ACTA CHEMICA SCANDINAVICA 1 (1947) 90—111

On the Relation between Nitrogen Fixation and Leghaemoglobin Content of Leguminous Root Nodules

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Some years ago certain observations were made in this laboratory which opened new possibilities of investigating the machinery indispensable to nitrogen fixation in the root nodules. Certain strains of legume bacteria were then discovered which truly form nodules on the roots of the host plant, but nodules which do not fix nitrogen.[1] A strain of this kind is, for instance, the pea *Rhizobium* H VIII of our laboratory. Comparison between the nodules formed by this strain and those formed by effective strains showed that a red pigment is absent from the H VIII nodules, whereas it is always present in the effective nodules.[2] In this way a distinct *chemical* difference between the effective and ineffective nodules was for the first time detected. This observation suggested that the red pigment is in some way connected with the nitrogen fixation in the nodules. Many different findings in continued investigations have consistently led to the same result. Nitrogen fixation could never be noted, unless the root nodules contained red pigment. Of particular significance is the observation [2,3] that the colour of the red active nodules changes to green when nitrogen fixation ceases in annual plants at the end of vegetative growth or even at an earlier stage if the plants are removed into the dark for some days.

The haemoglobin nature of the red pigment of the root nodules discovered by Kubo [4] just before the war was confirmed in our laboratory in 1944.[2] The pigment is able to store and carry oxygen. Keilin and Wang [5] also arrived at the same result, whereas Burris and Haas [6] were at first unable to ascertain the haemoglobin nature of the pigment although they found it to be a haemo-

protein. — The pigment can be brought into solution simply by crushing nodules. It can be purified by precipitating with ammonium sulphate. In this laboratory a preparation was first obtained the purity of which was 80—85 % on the basis of the iron and haematin contents [7] (assuming these to be the same as in the blood of the vertebrates). The molecular weight of this preparation was determined by Pedersen and found to be 34 100,[7] which indicates that the molecular weight of the protein is about a half of that of haemoglobin. The experiments so far made thus indicate that the protein of the leghaemoglobin is different from that of the haemoglobin. Later the purification was carried so far that the iron content of the pigment was the same as that of pure haemoglobin.

Leghaemoglobin (the haemoglobin of the leguminous root nodules) differs from the haemoglobin of the blood of vertebrates in that it is autoxidized more easily. While normal blood contains at the most only a few fractions of per cent methaemoglobin, the root nodules, on the other hand, often contain considerably legmethaemoglobin.[3] This may in part be due to the action of oxidases in nodules, but in part at least also to autoxidation of leghaemoglobin which takes place fairly rapidly also in highly purified preparations. Accordingly, it may be assumed that the protein component of leghaemoglobin is linked to the prosthetic group in a somewhat differing way than in haemoglobin of blood.

The green pigment can be isolated from the green nodules in the same way as leghaemoglobin from the red ones. It is a chromoprotein (probably a mixture) and contains still iron. It has no longer the absorption bands of leghaemoglobin or of its O_2 and CO-compounds with distinct maxima between 500 and 600 mμ.[3] The iron is liberated from the green pigment by boiling with dilute hydrochloric acid. It is thus obvious that the porphyrin ring has been broken in the green pigment and simultaneously the iron has become more easily cleavable. Accordingly, the green pigment would be an intermediate stage between the leghaemoglobin and the bile pigments,[3] resembling substances which Barkan *et al.*[8] and Lemberg *et al.*[9] have prepared from haemoglobin. Such a formation of chromoprotein from the leghaemoglobin in the root nodules shows that leghaemoglobin is decomposed in approximately the same way as haemoglobin in animal organism. After all the nodules on the roots have turned green nitrogen fixation has completely ceased.

This paper presents an experimental material a part of which describes our recent findings, a part again is connected with the results published in preliminary communications. A critical review of the biology and chemistry of the nitrogen fixation by legume bacteria has recently been written by Virtanen.[26]

EXPERIMENTS

Detection of the ineffectiveness of H VIII strain

The pea bacterial strain H VIII was isolated in 1942 from the root nodules of pea in a field cultivation. In pot experiments with quartz sand as substrate pea did not grow when inoculated with this organism. All the nodules were white. Under these conditions, however, it is not possible to detect a few milligrams' fixation of nitrogen per plant. In order to prove the ineffectiveness of the strain as accurately as possible two series of experiments were carried out in the sterile culture system used in this laboratory. The results were very similar in both cases. Details of the second one are given in the following.

Seeds of Torsdag pea (of Svalöv) weighing 189—193 mg were selected for the experiments. Ten seeds of the same lot, 190 mg each, contained nitrogen according to the Kjeldahl determination as follows: 6.86, 6.02, 7.14, 6.44, 6.72, 6.44, 7.14, 6.72, 6.86, and 6.86 mg, on the average 6.72 mg. When nitrogen was determined from 5 samples of seeds each containing 10 seeds of equal weight, the analysis of each lot gave the following values for the average nitrogen content of one seed: (seeds weighing 188 mg) 7.14, 6.99, 7.42, 6. 71, 6.50, mean value 6.96 mg; (seeds weighing 194 mg) 7.21, 6.78, 7.49, 7.77, 7.21, mean value 7.25 mg. These figures alone reveal that fixation of about 1—2 mg nitrogen cannot be demonstrated by two parallels.

Sterilization of seeds and the technique of preparing sterile cultures have been described in previous papers.[10] Sterilized seeds were placed in tubes 31. VIII. 1943 and the seedlings were transferred to culture flasks 9. IX. For culture flasks we used 1 litre suction flasks connected with rubber tube to reserve flasks. The experiment comprised 9 flasks with one inoculated pea plant in each and 9 flasks with one uninoculated pea plant in each. The composition of the nutrient solution in the culture flasks was: KCl 250 mg, $Ca_3(PO_4)_2$ 250 mg, $CaSO_4$ 250 mg, $MgSO_4$ 394 mg, 3 drops of 5 % $FeCl_3$ solution, tap water to 1 litre. The reserve flasks contained tap water only. On transfer of the seedlings to the culture flasks the nutrient solution in 9 of these was inoculated with 1 ml of a water suspension of H VIII strain prepared by suspending the bacteria in water from the surface of agar. This amount of bacterial suspension contained 0.01 mg N, an insignificant amount in these experiments.

The peas grew in the greenhouse under natural light conditions. Nodules appeared on the plants inoculated with H VIII strain 17. IX, 8 days after inoculation. The results are given in table 1.

