haemoglobin to determine its localization within the nodular tissue proper. If an ethanolic solution of benzidine together with hydrogen peroxide is added to an acid haemoglobin solution, the benzidine is oxidized to a substance with a quinonoid structure which polymerizes giving a deep blue compound. While some very strong oxidizing agents bring about a similar reaction, no such substances are found in living cells, and the formation of a blue colour under these conditions indicates the presence of haemoglobin. Under acid conditions peroxidase itself will not bring about this reaction and its presence in plant cells will not interfere with the detection of haemoglobin by this method. This reaction was tested on thin sections of soya nodules cut with a hand razor. In these sections the cytoplasm and often the nuclei of the large bacteria-containing cells were stained blue, while all other cells were unstained (Fig. 2).

Haemoglobin within the nodule is thus confined to those cells which contain bacteria and which are probably specifically concerned with nitrogen fixation.

If nodules are ground up in water, and the bacteria and insoluble debris centrifuged down, spectroscopic examination shows all the haemoglobin to be in solution. The pigment is not present in the bacteria, but diffused throughout the vacuole and cytoplasm.

The appearance of haemoglobin in young nodules

In the early development of the nodule there is a period when the meristem has begun to cut off the cells which will make up the bacteria-containing tissue, but during which the differentiation of these cells is still incomplete. At this time the future bacteria-containing cells are small, round and little distinguished from the interstitial cells. Observations were made to find out at what stage in nodular development detectable quantities of haemoglobin appear.

Soya nodules (*Rhizobium*, strain 505) were sampled 4 weeks after plant inoculation. Nodules of various sizes were sliced, and the slices examined for haemoglobin by direct observation of the presence or absence of a red colour (the nodules were too small to be examined under the microspectroscope). They were divided into two groups, those which were red on slicing and those which were white. Nodules from each group were fixed, sectioned and stained with iron haematoxylin.

All those nodules which possessed a detectable amount of haemoglobin also showed a well-developed bacteria-containing tissue, the large cells of which contained many bacteria and were easily distinguished from the interstitial cells. In nodules in which no haemoglobin could be observed, differentiation of the bacteria-containing region had not yet begun or was just commencing. The cells in such nodules contained few bacteria (Figs. 3 and 4).

Using this crude method of detecting haemoglobin it was possible to show that the time of appearance of a large amount of haemoglobin in the nodule more or less coincides with the differentiation of the bacteria-containing region and never precedes it.

The amount of haemoglobin in effective nodules

When it had been shown that the haemoglobin is restricted to the bacteria-containing cells, the concentration of haemoglobin within these cells was measured.

Haemoglobin in nodules of bean (Phaseolus vulgaris *dwarf variety*) *and soya*

Using the microspectroscope and wedge trough the amounts of haemoglobin in effective nodules from two species of legumes were measured. The

bean plants were harvested in April 1947 when flowering and bearing fairly large nodules. The soya plants (inoculated with *Rhizobium*, strain 505) were harvested in June 1947, just before flowering, and the nodules were placed according to size into two groups. Table 2 gives the data and the results of

Haemoglobin and haematin compounds in ineffective nodules

Different strains of *Rhizobium* may produce nodules on the same host plant species with greatly varying nitrogen-fixing activity; some strains giving rise to nodules which fix practically no nitrogen. Such strains are called ineffective strains and the nodules they produce will be referred to as ineffective nodules. Ineffective nodules are usually smaller than effective nodules and are distributed more evenly over the entire root system. Examined cytologically the central tissue is seen to be quite different from that of normal nodules; while both cortex and interstitial parenchymatous cells are entire, the large bacteria-containing cells appear in a state of breakdown. Usually the nuclei have disappeared, and the cytoplasm is rounded off into large masses containing many bacteria, mainly small cocci. The ratio of uninfected cells to infected cells in the central tissue of such nodules is characteristically greater (Fig. 5).

Thornton (1939) finds that in the early stages of development of the most ineffective nodules there is some organized bacteria-containing tissue, but that the difference between these and effective nodules lies in the stability of this central tissue. Once disintegrated it ceases to fix nitrogen. This is borne out by Thornton's experiments, in which, by cytological studies and Kjeldahl analyses on nodules and plants grown under sterile conditions (ensuring purity of the infecting *Rhizobium* strain), he showed that the amount of nitrogen fixed by nodules of varying effectiveness is proportional to the amount of organized central tissue and its duration. The nodules produced by strains of *Rhizobium* isolated by Virtanen (1947), which he claims fix no nitrogen, are probably distinguished by the extreme rapidity with which the cells of the central nodular tissue undergo disintegration. Virtanen's suggestion that the low nitrogen-fixing power of ineffective nodules is due to the absence of the irregularly shaped bacterial forms, usually referred to in the literature as bacteroids, has been criticized by Thornton who points out that

