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

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In the previous paper under the same title Virtanen *et al.* [1] reported on some results which suggest that the rate of nitrogen fixation depends on the haemoglobin content of the root nodules. In order to obtain more elucidation on this correlation we made parallel determinations of the nitrogen content of peas and horse beans, inoculated with bacterial strains of different effectiveness, and of the haematin * content of the nodules. In addition, in the experiments on soya bean, the nitrogen content of the plants and the haematin content of the nodules was determined at different stages of growth. In the following we shall describe our experiments. The results of the pea experiments were already briefly discussed at the IV. International Congress for Microbiology in Copenhagen [2].

EXPERIMENTAL

Methods

In the experiments 1 to 3 we examined the haematin content of nodules formed by bacterial strains with different activity. Pea and horse bean were grown in unglazed pots. Culture medium was quartz sand, pH about 6.5. Watering solution was free of combined N, pH 6.5. Its composition was: 3.6 g $MgSO_4$, 4.5 g KCl, 4.2 g KH_2PO_4, 4.0 g $CaSO_4$ in 60 litres of tap water. Each pot contained 3 plants. Seeds in 3 pots were inoculated with the same bacterial strain. When the experiment was interrupted the 3 parallel pots, 9 plants altogether, were taken for each determination. — In fine weather the plants were kept out in the open air, in the night-time and on rainy days in the greenhouse.

The plants were cut just above the sand and their dry matter and nitrogen contents were determined by drying the plants in an air-oven after which they were weighed

* Haematin has been used to designate the prosthetic group of haemoglobin independent of the valency of iron.

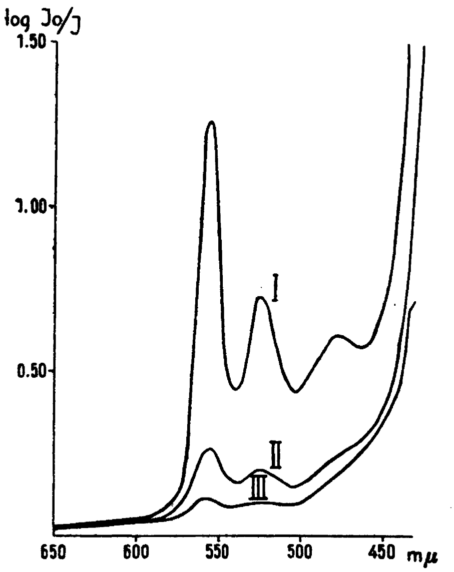

Fig. 1. Absorption spectrum of the pyridine extraction from different root nodules of pea.
I. H 7 nodules, red (experiment 28. VI—6. VIII. 1947).
II. H 7 nodules, partly green (experiment 29. VIII—17. X. 1947).
III. H 5 nodules, green (experiment 29. VIII—17. X. 1947). Plants were kept in the dark for 66 hours after the end of the experiment.

and ground in a mill to fine powder. The Kjeldahl-determinations were made from the powder. The results of the analysis are expressed per plant.

The roots were carefully separated from the sand by pouring the contents of the pot upon a large sheet of paper. As far as possible all nodules were removed. Haematin was determined as pyridine hemochromogen as follows.

A sample of 500 mg was taken from the nodule mass, washed and quickly dried between sheets of blotting-paper, pyridine and some sodium hydrosulphite were added, the mixture was crushed in a mortar, and centrifuged. Extraction with pyridine was repeated twice, after which red colour was no longer detected in the solution. The extinction of the clear extract was determined in the experiment 1 (pea) by Pulfrich photometer using the filter S 53 and the cell 0.25 cm. In the experiment 2 (pea) extinction was determined in the same way. In addition, the absorption between 650 and 440 mμ was measured by the Beckman spectrophotometer. Determination of haematin was even then made on the basis of the absorption at 530 mμ and the haematin content was expressed as chlorhaematin. As standard solution we used the pyridine solution prepared from haematin-HCl-compound isolated from blood.