On the basis of the results it can be stated that no nitrogen fixation could be ascribed to H VIII strain. As the nutrient solution was the same in both series and the plants took up during the growth practically the same amounts of tap water the small amount of nitrogen (0.28 mg per litre) has not affected the results. In order to supply the nodules with sufficient oxygen the level of the water was allowed to lower to middle height of the flask thus providing excellent conditions for nitrogen fixation. The experiment included in the table for comparison shows that growth and nitrogen fixation were good in these circumstances when the plant was inoculated with the effective H 6 strain.

In the nodules formed by H VIII strain no traces of red pigment could ever be noted although the nodules could be examined continuously in the culture flask. On the other hand, the nodules formed by H 6 strain turned reddish in a few days after their appearance. Especially the nodules above the surface of the water had a beautiful reddish tinge.

Table 1. Experiment illustrating the nitrogen fixing ability of H VIII strain of pea Rhizobium. Test plant: Torsdag pea. Substrate: nutrient solution without combined nitrogen. Period of growth: 9. IX—6—11. X. 1943. Nodules appeared 17. IX.

	Tops			Roots			Nodules			Total dry weight, mg	Total N, mg	Tap water per plant, litres
No. of expt.	Dry weight, mg	N, mg	N, %	Dry weight, mg	N, mg	N, %	Dry weight, mg	N, mg	N, %			
Inoculated												
1	181	5.0	2.7	85	1.7	2.0	12	0.8	6.7	278	7.5	1.340
2	210	4.7	2.2	69	1.1	1.6	18	1.5	8.3	297	7.3	1.350
3	218	6.8	3.1	67	1.6	2.3	18	0.6	3.3	303	9.0	1.305
4	183	4.4	2.4	84	1.7	2.0	11	0.3	2.8	278	6.4	1.310
5	182	4.7	2.6	63	1.5	2.3	16	0.8	5.0	261	7.0	1.330
6	233	5.8	2.5	104	1.9	1.8	25	0.7	2.8	362	8.4	1.320
7	208	4.9	2.3	68	1.5	2.2	27	0.8	3.0	303	7.2	1.320
8	218	5.1	2.3	89	1.7	1.9	24	0.6	2.5	331	7.4	1.180
9	215	4.9	2.3	114	2.1	1.8	8	0.3	3.7	337	7.3	1.320
Average	205	5.1	2.5	83	1.6	2.0	18	0.7	4.2	306	7.5±0.77	1.308
Uninoculated												
1	219	6.4	2.9	111	2.3	2.1				303	8.7	1.300
2	185	5.1	2.7	119	2.4	2.0				304	7.6	1.340
3	175	4.7	2.7	120	2.1	1.8				295	6.8	1.330
4	189	4.9	2.5	130	2.4	1.8				319	7.3	1.270
5	178	4.4	2.4	115	2.4	2.1				293	6.8	1.350
6	188	4.6	2.4	124	2.3	1.9				312	6.9	1.100
7	186	5.0	2.7	127	2.5	2.0				313	7.5	1.100
8	182	4.3	2.3	119	2.5	2.1				301	6.8	1.330
9	188	4.4	2.3	140	2.7	1.9				328	7.1	1.340
Average	188	4.9	2.5	123	2.4	2.0				308	7.3±0.62	1.273
Inoculated with strain H 6												
	3430	94.9	2.8	247	5.0	2.0	98	4.6	4.7	3775	104.5	

Deeper under the water the colour was paler. The indispensability of sufficient supply of oxygen to the growth of inoculated legumes, which has been previously shown in this laboratory[11] is probably associated with the formation and action of leghaemoglobin in the nodules. — Leghaemoglobin is found in the interior of the nodule as well as the active bacterial mass. The easily accomplished passing of the pigment to solution on crushing of nodules implies that it is not in bacterial cells. In microscopic examination the H VIII nodules were found to contain exclusively rods surrounded by a slimy layer. In nodules formed by effective strains, such as H 6, only bacteroid forms[2] were found when the nitrogen fixation was at highest whereas in green nodules rods again were noted.

Through the courtesy of Prof. A. Wilska we were offered the possibility of examining the bacteroids in living state by the new micro-optics invented by him. By means of his microscope the differences in the refraction index are reflected in the darkness of the colour. The drawing in fig. 6 shows the bacteroid types observed. The large black granules are characteristic of bacteroids. The spaces between them seem to be optically almost empty.

The colour photographs (figs. 1—3) on the plate show nodules formed by the ineffective strain H VIII, red active nodules formed by the effective strain H 6, and nodules of the same strain which have changed to green and inactive in the dark. Fig. 4 on the same plate illustrates different shapes of bacteria in white, red, and green nodules, drawn according to the microscopic dyed preparation. Fig. 5 is a drawing of the living bacteroids visible under the microscope of Prof. Wilska.

Influence of nitrate on nitrogen fixation by peas

The much discussed question as to whether the root nodules fix nitrogen if the host plant is supplied with nitrate or with some other suitable form of combined nitrogen has not been solved. The experiments so far made had failed to give any solution owing to inadequate methods.[12] In connection with the experiments which we undertook to elucidate the indispensability of leghaemoglobin to nitrogen fixation solution was also found to the open question.

The experiments were made in the sterile culture system by using the so-called branched tube system which has been previously described in detail.[13] In this system the roots are divided into two culture flasks (fig. 7). This arrangement makes it possible to supply both branches with different nutrient solutions.

The experiment lasted from 25. V. to 25. VI. 1946. One culture flask contained 119 mg NO_3-N (in the form of $Ca(NO_3)_2$), the other was left without combined nitrogen, but inoculated with effective strain H 7. The composition of the nutrient solution was otherwise the same in both flasks.

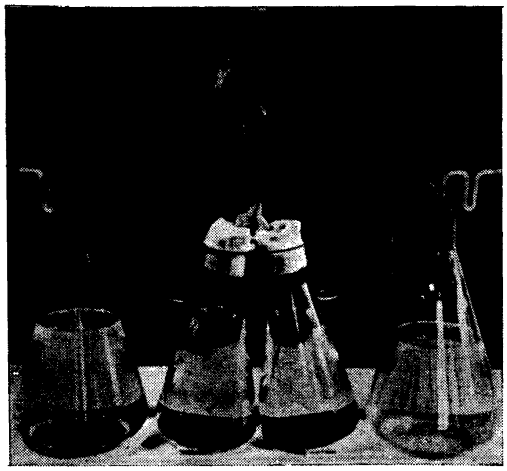


Fig. 6. The sterile branched tube system for investigation of nitrogen fixation and nutrient uptake in general.

