CHAPTER VI

HAEMOGLOBIN CONTENT OF LEGUME ROOT NODULES.

Considerations of amounts of nitrogen fixing tissue concern directly nodule size and abundance in legumes. One important fact with reference to nodulation relates to the inverse relationship between nodule size and abundance (Nutman, 1958). In his studies on nitrogen fixation in legume symbiosis, Nutman (1956 b) has clearly shown that there is a limitation to the number of nodules a host plant could form. This limitation has its origin in host metabolism. Besides, it is now known that the specific nodule volume is a function of the host and is independent of bacterial variation or virulence (Nutman, 1958).

Thornton (1939) and Chen and Thornton (1940) have concluded from a study of effective and ineffective nodule structure in various legumes that the amount of nitrogen fixed was a function of the central nodule tissue and the time for which this tissue persisted. This tissue in effective nodules contains the pigment haemoglobin. That there was a direct positive correlation between haemoglobin

content and nitrogen fixation as shown by Virtanen (1955). Recently, Bergersen (1961) demonstrated that the haemoglobin content of root nodules was well correlated with the effective central tissue volume, as haematin concentration and nitrogen fixation are correlated as was suggested by Virtanen (1955).

It, therefore, follows that a study of nodule formation and haemoglobin content might reveal the effectiveness or ineffectiveness of symbiotic associations among legumes.

This needs to be studied since wide variations in the effectiveness of the tropical cow pea type of Rhizobium have been reported (Allen and Allen, 1939, 1940; Bonnier, 1957 a; Bowen, 1956 a, 1956 b; Bumpus, 1957; Hussein, 1955; Masefield, 1952, 1957; Norris, 1956; Savic, 1956).

PLAN OF WORK:

The legumes studied included:

Arachis hypogaea (varieties TMV 2, 3, 5 and HG 1)
Vigna catiang;
Dolichos lab lab;
Phaseolus mungo;
Cyamopsis tetragonoloba;
Dolichos biflorus

and

Crotalaria juncea.

Field soil under grass cover from an uncultivated virgin area was procured, sieved to remove sods and larger particles, and the soil was dispensed into large-sized glazed pots. Seeds were sown.

A heavy rhizobial inoculum (R_4) was supplied to the germinated seedlings. The Rhizobium strain R_4 was an effective isolate from Arachis hypogaea. This strain was used since it could induce heavy nodulation of the species studied under field conditions of cultivation.

Each pot contained 5 to 10 plants depending on whether the legume in question was a small seeded or a large seeded variety, and 5 replicate pots were maintained for each species or variety of host plant. The plants received nutrient solution (vide Materials and Methods) once in every fortnight, while watering was done regularly. The pots were set on a clean platform outside the green house in full broad day light. The plants grown were vigorously healthy and had dark green foliage.

HARVESTING OF NODULES:

Nodules were excised from various legumes using a total of 20 plants in each species or variety of host