Table 2. *Haemoglobin content of nodules as determined in aqueous extracts*

Plant	Fresh wt. of sample (g.)	Mean fresh wt. of single nodule (mg.)	Volume of nodule sample (ml.)	Haematin in extract (mg.)	Mean value of the ratio: Volume of bacteria-containing cells / Total volume	Concentration Hb in bacteria-containing cells (as haematin) (10^{-4} M)	$\frac{Hb_{17,000}}{\text{Fresh wt. nodule}}$* (mg./g.)	Mean* $\frac{Hb_{17,000}}{\text{nodule}}$ (mg.)
Bean	1·7	16·8	1·7	0·072	0·59	1·09	1·09	0·0183
Soya (medium-sized nodules)	0·866	8·7	0·85	0·088	0·38†	4·2	2·68	0·0233
Soya (small nodules)	0·404	4·9	0·40	0·050	0·38†	5·0	3·25	0·0157

* The amount of haemoglobin is calculated as haemoglobin units of molecular weight 17,000.
† Independent of nodule size. Standard error in each case is ±9·7% of mean value.

Table 3. *Haemoglobin (including haematin from cytochromes) as determined in bean nodule slices*

Nodule no.	Concentration of Hb as haematin in bacteria-containing cells ($\times 10^{-4}$ M)
1	2·5
2	1·72
3	3·76
4	1·28
5	2·17
6	2·59
	Mean **2·34** (S.E. ±15%)

measurements carried out in aqueous extracts, and Table 3 records measurements made on individual nodule slices from a single sample of bean nodules.

While the concentration of haemoglobin in different nodules is subject to considerable variation the values are all of the same order of magnitude. Those obtained from measurements of total haematin in nodule slices give an upper limit to the amount of haemoglobin in the nodule, and are consequently greater than values from measurements in aqueous extracts. The bean nodules were grown in April 1947, those of soya in June 1947. Because of this difference in time of measurement, it is not certain whether the greater amount of haemoglobin in the soya nodules should be attributed to differences in cultural conditions or to a difference between the two genera. Within the range examined there appears to be little variation in haemoglobin content of nodules of different sizes. It is probable that the haemoglobin content of the bacteria-containing cells remains constant after their differentiation until the onset of their disintegration, but when the latter occurs the haemoglobin eventually disappears and may be replaced by a green pigment (Virtanen, Laine & Linkola, 1945; Virtanen & Laine, 1946).

bacteroids may be observed in young ineffective pea and clover nodules, and furthermore that bacteroids are absent in all nodules formed on soya plants, whether effective or ineffective. There is no good reason to connect bacteroids with the process of nitrogen fixation. However, the low rate of nitrogen fixation by ineffective nodules is always associated with the breakdown of the nodular tissue.

Virtanen (1945) has shown that haemoglobin cannot be detected in ineffective nodules. Instead a green pigment is often found which, as Virtanen *et al.* (1945) and Virtanen & Laine (1946) have shown, may be similar to certain of the bile pigments formed during haemoglobin breakdown in animals. This green pigment is of little interest in the study of the nitrogen fixation process since it is invariably formed after the nodule has ceased to fix nitrogen. Since it appears in considerable quantities in nodules which have never contained detectable amounts of haemoglobin, it is difficult to understand how it originates from the breakdown of haemoglobin, unless this is being produced and broken down almost simultaneously. Alternatively, haemoglobin may not be produced, but instead quantities of haematin which are transformed to the green pigment.

To find out in what way ineffective nodules are distinguished from effective nodules some measurements of the total amounts of haematin in effective and ineffective nodules and of haematin production by both types of strain of the nodule bacteria were made.

Haemoglobin and total haematin in effective and ineffective soya nodules

Three groups each of forty soya plants were grown in open pots in sterilized soil, each group in a separate part of the greenhouse. One group was inoculated with *Rhizobium* strain 505 (effective), the second group with strain 507 (ineffective), while the third (control) group of plants was left uninoculated. None of the control plants produced nodules, consequently it may be assumed there were no contaminant infections. The plants were harvested 4 weeks after inoculation (June 1947).