The seedling was transferred to the system 25. V. The first nodules appeared on the inoculated root branch 1. VI and the nodules began to turn red after 4 days. The nodulation thus occurred as rapidly as in the experiments where the whole root system grew in the nutrient solution without combined nitrogen. The experiment succeeded in so far that the root branches grown into both flasks were approximately as well developed. The shape of the inoculated root system differed from that of the nitrate roots. In another paper we shall describe the effect of different nitrogen nutritions on the shape of roots. The experiment was interrupted 25. VI, at the start of flowering. The inoculated root system had then 68 red nodules, 22 green nodules, and 5 white nodules; a total of 95 nodules. The results of the analyses are summarized in table 2.

Table 2. Experiment with Torsdag pea in sterile branched tube system. Period of growth: 25. V— 25. VI. 1946. One culture flask contained 119 mg NO_3-N, the solution in the other was without combined nitrogen, but inoculated with the effective H 7 strain. Red nodules formed on this branch. Nitrogen balance at the end of the experiment appears from the figures in the table.

	Dry weight, mg	N, mg		mg
Nodules	22	1.1	Initial N of the nutrient solution	119.0
Roots, inoculated branch [1]	217	6.6	N in seed [2]	7.5
Roots, nitrate branch	180	7.0	N in 2 litres of tap water [2]	1.0
Green parts	2 055	74.9		
Total	2 474	89.6	Total initial N	127.5
Left in the nutrient solution		56.7	**Increase of N in the system**	**18.8**
Total final N		146.3		

The experiment proves convincingly that even when the pea was supplied with sufficient nitrate, red active nodules were formed in the roots and nearly 20 mg nitrogen were fixed. On the other hand, nitrate has evidently caused a marked decrease in the number and size of nodules compared to the plants grown without nitrate, and the amount of fixed nitrogen represents therefore only perhaps one third of that which would have been fixed had even the uninoculated branch grown in nitrate-free solution. Such an experiment was not carried out as parallel here but on the basis of the corresponding experiments previously made the influence of nitrate can be approximately estimated.

In another experiment where the ordinary sterile culture system was employed and the whole root system grew in the same culture flask, the following was noted. If the concentration of nitrate in the nutrient solution is comparatively low, less than 25 mg NO_3-N per litre, red normal-sized nodules are formed in the upper part of the roots, though they are few in number, and fixation of nitrogen takes place. If the concentration

[1] Nodules separated.

[2] Estimated by the control.

of nitrate is high, e. g. 100 mg NO_3-N per litre, in most cases no nodulation occurs. Exceptionally a few very small nodules are formed. No nitrogen is then fixed. In some experiments in which the nutrient solution contained 30—50 mg NO_3-N per litre a few quite small nodules were at first formed in the upper part of the root system, a part of them showing red colour. No nitrogen fixation was detected. Whether fixation has occurred in minute quantities can be demonstrated only by isotope method. It is possible that a compound of nitric oxide-leghaemoglobin is then formed preventing the action of leghaemoglobin. This has, however, not yet been experimentally proved.

When ammonium sulphate forms the source of nitrogen large red nodules are formed in concentrations where the nitrate nitrogen reduces the size of nodules to quite small and prevents nitrogen fixation. For example, in an experiment where the nutrient solution contained in the beginning 50 mg and at the end 31 mg NH_4-N per litre, the pea took up from the solution 42.4 mg NH_4-N (total amount in the solution 100 mg N) and received almost an equal amount of nitrogen fixed in the root nodules, since the whole plant contained 87.4 mg N. There seems thus to exist a great difference between the nitrate and ammonium nitrogens in their relation to the nitrogen fixation. The result obtained in the branched tube system indicates that the NO_3-concentration in the roots has an inhibiting effect on the action of nodules.

Purification of leghaemoglobin

As a starting material we used samples of 200—500 g of the root nodules of soya grown in large wooden boxes of sand or in the experimental field of our laboratory. The nodules were detached from the roots of the test plants when in best growth. In trying to obtain as pure preparation of leghaemoglobin as possible we collected the fraction which precipitates when ammonium sulphate is added up to 66—75 % saturation,[7] the solution neutralized and kept overnight in cold. The nodules were crushed in a mortar,. a large volume of water was added, and the suspension was centrifuged. To the red solution ammonium sulphate was first added to 60 % saturation. After addition of ammonium sulphate the solution was always neutralized with sodium hydroxide. After standing overnight in cold, the precipitate was separated by centrifuging, and ammonium sulphate was added to the solution to make the saturation 80 %. After a further standing overnight in cold the precipitate was again separated by centrifuging, dissolved in water, and precipitated first by adding ammonium sulphate to 66 % saturation and then after removal of the precipitate up to 66—75 % saturation. The first purifications in 1945 of the leghaemoglobin from the nodules of soya led after five successive precipitations (66—75 % saturation) to a preparation which was chiefly legmethaemoglobin and which after dialysis against running water until free from ammonium sulphate contained 0.27 % Fe in the dry matter. The haematin content of the preparation was determined as pyridine haemochromogen. The extinction was determined by filter S 53. Haemochromogen prepared in the same way from the haemoglobin of blood was used as control. The haematin content of the preparation was 3.8 %. On the basis of these values the purity of the preparation was 80—85 %[7] presuming that the iron and haematin contents are the same as those of the haemoglobin of blood.

With this preparation K. O. Pedersen in Uppsala determined in November 1945 the sedimentation and diffusion constants. We are greatly indebted to Dr. Pedersen for his valuable determinations. On the basis of his values the molecular weight of the leghaemo-

globin would be 34 100 or a half of that of the haemoglobin of blood. Leghaemoglobin will accordingly contain two haem groups in its molecule.

In autumn 1946 we made a preparation of leghaemoglobin from the root nodules of soya bean by passing carbon monoxide into the solution and by taking care that after each precipitation and dissolution of the precipitate the solution was treated with carbon monoxide. In this way it was attempted to prevent the oxidation of the preparation to legmethaemoglobin. After the fifth precipitation with ammonium sulphate the preparation was dialyzed and no precaution was then taken to avoid its autoxidation. So, after dialysis, the preparation was for the most part legmethaemoglobin. Its Fe-content was now 0.34 % corresponding with fair accuracy to that of the crystalline haemoglobin of blood. Accordingly, the purity of the preparation would have been nearly 100 %.

Haematin and leghaemoglobin and/or legmethaemoglobin contents of the nodules

In order to determine the haematin content of the nodules formed by different strains and consequently varying in effectiveness we used the following technique. A weighed amount of the nodules of pea was crushed in a mortar with pyridine. The precipitate was separated by centrifuging and washed three times with a small volume of pyridine. The extinction was determined from the combined solution of pyridine which was deep red in colour. The results thus obtained gave a rough idea of the haematin content of the nodules in the early part of growth up till flowering. When the formation of the green pigment starts to a greater extent the method gives very erroneous results because the green pigment interferes with the results.