The curves in Fig. 1 indicate the absorption between 440 and 650 mμ of the pyridine solutions obtained from red and green nodules. As shown by curve I the pyridine extraction of the red nodules has a very distinct maximum at about 555 mμ and another pronounced maximum at about 525 mμ. When the plants come to full bloom and the pods are partly formed the red pigment begins to turn green. The absorption maxima are then rapidly lowered as shown by curve II. The velocity of the change in the colour depends on the intensity of light and, on the whole, upon the factors affecting the growth. In unfavourable conditions the nodules may start to turn green already at the early part of flowering or even before flowering, whereas in more favourable conditions the change does not take place until pods begin to form. Great differences are also noted between the nodules of different bacterial strains in respect to the rapidness of the change.

When the plants are kept in the dark for a couple of days the nodules turn green, as previously reported by Virtanen [3] and the absorption maxima almost entirely disappear. This is clearly seen from the curve III.

The curves in Fig. 1 seem to suggest that for determination of haematin the absorption range from 550 to 560 mμ is the most suitable. But as we at first had available only the filter S 53 of Stufen photometer we had to determine the extinction by that and therefore, when we later obtained the Beckman spectrophotometer we chose the same wavelength (530 mμ) for the basis of the measurements to facilitate comparison.

The curves reveal that the method employed by us for determination of haematin gives the less reliable values the more abundant the formation of green pigment since the green pigment has an even, though weak, absorption between 500 and 560 mμ, and this raises the total absorption. Therefore in comparing the haematin content of the nodules of different strains the determination has to be made at a rather early stage when the red pigment has not yet turned green to any greater extent. Even then there may be in certain cases so much green pigment as to slightly affect results.

According to the spectrophotometric determinations the nodules formed by H VIII strain do not contain leghaemoglobin and therefore the very weak absorption of the pyridine solution from the H VIII nodules at 530 mμ has been subtracted from the extinction value of the nodules of other strains. The haematin content of H VIII nodules is thus marked as 0 in the figures. In this way the haematin values can be considered to have nearest correspondence to the haematin groups found in leghaemoglobin.

In experiment 3 (horse bean) the absorption was determined by Beckman spectrophotometer at 530 mμ.

In experiment 4 (soya bean) the haematin content of the nodules was examined at different stages of growth. Inoculated soya beans were grown in large boxes of sand out-of-doors and five times during the growth period (73 days) 20 plants were taken for analysis. After separation of the nodules from the plants dry matter and nitrogen were determined in them. From the nodule mass haematin was determined by the Beckman spectrophotometer in the manner described above in connection with the pea experiments. The remark on the effect of the green pigment on the haematin values refers also to this experiment.

Experiments

The 1st set of experiments on Torsdag pea was carried out 23. IV—17. VI. 1947. The experiment comprised 39 pots, each 3 of them were inoculated with the same strain. Thus the whole set came to include 13 parallel experiments with 13 different strains. The strain H VIII forms completely ineffective nodules, H 130 is slightly effective, H 9, too, is weak. The other strains are effective or very effective. The experiments were interrupted at the same time at the early part of flowering. The nodules were, on the whole, red, but some green pigment could be seen in the nodules of certain strains.

The nitrogen content of the plants (3 in each pot) and the haematin content of the nodules are given in Table 1 and Figs. 2 and 3.

The 2nd set of experiments on pea was conducted 28. VI—5. VIII. 1947. The experiment was otherwise similar to the previous one, except that it was interrupted before flowering. The nodules were still red, hardly any green nodules were perceptible. Table 1 and Fig. 4 show the results of this experiment.

The 3rd set of experiments was made on horse bean. For inoculation 7 different strains were used, 2 of them were very weak. Period of growth was 11. VII—27. VIII. 1947.