The haemoglobin was extracted in water from samples (1–2 g.) of both types of nodule and estimated as pyridine haemochromogen as previously described. Only bands of haemoglobin could be detected when the aqueous extract from effective nodules was examined under the microspectroscope. No spectroscopically distinguishable components were visible in the aqueous extract from ineffective nodules although this extract contained a green pigment. As will be seen, however, haematin compounds were present in small amounts in this extract. When the solid debris remaining after centrifuging was examined under the microspectroscope in the presence of a little reducer ($Na_2S_2O_4$), only bands of the cytochromes could be seen in each case, but these bands were much stronger in the debris from effective nodules. The amounts of haematin in these solid residues were also measured. Table 4 lists all these results. The haematin in the solid residue in both cases comes from the cytochromes and small amounts of other haemoproteins. The haematin in the aqueous extract of 505 nodules is derived almost entirely from haemoglobin. That found in the 507 aqueous extract may originate in any of three ways: (*a*) certain of the cytochromes may pass more readily into solution because of the tissue breakdown in these nodules; (*b*) free haematin or unidentified haematin compounds may be present; or (*c*) there may be in 507 nodules a quantity of haemoglobin too small to be detected by direct observation.

Table 4. *Haematin content of effective and ineffective nodules*

	Effective nodules (*Rhizobium*, strain 505)		Ineffective nodules (*Rhizobium*, strain 507)	
	(mg. haematin/g. fresh weight of nodules)	Percentage of total	(mg. haematin/g. fresh weight of nodules	Percentage of total
Haematin in aqueous extract	0·071	92·2	0·0068	78·2
Haematin in residue	0·006	7·8	0·0019	21·8
Total haematin	0·077	—	0·0087	—

The ratio (total haematin in nodules of strain 507/total haematin in nodules of strain 505) was 0·113.

The cytology of ineffective soya nodules (Rhizobium, *strain* 507)

In sections of ineffective nodules the general disintegration of the whole nodule tissue was very marked, more especially affecting the large bacteria-containing cells. The nuclei of these had disappeared and the cytoplasm was breaking up into fragments containing large numbers of bacteria. The cytoplasm of the interstitial cells was also undergoing disorganization and groups of bacteria were associated with the cell walls which were breaking down. The relative number of interstitial cells was much greater in this type of nodule. Younger ineffective nodules

showed only the initiation of these processes, but haemoglobin could not be detected in these, however young.

In the effective nodules produced by *Rhizobium*, strain 505, no such disorganization could be seen even in large nodules (Figs. 1 and 5).

Haematin and cytochrome in pure cultures of Rhizobium

The great difference in total haematin contained in effective and ineffective nodules suggested the possibility that *Rhizobium* strains might differ in their capacity to produce haematin.

In one experiment pure cultures of the *Rhizobium*, strains 505 and 507, were grown at 28° on an agar medium containing inorganic salts and yeast extract. After 4 days (the soya organism belongs to the slow-growing group of Rhizobia) the cells were harvested and washed in 0·1 M–phosphate buffer, pH 7·3.

The intensities of the reduced cytochrome bands appeared about the same in both suspensions. Examination of these bands showed in each case the presence of cytochromes *a*, *b* and *c* with the α bands of components *b* and *c* extremely close together. On samples of the suspension the total haematin was determined as pyridine haemochromogen, and the nitrogen content by the micro-Kjeldahl method using the Markham steam-distillation apparatus. These results are given in Table 5.

Table 5. *Haematin content of pure cultures of effective and ineffective strains of* Rhizobium

Strain no.	Haematin content of suspensions (mg./100 ml.)	Nitrogen content of suspensions (mg./ml.)	Haematin (mg./mg. N)
505	1·3	8·6	$1·5 \times 10^{-3}$
507	0·86	8·6	$1·0 \times 10^{-3}$

The amounts of haematin produced by these two strains are of the same order. The difference is small and does not account for the great difference in haematin content of the two types of nodule.

DISCUSSION

In nodules produced by ineffective strains of *Rhizobium*, not only is the absence of haemoglobin paralleled by a correspondingly lower amount of extractable haematin as compared with that of effective nodules, but the amount of non-extractable haematin compounds is also lower. This latter difference exists when the haematin content is calculated on a fresh weight or a dry weight basis (the ratio fresh weight/dry weight being the same in each type of nodule). There is also less cytochrome in ineffective nodules. This may probably be accounted for by the smaller number of bacteria in such nodules.

When grown in pure culture, effective and ineffective strains of the root nodule bacteria do not differ greatly in the amounts of haematin they produce. The absence of haemoglobin and the failure to fix nitrogen are doubtless directly connected with the breakdown of the bacterial tissue, as these three phenomena occur together in nodules produced under other circumstances. Such types of nodule are: (1) Nodules formed by effective strains of *Rhizobium* on certain varieties of host plant (Nutman, 1946). These nodules have a white-green colour and thus can possess little haemoglobin (Nutman, private communication). (2) Nodules formed on boron-deficient plants (Brenchley & Thornton, 1925). It is not known whether these nodules contain haemoglobin. (3) Effective nodules borne on plants nearing the end of their life cycle. (4) Nodules on plants which have been kept in the dark for a period of at least 11 days (Keilin & Smith, 1947).