Table 3. Experiment in the sterile culture system with pea (Torsdag) in nutrient solution without combined nitrogen. 12 Woulff-bottles, 2 peas in each, inoculated with different strains of pea Rhizobium as follows:

5 bottles with strain H 7 (very effective)
5 » » » H 2 (less effective)
2 » » » H VIII (ineffective)

The seedlings were transferred to bottles 27—28. VIII. 1946. The first nodules appeared 5—7. IX. On appearance of the first buds (8. X) two plants, inoculated with different strains, were simultaneously taken for haematin determination.

	In 2 plants				Haematin in nodules		Leghaemoglobin in nodules	
Inoculation	Dry matter, mg	N, mg	Number of nodules	Dry weight of nodules, mg	γ per g dry matter	γ per plant	% of dry matter	γ per plant
Strain H 7	1 382	51.0	831	117	1 130	66	2.9	1 700
Strain H 2	1 023	36.5	678	77	910	35	2.3	900
Strain H VIII	664	17.3	420	25	0	0	0	0

These preliminary results, too, seem to suggest that the rate of nitrogen fixation depends on the haemoglobin content of the root nodules. In the future experiments this point will be taken into closer examination.

Since, however, the haematin component in the nodules seems, at any rate in the early days of growth, to belong mainly to the haemoglobin or methaemoglobin — denatured haemoprotein is then found only little — the approximate amount of leghaemoglobin + legmethaemoglobin in the nodules can be calculated on the basis of the haematin content provided that good growth conditions prevail until flowering. At a later stage of growth the amount of denatured haemoprotein increases and the calculation leads therefore to very erroneous results. By assuming the haem content of the pure leghaemoglobin to be 3.9 % we obtained the following values in a preliminary experiment for the haematin and leghaemoglobin + legmethaemoglobin contents of the nodules (table 3).

Iron content of the nodules

Some experiments have been made to determine the iron content of the nodules. The total iron was determined from the ashes. Some drops of conc. nitric acid were added to the ashes, and after evaporation 1 ml of 6 *N* hydrochloric acid was added and heated to boil. The solution was washed to a small measuring glass, made up to 2 ml, whereupon 1 ml of 4 % ammonium thiocyanate solution and 2 ml of ethylacetate were added and the mixture was shaken. The intensity of the red colour of the clear ethylacetate solution was determined by the Pulfrich photometer. By comparing the extinction values with the values obtained with known amounts of iron it is possible to determine the iron content. Table 4 gives the results.

Table 4. Two plants, inoculated with different strains were simultaneously taken for iron determinations from the sterile culture experiments described in table 3. The figures in parentheses indicate the haematin iron. Determinations were made on following dates:

I. *8. X on appearance of the first buds*
II. *15. X during flowering*
III. *22. X at the end of flowering*
IV. *25. X when pods had developed*

	Fe, γ per g dry nodules			Fe, γ per g dry roots (nodules separated)		
	H 7	H 2	H VIII	H 7	H 2	H VIII
I	850	685	—	—	—	—
II	825 (100)	580 (80)	440 (0)	315	270	340
III	680 (108)	580	—	315	310	—
IV	540	—	—	—	—	—

The data show that the iron content of the nodules is considerably higher than that of the roots and that the difference is not caused by leghaemoglobin alone. According to our preliminary experiments the other iron compounds of the nodules, excluding the leghaemoglobin iron, can for the most part be dissolved by boiling with 0.5 % hydrochloric acid. The iron in the roots is then likewise almost entirely dissolved.

Isolation and purification of the green pigment

The formation of the green pigment was started by removing the sterile cultures of peas, inoculated with effective bacterial strains, into a completely dark room for a few days. As a rule the colour of the nodules changes to green in 3—4 days. The nodules were then separated from the roots and crushed in a mortar with water. The purification of the green pigment was performed by the same method as that of the red one. In precipitating the green pigment the precipitate was collected which was formed by 50—75 % saturation with ammonium sulphate. The preparation thus obtained was dialyzed. The iron contents of three different preparations were 0.22 %, 0.27 %, and 0.29 %.

With the preparations of green pigment isolated from the root nodules of soya and precipitated with ammonium sulphate some experiments were made for liberation of the iron with hydrochloric acid. The preparation was boiled in two tubes for four hours in a 1 % solution of hydrochloric acid, a current of carbon monoxide was led to one tube during boiling, a current of air or oxygen to the other. Different amounts of preparation were used in both experiments. The iron was determined in the manner described above by using ethylacetate.

	Atmosphere of air	Atmosphere of CO
Experiment 1	Extinction 72	Extinction 78
	Atmosphere of O_2	Atmosphere of CO
Experiment 2	Extinction 33	Extinction 31

The experiment shows that CO has not prevented the liberation of iron during boiling. In view of the fact that approximately the same amounts of iron were found both in atmosphere of CO and in that of oxygen it is likely that the iron in the pigment is trivalent. Whether iron becomes oxidized on isolation and purification or whether it is trivalent already in the nodules, is unknown. According to a previous observation preliminarily communicated [3] carbon monoxide seemed to prevent at least to some extent the cleavage of iron. The possibility that the iron of green pigment would occur in the nodules both in di- and trivalent forms must therefore be considered.

With regard to the preparations of green pigment so far made it is highly probable that the question is about a mixture of several chromoproteins. The green pigment isolated from the nodules which have turned green after the cessation of growth resembles in its properties the pigment formed in young nodules in the dark. On the basis of the findings so far made it seems that the green pigment becomes more easily denatured than the leghaemoglobin.

Leg- and legmethaemoglobin turns greenish in a solution containing ascorbic acid and hydrogen peroxide. Absorption maxima between 500—600 mμ disappear at the same time. In a leghaemoglobin-containing solution (6.0 ml), the pH of which was adjusted to about 6.5 by means of phosphate buffer and which was 0.03 % in regard to l-ascorbic acid, a 0.4 ml addition of 3 % H_2O_2 affected at room temperature the change of the colour from red to greenish and the disappearance of absorption maxima in 1 minute. With an addition of 0.04 ml of 3 % H_2O_2 the change took place in about half an hour.

The Gmelin test was made with the green nodules of soya as follows: the nodules were crushed in glacial acetic acid, acetone was added and the solution evaporated to a small volume, whereby precipitate was formed. A small volume of the solution was carefully poured on nitric acid in a test tube. Various coloured rings, though faint, formed at the junction. The extract of green nodules also gave a similar reaction.