Table 1. 1st experiment on different bacterial strains. Test plant: Torsdag pea in full bloom. Period of growth: 23. IV—17. VI. 1947, 55 days.

Strain	Number of plants	Plants (green parts)		Nodules							
							Haematin				
		Dry matter g/plant	N mg/plant	Fresh weight mg/plant	Dry matter %	Dry weight mg/plant	γ/g fresh weight**	γ/g dry weight**	γ/plant	N mg/g dry weight	Fe γ/g dry weight
H 1	9	1.006	32.4	693			136 (116)		78		
H 2	9	1.031	42.3	380	10.6	40.3	172 (152)	(1210)	39	71.1	592
H 3	9	1.162	49.3	400	9.5	38.0	176 (156)	(1540)	59	78.8	662
H 4	9	0.651	33.1	503	10.0	50.3	104 (84)	(730)	37	86.5	542
H 5	9	1.201	49.0	351	9.7	34.0	160 (140)	(1330)	45	89.5	628
H 6	9	0.054	42.4	345	9.2	31.8	144 (124)	(1260)	40	98.6	592
H 7	9	0.929	36.7	294	10.5	30.8	127 (107)	(900)	28	74.3	662
H VIII	9	0.267	5.5*	430	6.3	27.1	20 (0)	(0)	0		
H 9	9	0.492	20.6	456			120 (100)		41		
H 10	9	0.802	34.6	606	10.4	63.0	116 (96)	(800)	50	102.6	482
H 43	9	1.023	38.1	332	7.7	25.6	147 (127)	(1600)	41	77.1	654
H 47	9	0.913	33.1	199	9.7	19.4	129 (109)	(1020)	20	94.6	798
H 130	6	0.349	8.9	100	7.5	7.5	44 (24)	(280)	2	47.1	370
2nd experiment. Torsdag pea in bud. Period of growth: 28. VI—5. VIII. 1947, 39 days.											
H 1	9	0.588	21.4	506	8.3	41.4	108 (83)	(1040)	43		510
H 2	9	0.579	24.6	316	8.5	26.8	136 (111)	(1340)	36		568
H 3	9	0.600	25.6	309	9.8	30.3	177 (152)	(1550)	47		565
H 4	9	0.457	19.9	453	10.6	48.0	142 (117)	(1080)	52		411
H 5	9	0.557	24.7	346	10.6	36.7	156 (131)	(1200)	44		512
H 6	9	0.647	29.2	338			151 (126)		39		
H 7	9	0.676	30.6	359	10.1	36.2	172 (147)	(1450)	53		551
H VIII	9	0.214	4.8*	318	9.6	30.6	25 (0)	(0)	0		384
H 9	9	0.261	7.3	557	8.9	49.6	73 (48)	(550)	27		396
H 43	9	0.891	37.8	445	9.8	43.6	170 (145)	(1480)	65		602
H 47	9	0.740	29.8	370	9.9	36.6	177 (152)	(1550)	57		541
H 130	6	0.246	6.1	251	8.3	20.8	38 (13)	(200)	4		373

* No N-fixation. N originates from seed. N-content of a seed of Torsdag pea is about 7—8 mg.

** The figures in brackets are after subtraction of »haematin» in H VIII nodules.

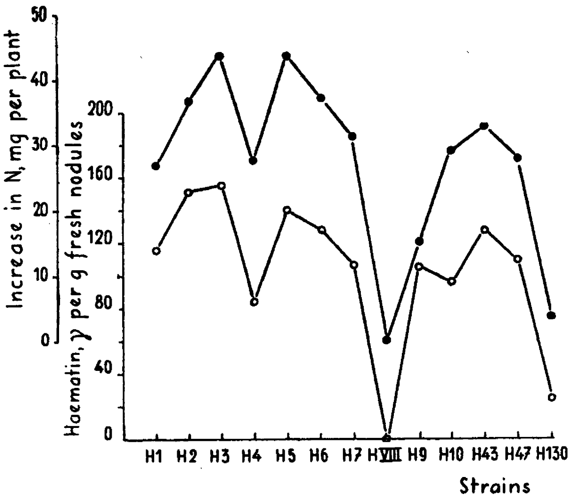

Fig. 2. Haematin content of the nodules of Torsdag pea inoculated with different bacterial strains. Period of growth: 23. IV—17. VI. 1947. Plants in full bloom at the end of the period, nodules chiefly red, a few green nodules in some experiments. The low absorption of inactive H VIII nodules is subtracted from each experiment.