PLATE VII

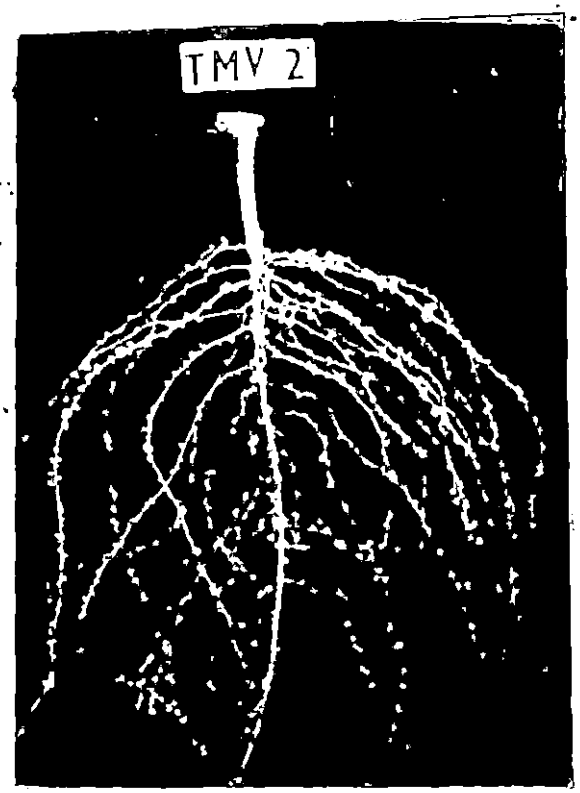
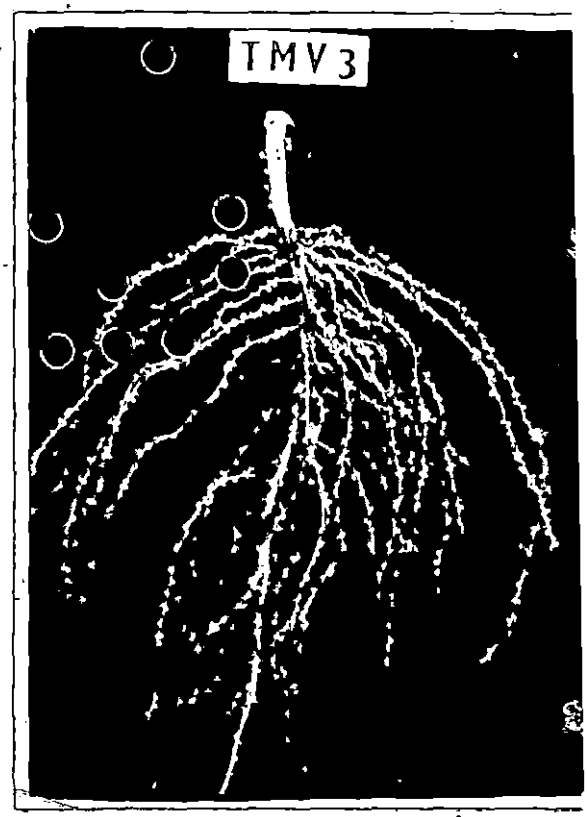


Fig.a



Fig,b

Fig.c

d

PLATE VII
(Continued)

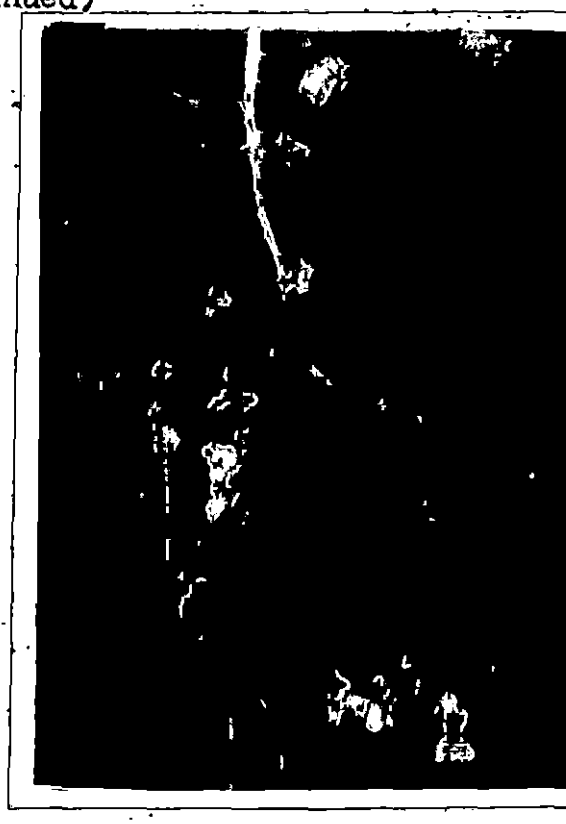


Fig.e

Fig.f

Fig.g

PLATE VII

The figures present root and nodule development in a few tropical legumes when inoculated with an effective Rhizobium strain (R4)

a. Arachis hypogaea TMV2 (Bunch)
b. Arachis hypogaea TMV3 (Spreading)
c. Dolichos biflorus
d. Vigna catiang

PLATE VII
(Continued)

The figures present root and nodule development in a few tropical legumes when inoculated with an effective Rhizobium strain (R4)

e. Crotalaria juncea
f. Cyamopsis tetragonoloba
g. Phaseolus mungo

plant prior to the time of flowering. After determining the number, weight and volume of nodules carefully, the haemoglobin content was estimated as described earlier. A weight of 2-5 grams of nodules was taken for haemoglobin determination from each species or variety depending on the comparative abundance of nodule material.