Nitrogen fixation with free-living legume bacteria

As the indispensability of leghaemoglobin to nitrogen fixation in the root nodules seemed very plausible some experiments were made in our laboratory in order to find out whether it is possible to bring about nitrogen fixation with free-living legume bacteria by supplementing the nutrient solution with a water extract of red nodules or with purified leghaemoglobin. In the first experiments we used a raw extract of nodules which was filtered through a bacterial filter and contained both leghaemoglobin and legmethaemoglobin (and naturally many other substances). The sterile nutrient solution without combined nitrogen, containing mannite 20 g, K_2HPO_4 1.2 g, $CaCO_3$ 1.0 g, $MgSO_4 \cdot 7\,H_2O$ 0.5 g, $MnSO_4 \cdot 7\,H_2O$ 0.5 g, Na_2MoO_4 0.005 g, $FeSO_4 \cdot 7\,H_2O$ 0.1 g, and tap water to 1 000 ml, was divided into Roux-flasks, 150 ml to each, and each flask was inoculated with a loopful of H 7 organism and stored for 3 days at 25°. After that nodule extract was added to 2 of the flasks, to 2 again nodule extract plus oxaloacetic acid, while 2 were kept as controls without any additions. The flasks were kept at 25° for 3 days, after which nitrogen was determined. Before Kjeldahl determination an equal amount of the root nodule extract, stored in the ice box, was added to the controls as had been added to the test flasks 3 days earlier. The added amount of combined nitrogen thus came to be the same in all flasks. In the first experiments[14] an increase of nitrogen was found in amounts far beyond experimental error, particularly in the flasks containing oxaloacetic acid and nodule extract, but also in some flasks with nodule extract alone. On the other hand, no increase of nitrogen was affected by the purified pigment.

In repeating these experiments in winter 1945—46 we failed to confirm the positive results of the first experiments[15]. In summer 1946 altogether seven further series of experiments were carried out by employing the same bacterial strain for inoculation as in the preceding summer. In the most experiments the nodule extract was prepared from the nodules of soya, in one again from those of pea. The oxaloacetic acid solution was neutralized, filtered through a bacterial filter and added to the flasks in four lots during a period of 3 days, 20 mg at a time. The bacteriological purity of the control cultures was tested at the end of the experiment microscopically and in sterile milk and on slanted agar. No contamination was noted in any experiments.

As can be seen from table 5 no nitrogen fixation was noted when to the culture of legume bacteria either a raw nodule extract or purified leghaemoglobin was added. Only in the culture 4 in experiment 5 a considerable increase of nitrogen seemed to occur, but this single exception is evidently due to an experimental error. The nitrogen analyses in parallel experiments have generally given concordant values. In the experiment made for comparison with *Azotobacter* (no. 10) a powerful nitrogen fixation was observed. — What accounts for the positive results obtained in summer 1945 has remained unrevealed.

Transfer of nitrogen from nodules to host

In order to follow the transfer of nitrogen from nodules to host at different stages of growth as well as the changes in the colour of the pigment we undertook several experiments on pea. A typical example is given below.

An experiment on inoculated Concordia pea was made 17. V.—30. VII. 1941. The plants were grown in quartz sand in unglazed clay pots 8 inches in diameter. The experiment comprised 15 pots, 3 plants in each. The watering solution contained: 3.6 g $MgSO_4$,

Table 5. Experiments in 1946 with free-living legume bacteria for ascertainment of nitrogen fixation. The quantity of nodule extract given in the table was equally divided between the individual culture flasks of each experiment. To the control the extract was added at the end of the experiment prior to determination of nitrogen. The volume of nutrient solution was 100 ml per flask, the additions appear from the table. The figures express ml of 0.1 N *sodium hydroxide used for back titration of 50 ml 0.1* N *sulphuric acid, except in experiment no. 7 where they express N in mg. Duration of experiment was 3 days.*

No. of expt.	Added	Extract and oxaloacetic acid		Extract		Control	
		Flask 1	Flask 2	Flask 3	Flask 4	Flask 5	Flask 6
1	55 ml water extract from 20 g of nodules of pea	—	47.1	47.2	47.1	47.2	47.2
2	20 ml water extract from 12 g of nodules of soya	47.9	48.4	47.4	48.2	48.2	49.6
3	26 ml water extract from 13.5 g of nodules of soya	47.8	—	47.8	47.8	47.8	47.8
4	16 ml water extract from 15 g of nodules of soya	46.7	47.1	46.8	47.1	46.9	46.9
5	26 ml water extract from 22 g of nodules of soya	45.7	—	—	38.7	46.1	46.4
6	26 ml water extract from 25 g of nodules of soya	44.6	44.6	44.9	45.0	—	45.6
7	50 ml extract[1] from 271 g of nodules of soya	16.9	—	17.4	—	17.7	—
8	10 ml purified leghaemoglobin[2] solution	47.6	47.3	—	—	47.6	47.5
9	4 ml sterile blood of sheep	29.2	29.3	—	—	28.3	29.8
	Azotobacter chroococcum (experiments inoculated, but no extract added; controls uninoculated)	41.9	40.4	—	—	49.1	48.9

[1] Nodules were crushed without addition of water and the juice was pressed through a cloth, centrifuged and filtered through a bacterial filter. Experiment comprised 15 flasks. Each figure represents the mean value of analyses from 5 flasks. (Experiment was carried out in August 1946 by J. Erkama to whom we express our thanks.)

[2] Leghaemoglobin was isolated from a 100 g sample of nodules of soya, and purified by precipitating twice with ammonium sulphate (66—75 % saturation). After dialysis the preparation was used to the experiment. (Experiment was carried out in autumn 1945 by T. Laine.)

4.5 g KCl, 4.2 g KH_2PO_4, 4.0 g $CaSO_4$ in 60 litres of tap water. Besides, at the time of seeding the sand was watered with the minor element solution of Hoagland and Broyer. The effective strain H 11 was used for inoculation. In different periods of growth plants from 3 pots were analyzed at a time, accordingly, the mean values of each period are based on analyses from 9 plants. The periods of growth are apparent from table 6.

Table 6. Experiment with Concordia pea in open pots. Substrate: quartz sand without combined nitrogen. Period of growth: 17. V—30. VII. 1941.

No. of expt.	No. of cultures	Period of growth	Duration of growth, days	Length of period, days	Stage of growth
I	1—3	17. V—30. VI	45	45	Before flowering
II	3—6	17. V—12. VII	57	12	Start of flowering
III	6—9	17. V—21. VII	66	9	End of flowering
IV	9—12	17. V—30. VII	75	9	Pods well developed

Table 7. Experiment belonging to that presented in table 6. Dry weights and N-amounts of plants and nodules during different periods of growth.