●———● *Increase in N, mg/plant.*
○———○ *Haematin, γ/g fresh nodules.*

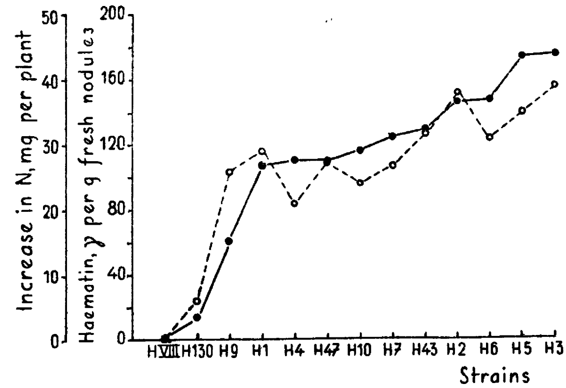

Fig. 3. Similar experiment as in Fig. 2 but interrupted before flowering.

●———● *Increase in N, mg/plant.*
○— — —○ *Haematin, γ/g fresh nodules.*

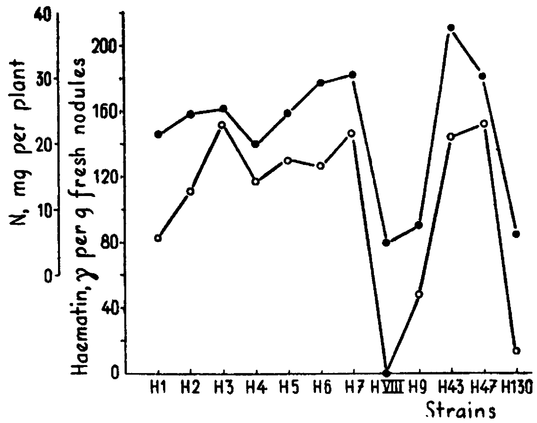

Fig. 4. Haematin content of the nodules of Torsdag pea inoculated with different bacterial strains. Period of growth: 28. VI—5. VIII. 1947. Harvested before flowering, nodules red. The low absorption of H VIII nodules is subtracted from each experiment.

●———● *N, mg/plant.*
○———○ *Haematin, γ/g fresh nodules.*

Table 2. 3rd experiment on different bacterial strains. Test plant: horse bean. Period of growth: 11. VII—27. VIII. 1947, 47 days.

Strain	Number of plants	Plants (green parts)		Nodules					
				Fresh weight mg/ plant	Dry matter %	Dry weight mg/ plant	Haematin		
		Dry matter g/plant	N mg/ plant				γ/g fresh weight	γ/g dry weight	γ/plant
F 1	6	2.472	90.0	796	12.3	98.1	182	1480	145
F 2	6	2.705	99.0	933	15.5	140.0	195	1260	176
F 3	6	2.405	85.4	784	13.0	102.0	152	1170	119
F 4	6	2.420	87.6	927	14.3	132.6	195	1350	180
F 5	6	3.040	112.8	776	12.6	97.8	216	1710	167
F 6	6	0.526	14.3	100	13.8	13.8	68	495	68
H 5*	6	0.718	20.9	192	11.8	22.6	32	272	62

* Strain of pea bacterium.

We are indebted to Mr. P. I. Forsius for determinations of haematin in this experiment.