Under the last three conditions the factor causing the breakdown of the nodular tissue is apparently shortage of carbohydrate. Bond (private communication) notes that nodules formed on plants grown in the dark but supplied with glucose are red in colour (in contrast to nodules formed under these conditions without an external supply of carbohydrate).

Taking into account the structure and properties of all these different types of nodule, it may be concluded that the production of haemoglobin and the ability to fix nitrogen are properties not resulting from the mere haphazard association of bacteria and host tissue in the nodule, but are dependent on a very precise relationship between the bacteria and the large cells in the nodular tissue in which they are found. The presence of haemoglobin, and the ability to form with the rest of the plant a nitrogen-fixing system, are characteristics which do not arise until these cells reach a certain size, and both disappear when sooner or later in their development (depending on the conditions listed above) these cells undergo breakdown.

SUMMARY

1. Haemoglobin in legume nodules is contained only in the large bacteria-containing cells and does not appear in detectable amounts until these cells have been differentiated.

2. The concentration of haemoglobin in the bacteria-containing cells of nodules of different legume species varied between 1 and 5×10^{-4} M. After cell differentiation the concentration did not appear to vary with size of nodule.

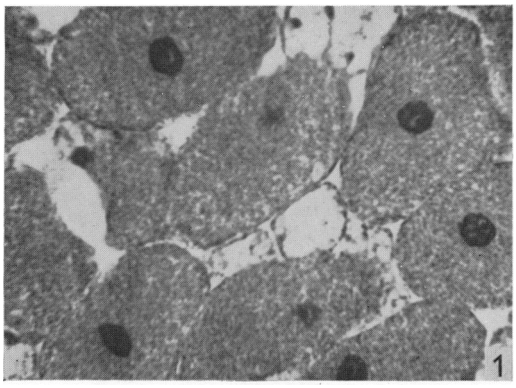

Fig. 1

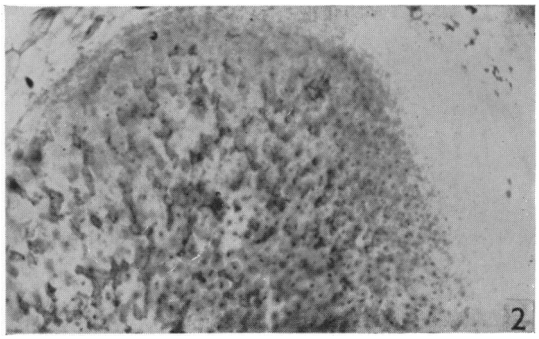

Fig. 2

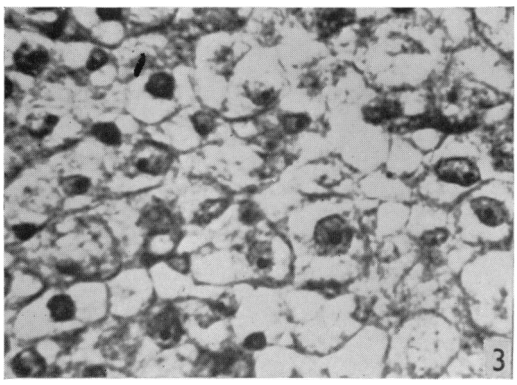

Fig. 3

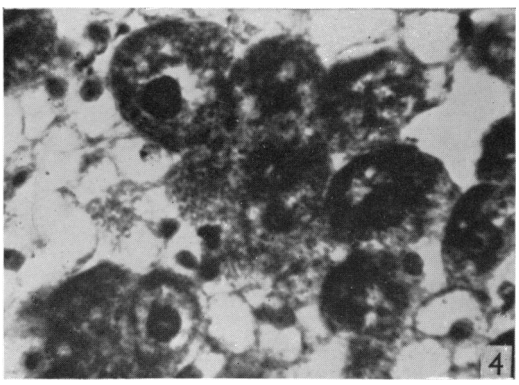

Fig. 4

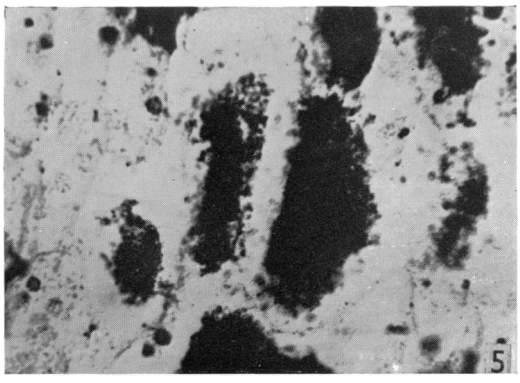

Fig. 5

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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.) (×800.)

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

Fig. 3. The central tissue of a soya nodule before the development of detectable amounts of haemoglobin. (Iron haematoxylin.) (×800.)

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

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