No. of expt.	No. of culture	Tops			Roots			Nodules		
		Dry weight, g	N, mg	N, % of dry weight	Dry weight, g	N, mg	N, % of dry weight	Dry weight, g	N, mg	N, % of dry weight
I	1	2.059	110.50	5.37	0.539	14.39	2.67	0.271	21.11	7.79
	2	2.013	80.40	3.99	0.679	16.97	2.50	0.274	22.84	8.34
	3	1.604	78.40	4.89	0.586	15.16	2.59	0.251	21.32	8.49
Mean value		1.892	89.77	4.75	0.601	15.51	2.59	0.265	21.76	8.21
II	4	4.670	211.92	4.54	0.647	18.04	2.79	0.424	28.94	6.83
	5	5.545	232.56	4.19	0.912	22.47	2.46	0.539	39.26	7.28
	6	7.452	273.34	3.67	1.091	34.99	3.21	0.865	61.87	7.15
Mean value		5.889	239.27	4.13	0.883	25.17	2.82	0.609	43.36	7.09
III	7	12.029	344.03	2.86	1.417	37.60	2.65	0.924	46.96	5.08
	8	8.853	339.96	3.84	1.269	44.59	3.51	0.788	53.30	6.76
	9	10.125	324.61	3.21	1.125	28.67	2.55	0.860	52.29	6.08
Mean value		10.336	336.20	3.30	1.270	36.95	2.90	0.857	50.85	5.97
IV	10	9.152	282.61	3.09	0.926	23.63	2.55	0.568	27.95	4.92
	11	12.520	388.62	3.10	1.317	30.25	2.30	0.709	37.63	5.31
	12	12.250	406.46	3.32	1.577	32.70	2.07	0.753	37.02	4.92
Mean value		11.307	359.23	3.17	1.273	28.86	2.31	0.677	34.20	5.05
Uninoculated		0.620	14.81	2.39	0.628	9.85	1.57	—	—	—

Table 8. Experiment belonging to that presented in tables 6 and 7. Nitrogen amounts and the ratio of N in the host plant to the N in the nodules at different stages of growth.

No. of expt.	No. of culture	Duration of growth days	N mg, control subtracted: Tops	Roots	Tops + roots	N in nodules, mg	Ratio of N in tops + roots to N in nodules	N in tops + roots, % of total N
I	1		95.7	4.5	100.2	21.1	4.75 : 1	82.6
	2	45	65.6	7.1	72.7	22.8	3.1. : 1	76.1
	3		63.6	5.3	68.9	21.3	3.24 : 1	76.4
Mean value			75.0	5.6	80.6	21.7	3.73 : 1	78.4
II	4		197.1	8.2	205.3	28.9	7.10 : 1	88.0
	5	57	217.8	12.6	230.4	39.3	5.89 : 1	85.4
	6		258.5	25.1	283.6	61.9	4.58 : 1	82.1
Mean value			224.5	15.3	239.8	43.4	5.86 : 1	85.2
		In 12 days	149.5	9.7	159.2	21.7		86.9
III	7		329.2	27.8	357.0	47.0	7.60 : 1	88.4
	8	66	325.2	34.7	359.9	53.3	6.72 : 1	87.1
	9		309.8	18.8	328.6	53.3	6.17 : 1	86.1
Mean value			321.4	27.1	348.5	51.1	6.83 : 1	87.2
		In 9 days	96.9	11.8	108.7	7.7		93.4
IV	10		267.8	13.8	281.6	28.0	10.10 : 1	91.0
	11	75	373.8	20.4	394.2	37.6	10.44 : 1	91.3
	12		391.7	22.9	414.6	37.0	11.23 : 1	91.8
Mean value			344.4	19.0	363.5	34.2	10.63 : 1	91.4
		In 9 days	23.0	—8.1	15.0	—16.9	—	—

The results are examined in detail in the discussion.

For analysis the plants were freed from sand. The tops and the roots were separated from each other. The roots were washed free from sand and the nodules were detached by means of forceps and knife. Tops, roots, and nodules were weighed fresh and dried at 105° to a constant weight. Nitrogen was determined separately from tops, roots, and nodules. The total quantity of nodules and roots was used for the analysis, of the tops a sample of 500 mg was taken. The results are given in tables 7 and 8.

DISCUSSION

The experimental results of this laboratory which have been recorded both in previous papers and in the present one show concordantly that the red pigment is always present in active N-fixing root nodules, whereas it is never

found in ineffective ones. It seems that factors of most various kind which inhibit nitrogen fixation effect harmfully on the formation or promotingly on the inactivation of the pigment in the root nodules. This is true, for instance, of the effect of nitrate. The question much discussed as to whether the nodules fix nitrogen when the plant is supplied with sufficient nitrate or ammonium nitrogens has got a positive solution in our experiments and it has been shown at the same time that the red nodules are then in action. In nitrate concentrations where the nodules remained white no nitrogen fixation took place. If the nitrate concentration of the nutrient solution is so high that it allows formation of a few small nodules only, it seems possible that a nitric oxide-leghaemoglobin compound may be formed preventing the action of leghaemoglobin. In one experiment on pea in which a part of the roots (inoculated) grew in nitrate-free nutrient solution, a part again in nutrient solution with very high nitrate concentration, active red nodules appeared on the roots in the nitrate-free solution and nitrogen was fixed considerably. It seems therefore likely that the concentration of nitrate in the roots is an inhibiting factor for the formation and action of the nodules. This is suggested by the fact that ammonium salts do not prevent formation of normal-sized leghaemoglobin-containing nodules nor ample N-fixation in such concentrations where the nitrate already completely prevents the N-fixation.

On the basis of these observations the hypothesis [12] that combined nitrogen reduces the level of carbohydrate in the host and thus prevents nitrogen fixation is insufficient to explain the specific effect of nitrate. The remarkable observations of Fred, Wilson *et al.*[16] of the inhibiting effect of too intensive continuous lighting upon the nitrogen fixation in soya are possibly also ascribable to the failure of the formation of leghaemoglobin.

Determinations of haematin and leghaemoglobin in the nodules imply that a positive correlation exists between the effectiveness of the nodules and their leghaemoglobin content. In the light of the ocular examination of the nodules in numerous parallel cultures (sterile system) it seems likely, that the more leghaemoglobin is found in nodules and the longer it remains undecomposed, the more nitrogen is fixed. The nodules formed by less effective strains often begin to turn green already at an early stage of growth. Under unfavourable conditions the same is also noted in nodules from effective strains.