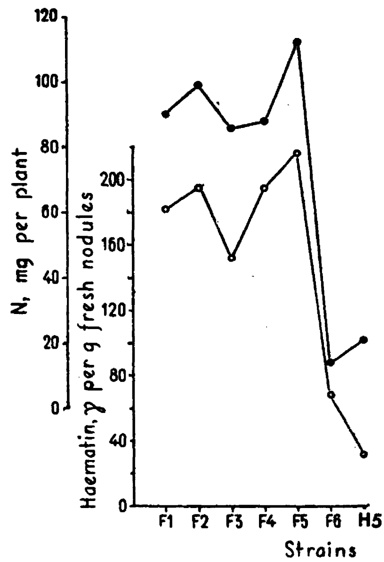

Fig. 5. Haematin content of the nodules of horse bean inoculated with different bacterial strains. Period of growth: 11. VII—27. VIII. 1947. Plants in the beginning of flowering, nodules red.

●———● *N, mg/plant.*
○———○ *Haematin, γ/g fresh nodules.*

Table 3. 4th experiment. Test plant: soya bean. Period of growth: 30. VI—12. IX. 1947. Haematin content of nodules and nitrogen content of plants determined 5 times in the course of growth.

Date	Age days	Stage of growth	Plants: Number	Average length cm	Fresh weight g/plant	Dry weight g/plant	N mg/plant
17. VII	18	Before flowering	5	15	6.94	1.30	42.2
26. VII	27	Flowering begins	5	25	20.8	3.92	134.2
11. VIII	43	Flowers and pods	5	38	44.2	8.81	261.0
22. VIII	54	Pods, some flowers	5	45	49.3	9.70	322.0
12. IX	73	Pods, no growth	5	45	55.4	13.38	412.4

Date	Nodules: Number	Colour	Fresh weight mg/plant	Dry matter %	Dry weight mg/plant	Haematin γ/g fresh weight	Haematin γ/g dry weight	N mg/plant	Fe γ/g dry weight
17. VII	20	red	309	21.1	65	155	730	3.15	340
26. VII	30	»	755	24.1	182	242	1000	9.40	300
11. VIII	46	»	1487	22.9	340	358	1560	17.0	410
22. VIII	75	»	1574	25.2	397	382	1560	19.2	360
12. IX	71	green	2084	19.0	397	176*		18.4	355

* Haematin value of no significance, since the nodules were green.

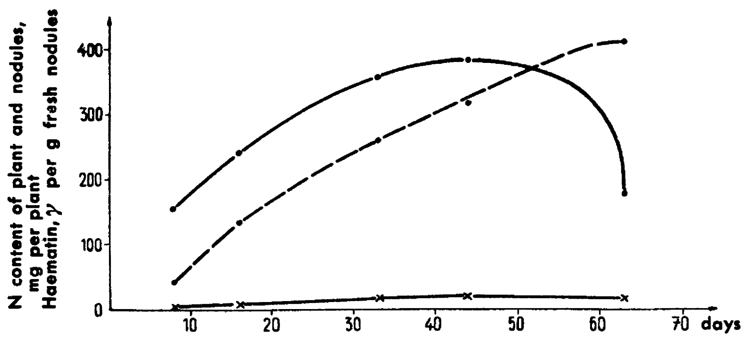

Fig. 6. Nitrogen content of plant and haematin content of nodules at different stages of growth of soya bean.

●——● *Haematin, γ/g fresh nodules.*
●— — —● *N, mg/plant without nodules.*
×——× *N in nodules, mg/plant.*

When the experiment was interrupted the plants had just started to bloom and the nodules had not yet even partly turned green. Table 2 and Fig. 5 illustrate the results.

The 4th set of experiments on soya bean was carried out 30. VI—12. IX. 1947. Samples were taken 5 times at different stages of growth. At the last time after 73 days of growth the soya beans had already ceased to grow and were producing seeds. The nodules had turned green and the haematin determinations had no longer significance in quantitative sense. On the former samples taken at the age of 18, 27, 43, and 54 days nodules were red. The results are given in Table 3 and Fig. 6.