The variation in nodule number and size are illustrated in plate VII (a, b, c, d, e and f). The effectiveness in symbiosis in terms of nodule weight and haemoglobin concentration are presented in Tables 7 and 8.

The values of mean weight of a nodule and haemoglobin (µg/nodule) were calculated from an analysis of the total nodule material from 20 plants.

TABLE 7.

EFFECTIVENESS IN SYMBIOSIS IN TERMS OF NODULE WEIGHT AND CONCENTRATION OF ROOT NODULE HAEMOGLOBIN IN LEGUMES. *

Host X Bacterial strain (R_4).	Mean Weight/Nodule (mg).	µg-Haemoglobin/Nodule (mg).
Arachis hypogaea TMV 2	6.0	1.707
Arachis hypogaea TMV 3	7.3	1.908
Arachis hypogaea TMV 5	4.5	1.404
Arachis hypogaea HG I	6.0	1.080
Phaseolus mungo	6.0	1.909
Vigna catiang	24.0	8.400
Cyamopsis tetragonaloba	30.5	9.404
Dolichos lab lab DL 231	20.8	22.300
Dolichos biflorus	70.10	23.005
Crotalaria juncea	225.00	66.205

* All the species were harvested just prior to the time of flowering. Rhizobium strain (R_4) isolated from Arachis hypogaea forms effective symbioses, cross-inoculating freely with all the species studied.

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There was a well defined correlation between the mean weight of a single nodule and the haemoglobin contained in it amongst the legumes examined. In this regard the species of Arachis and Phaseolus constitute a category where the mean weight per nodule and haemoglobin content per nodule are in low quanta, when compared to the category of species such as Vigna, Cyamopsis, Dolichos and Crotalaria. The increase in the weight per nodule and haemoglobin content in a single nodule in various species examined (Table 7) further emphasize the differences in nitrogen fixing potential of their nodules.

The effectiveness in symbiosis in terms of legume root nodule haemoglobin concentration and haemoglobin per unit nodule volume in legumes are illustrated in Table 8.

TABLE 8.

EFFECTIVENESS IN SYMBIOSIS IN TERMS OF LEGUME ROOT NODULE HAEMOGLOBIN CONCENTRATION PER UNIT VOLUME.

Host X Bacterial strain (R_4)	µg haemoglobin/ gram fresh weight of nodules.	Haemoglobin/unit nodule volume*
Arachis hypogaea TMV 2	295	0.230
Arachis hypogaea TMV 3	340	0.256
Arachis hypogaea TMV 5	320	0.281
Arachis hypogaea HG I	315	0.216
Phaseolus mungo	330	0.300
Vigna catiang	350	0.353
Cyamopsis tetragonoloba	275	0.275
Crotalaria juncea	340	0.265
Dolichos biflorus	325	0.316
Dolichos lablab	780	0.772

* Haemoglobin/unit nodule volume = $\frac{\text{µg haemoglobin/nodule}}{\text{nodule volume in µl/nodule}}$

The haemoglobin content (µg) per gram fresh weight of nodules as well as haemoglobin per unit nodule volume were a constant although the weight per nodule (Table 7) amongst the various legumes differed widely.

It is clear that the haemoglobin concentration per unit nodule volume amongst these legumes was a constant although nodule formation in terms of mean weight per nodule showed considerable variations.

Besides, in terms of root nodule haemoglobin concentration, the symbioses could be considered to be very highly effective, since haemoglobin content of nodules was far in excess of 200 µg/g fresh weight of tissue, which level was chosen as the threshold value for effectiveness in previous work.