The effective nodules contain more iron than the roots. The difference is due partly to the haematin iron, partly possibly to other iron compounds. The nodules of the ineffective strain H VIII contain only a little more iron than the roots. The iron of the roots and of the nodules, excluding

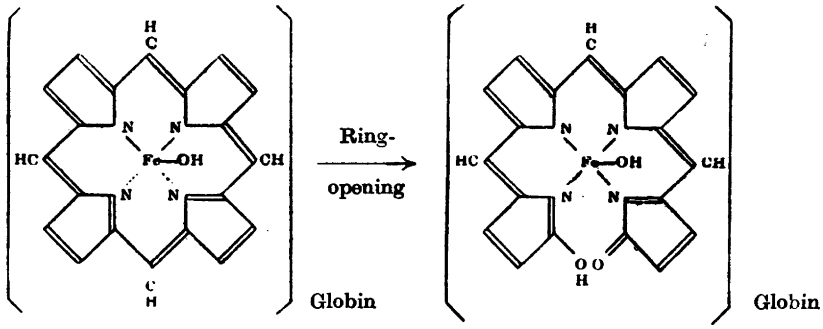
the leghaemoglobin iron, seems for the most part to dissolve on boiling with dilute hydrochloric acid. Since the iron content of the nodules seems to be the higher the more effective the nodules are, the extra iron has evidently accumulated in the interior of the nodule where the active bacterial mass is found.

The indispensability of leghaemoglobin to the function of root nodules may be entirely due to its ability to store and carry oxygen. This action of leghaemoglobin may promote the respiration of the nodule bacteria and it may also supply the oxygen for the oxidation of molecular nitrogen, if this is the first step in the nitrogen fixation. Since leghaemoglobin is autoxidized to legmethaemoglobin more easily than the haemoglobin of the blood of vertebrates it can also be assumed that the valency changes of iron in the leghaemoglobin may be involved in the nitrogen fixation.[3] This hypothesis is, however, fully speculative.

It is interesting to note, that Keilin and Wang [17] in their recent studies on the haemoglobin of *Gastrophilus*-larvae, which also is easily autoxidized, advanced the possibility »that the oxygen dissociating from its union with haem may possess a slightly higher level of reactivity than the ordinary molecular oxygen and that this level may vary with the haemoglobin». The easily autoxidizable haemoglobins, which seem to constitute the low-molecular haemoglobins (the molecular weight of leghaemoglobin is probably 34 000, of the haemoglobin of *Gastrophilus* 34 000, and of myoglobin 18 000), would thus occupy a position between the oxygen carriers and the oxidizing catalysts. A point of interest is that according to the determinations of Keilin and Wang the haemoglobin of *Gastrophilus* has a very low affinity for carbon monoxide ($K = [HbCO] \times pO_2 / [HbO_2] \times pCO = 0.67$), the corresponding value with phenoloxidase being 0.25—1, with cytochrome oxidase 0.1 and with the haemoglobin of vertebrates 120—550. With a very impure preparation of leghaemoglobin the said authors have obtained the value 37, which is of the same order as that with myoglobin.

Leghaemoglobin and legmethaemoglobin are easily changed to green pigment. A water-soluble green chromoprotein (probably a mixture of chromoproteins) which contains iron can be isolated from the nodules of plants kept some days in the dark as well as of plants which have normally ceased to grow. In addition, the nodules contain denatured green pigment. Ascorbic acid may have an important role in the formation of green pigment. The experiments *in vitro* have proved that leghaemoglobin and legmethaemoglobin are rapidly changed to green pigment at room temperature if the solution contains ascorbic acid and hydrogen peroxide. Thereby a considerable denaturation of protein also takes place. The ascorbic acid content of the nodules after several days' storage in the dark is still so high (0.01—0.02 % of the nodule juice after 4 days) that the formation of the green pigment may well

be linked to the ascorbic acid. As far as hydrogen peroxide is formed in the nodules in these circumstances all factors are provided for the formation of green pigment. The catalase activity of the nodules decreases if pea or soya is kept in the dark and consequently, the chances to formation of hydrogen peroxide in the nodules become greater. It is possible that the breaking of porphyrin ring may also occur without hydrogen peroxide at the presence of oxygen although the reaction is then very slow at 10—20°. Whether the opening of the ring is caused by the oxidation of leghaemoglobin and/or legmethaemoglobin is unknown. The latter alternative seems to be supported by the increase of legmethaemoglobin when the plants are kept in the dark. The reaction would then proceed in the following way.



Of course leghaemoglobin, too, may undergo the corresponding oxidation which leads to the opening of the porphyrin ring.

Lemberg [9] assumes that the precursor of bile pigments, choleglobin, is formed in animal tissues from oxyhaemoglobin in coupled oxidation with ascorbic acid (H_2A):

$$HbO_2 + H_2A \longrightarrow Hb \cdot H_2O_2 + A$$

$$Hb \cdot H_2O_2 \longrightarrow HbOH \text{ (methaemoglobin)}$$

$$Hb \cdot H_2O_2 \longrightarrow \text{choleglobin}$$

The mode of formation of the green pigment in the root nodules is still to a great extent obscure. It seems likely in any case that both in the dark and with cessation of growth either some substance disappears from the nodule juice which during the growth prevents the breaking of porphyrin ring in leghaemoglobin (or legmethaemoglobin) or a factor is formed which causes

the oxidation of the ring. According to our determinations oxaloacetic acid disappears in such conditions but so do probably some other active substances. The possible role of oxaloacetic acid in this connection has previously been pointed out.

Much attention was at first paid to Beijerinck's [19] observation of the occurrence of swollen and irregular »bacteroids» in the root nodules, since these were supposed to represent a type of nitrogen-fixing bacteria and the unchanged bacteria in the nodules were regarded as ineffective (for instance, Nobbe and Hiltner [20]). This conception was, however, later rejected. Especially the findings of Almon [21] according to which the bacteroids are not able to multiplication and do not cause nodulation indicated that the bacteroids are senile and biologically very inactive organisms. This does not, however, prove that bacteroids could not be active factors in the nitrogen fixation since this occurrence seems not to be associated with the propagation of bacterial cells (*cf.* below).

According to our observations the bacteria appear in the red nodules, formed by effective strains H 6 and H 7, chiefly as »bacteroids», in the ineffective white nodules, formed by H VIII strain, as rods which are surrounded by a slimy layer [2] (fig. 4). These observations thus support the old conception of the significance of »bacteroids» in the nitrogen fixation. The findings of some other workers (*e. g.* Pfeiffer [22]) of the absence of bacteroids from certain legume make, however, further investigation necessary and prevent generalization of our findings made with nodules of pea. The formation of red pigment and the development of bacteroids in the roots of pea seem to proceed, at any rate qualitatively, parallel.