DISCUSSION

All the experiments give the general impression that the ability of the nodules to fix nitrogen is correlated with the haemoglobin content of the nodules. Although haematin only was directly determined and not leghaemoglobin the values, nevertheless, chiefly refer to leghaemoglobin. In young peas the haematin of nodules originates for the most part from leghaemoglobin.

In examining the relation of the haematin content of the root nodules and the nitrogen fixation a distinctly positive correlation is observed between the nodules formed by weak and effective strains. If the differences in the effectiveness of strains are but slight the variations in the haematin content of the nodules do not always parallel the differences in the nitrogen fixed in the plants, as illustrated by Fig. 3. It is evident that a complete correlation between the nitrogen fixation and the haematin content of the nodules can hardly be expected since many various factors affect the growth and the nitrogen fixation. Such a parallelism as was found in our experiments is almost surprising in these conditions.

While a parallelism exists between the nitrogen fixation and the haematin concentration of the nodules (haematin γ per g nodules), a corresponding relation is not found, at least by far not to the same extent, between the nitrogen contained in the plant and the quantity of haematin in the total number of nodules. If the weight of the root nodules is great but the concentration of haemoglobin small (haematin γ per g nodules), the nitrogen fixation is weaker than in a reverse case (compare for instance, the nitrogen amount per plant and the weight and haematin of the nodules in experiment 1 on strains H 1 and H 10, on the one hand, and on strains H 3 and H 5, on the other). This can be possibly interpreted so that with the rise of the concentration of haemoglobin in the nodules its effect on the occurrences controling the nitrogen fixation increases more than is directly implied by the rise. This may be accounted for by the fact that because of the small concentration of haemoglobin and its great dilution in the nodule tissue the linkage of oxygen to the iron of leghaemoglobin is incomplete. The higher the haemoglobin concentration in the nodule the greater the chance of every haemoglobin molecule to participate in the transfer of oxygen.

The variations in the total iron content of the nodules do by no means regularly correspond to the variations in the nitrogen fixation although the iron content in the ineffective and slightly effective nodules is, as a rule, lower than in very effective nodules. Since the major part of the nodule iron does not belong to leghaemoglobin (Virtanen *et al.*[1]) a distinct positive correlation can hardly be expected between the nitrogen fixation and the total iron content, even when such a relation exists between the nitrogen fixation and the leghaemoglobin content.

In an experiment where the haematin content of the nodules of soya bean were examined at different stages of growth (Fig. 6) a pronounced parallelism was observed between the nitrogen fixation and the haematin content of the nodules almost until the end of the vegetative growth. After that the nodules turned green and the haematin disappeared. Simultaneously nitrogen fixation ceased.

SUMMARY

Root nodules formed by bacterial strains of different effectiveness were crushed with pyridine. Spectrophotometric examination of the extracts showed that the strong absorption maxima of the red nodules at 555 and 525 mμ disappear after nodules have turned green and leghaemoglobin is no

longer found. The strong absorption of the extracts from the red nodules is thus at least chiefly attributed to the prosthetic group of leghaemoglobin.

Comparative experiments with 13 different strains of pea bacterium and 6 different strains of horse bean bacterium indicated that a positive correlation exists between the haematin concentration of nodules (haematin γ per g nodules) and nitrogen fixation. The absolute quantity of pigment in the total number of root nodules (haematin γ per plant) is not by far so decisive as is the concentration of pigment in the nodules.

In experiments on soya bean where the haematin content of the nodules was determined at different stages of growth, a clear parallelism was noted between the nitrogen fixed in the plant and the haematin content of the nodules.

Correlation is not distinct between the total iron of the nodules and the nitrogen fixation although the nodules formed by very effective strains, as a rule, contain more iron than do the nodules of ineffective or poor strains.

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