An experiment recorded in the experimental part on the transfer of nitrogen from the nodules to the host over successive periods of growth after the appearance of nodules deserves still a closer examination. For this purpose we give below a graphical illustration of the results (fig. 7).

In the process of fixation and transfer of nitrogen several different phases can be distinguished. These have been marked in the graph by dotted vertical lines. Of course there is no sharp distinction between the different phases.

In the first few days the nodule-forming bacteria receive their nitrogen from the host plant (phase 1). The nodules are then white. However, after two-four days red pigment and the requisite machinery for nitrogen fixation begin to form in the nodules. Nitrogen fixation now starts and simultaneously begins the passage of nitrogen from the nodules to the host plant and a great number of new nodules appears. Lively multiplication of bacteria accompanied by protein synthesis still continues in the nodules for some time, and therefore appreciably nitrogen is retained in the nodules (phase 2). However, the

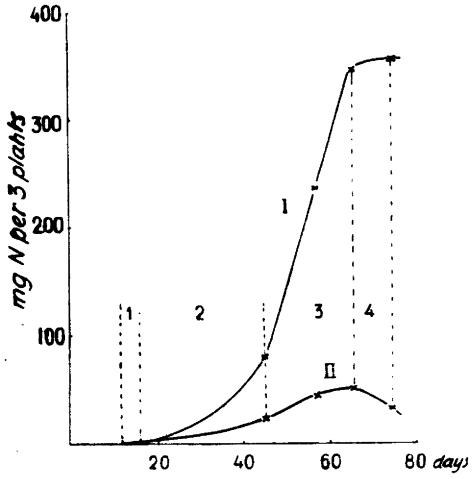


Fig. 7. Fixation of nitrogen in root nodules of pea and its transfer to host plant. Curve I: N in plants. » II: » » nodules.

percentage of nitrogen retained by the nodules decreases continuously during this phase. Nitrogen is fixed at a higher rate than new bacteria are formed within the nodules and as a consequence, the transfer of nitrogen to the host is proportionally increased. In the root nodules of pea the bacteria are now present chiefly as bacteroids. The transfer of nitrogen from nodules to host reaches gradually its maximal rate at phase 3. This phase during the middle and the later half of which about 90 % of the fixed nitrogen is regularly past to the host can be compared to the fermentation brought about in sugar solutions with »ready» bacterial or yeast masses (resting cells) when the enzyme machinery of the cells ferments sugar at an even rate. Nitrogen fixation is at phase 3 largely independent of the multiplication of bacteria and of the formation of bacterial proteins. The products of nitrogen fixation are probably excreted to the cytoplasm and used as nutrition by the host plants. The occurrence may be regarded as excretion especially as nitrogen compounds may also be excreted from the nodules to the culture medium. In connection with the latter phenomenon it was for the first time suggested [23] that the host plant obtains through excretion the amino acids (amino dicarboxylic acids) formed in the nitrogen fixation and that the earlier conception according to which proteins would first be formed in the nodules and only their decomposition products used by the host plant does not hold good.

Bond [24] has in his interesting experiments on soya arrived at the result »that a very high proportion probably in the region of 80 to 90 per cent is regularly liberated without appreciable delay into the host cytoplasm and that there is no retention or storage to any considerable extent of the fixed nitrogen within the bacteria or the nodules».

Wilson and Umbreit [25] have noted the same although they do not regard it as a proof of excretion.

The steady increase of nitrogen compounds in the host plant ceases during the last phase (phase 4) after the vegetative growth of the pea has come to an end. True, even after that the nitrogen content of the host plant is probably slightly increased (this is not easy to prove convincingly because of the variations in individual plants) and that of the nodules correspondingly decreased. The fixation of nitrogen has, however, completely ceased and soluble nitrogen compounds are passed from nodules to plant. This occurs possibly in certain measure already at the third phase especially towards the end of it as the nodules turn green. At the beginning of the fourth phase the red pigment has disappeared and the nodules have turned green. The association of leghaemoglobin with the nitrogen fixation is particularly apparent from experiments where the colour of the nodules and the increase of nitrogen are followed over the whole period of growth.

Nitrogen fixation could not be accomplished with free-living legume bacteria by adding to the nutrient solution an extract of crushed red nodules filtered through bacterial filter. The experiments made in winter and summer of 1946 failed to confirm the first positive results. In no experiment did the preparation of purified leg- and/or legmethaemoglobin cause nitrogen fixation in cultures of legume bacteria. The machinery which is required for nitrogen fixation has thus not been devised with free-living legume bacteria. It is possible that the occurrence is connected with a definite intranodular structure.

SUMMARY

The inability of the nodules formed by pea bacterial strain H VIII to fix nitrogen, at any rate in amounts detectable by ordinary analytical methods, has been shown. The H VIII nodules are devoid of leghaemoglobin.

Fixation of nitrogen by pea growing in nutrient solution with moderate concentration of nitrate has been demonstrated. Under such conditions the nodules are red. Nitrate prevents nitrogen fixation already in such concentrations which in regard to ammonium salts have no inhibiting effect on the formation of active red nodules of normal size. The causes of the harmful effect of nitrate nitrogen have been discussed.

Leghaemoglobin has been purified in so high degree that its iron content is the same as that of the haemoglobin of blood.

In parallel experiments on pea a positive correlation seems to exist between the leghaemoglobin content of the nodules and their N-fixing ability. More experiments are required in this respect.

The iron content of the effective nodules is considerably higher than that of the ineffective nodules and of roots.

The iron content of the purest preparation of the green pigment has been 0.29 %.

Nitrogen fixation has not been accomplished with free-living legume bacteria in nutrient solutions to which had been added either raw extract of nodules containing leghaemoglobin and/or legmethaemoglobin or purified preparation of these chromoproteids.

Transfer of nitrogen, fixed in the nodules, to the host has been examined over the successive stages of growth. During the main period when the major part of the nitrogen is fixed, on the average about 90 % is continuously past to the host. Nitrogen fixation is then largely independent of the multiplication of bacteria and of the formation of bacterial proteins. The bacteria appear then as swollen bacteroids at least in the nodules of pea. Towards the cessation of growth the red and brown pigments of the nodules begin to turn green. At the same time rod-shaped bacteria again occur in the nodules. At the end of nitrogen fixation the red pigment disappears and the nodules turn green. The plant then probably still receives some nitrogen nutrition from the nodules owing to the fact that soluble nitrogen compounds are past to the host and the nitrogen content of the nodules decreases.

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Received February 17, 1947.