The observed constancy in root nodule haemoglobin per unit nodule volume further stresses on the fact that there is a basic uniformity in nitrogen fixing systems underlying the remarkable diversity of nodule size and abundance in legumes.

THE ABSORPTION SPECTRA OF SOLUTIONS OF PYRIDINE HAEMOCHROMOGEN FROM THE ROOT NODULES OF VARIOUS LEGUMES.

A study was made of the absorption spectra of the pigments as obtained from fresh extracts of nodules after conversion to solutions of pyridine haemochromogen. Since the haemoglobin in the root nodules of leguminous plants of the tropics have yet to be studied fully, it was thought that this study should be made in order to understand if the pigments of various legume nodules differ in any way from one another.

The freshly collected nodules of various legumes were employed for the purpose. The nodules were harvested prior to the time of flowering of the host plants. The procedure of extraction of haemoglobin from root nodules and conversion to pyridine haemochromogen are previously described.

The absorption maxima and minima are listed in Table 9. For purposes of comparison, similar data of Kubo(1939) on peas and of Steinberg and Virtanen (1952) on soya beans are listed alongside.

TABLE 9.

ABSORPTION MAXIMA & MINIMA OF PYRIDINE HAEMOCHROMOGENS FROM HAEMOGLOBIN OF THE ROOT NODULES OF VARIOUS LEGUMES mμ.

Host plant	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.
rachis hypogaea	560	542	525	510						
alanus cajan	560	545	530	510						
olichos lablab	560	500	530	510						
igna catiang	560	543	530	510						
yamopsis tetragonoloba	560	545	530	510	480	470				
haseolus mungo	560	540	527	550	580	575	570	565	560	455
eas *	557	530								
oya beans *	555	539	525	501	481	460				

The earlier determinations on peas by Kubo (1939) as well as on soya beans y Virtanen (1952) are included for comparison.

The study revealed that the haemoglobin of the root nodules of the species showed heterogenity in terms of absorption spectra.

CHAPTER VII.

THE EFFECT OF VIRUS INFECTION ON NODULE FORMATION.

As pointed out in an earlier section, the influence of day length in regulating haemoglobin formation, nitrogen fixation and the parallel development of haemoglobin in nodules and chlorophyll in leaves suggest that nodulation proceeds parallel with the accumulation of chlorophyll in the leaves. Such a physiological connection in the development of haemoglobin and chlorophyll was also suggested by Bonnier et al., (1957) working on soya bean.

Since haemoglobin and chlorophyll are known to have common biosynthetic pathways of development (Granick, 1951; Rimington, 1957) a marked reduction in chlorophyll such as occur in mosaic virus diseases might affect nodule development through derangement in host function. For instance, a low carbohydrate content and a low C/N ratio are known to be the effects of virus infection in mosaic diseases (Bawden, 1950). This can impair nodule formation through inadequate carbohydrate supply to the roots and this, in spite of the fact that mosaic diseases do not affect the phloem as do the leaf rolls (Bawden, 1950).

Another aspect of nodule physiology in relation to virus infection hinges on the formation of haemoglobin in root nodules. Since haemoglobin bears a causal relationship to nitrogen fixation (Virtanen, 1955; Hamilton, Shrug and Wilson, 1957) one determinant of an effective symbiosis constitutes the synthesis of haemoglobin in root nodules. For this synthesis to take place it was suggested that pyrroles or their percursors may form in the shoot and be transported to the roots (Falk, Appleby and Porra, 1959). However, in view of the fact that viruses alter metabolism through their effects on translocation, ion balance, chlorophyll synthesis, respiration, carbohydrate formation, protein synthesis and growth in general, it appears tenable to state that virus infection might affect this process. It is all the more evident, since virus diseases are regarded essentially as a change in the protein metabolism of the host cells (Bawden and Pirie, 1956).

It was, therefore, deemed desirable to study the influence of virus infection by Dolichos Enation mosaic virus on nodulation in Dolichos lablab.

PLAN OF WORK:

Healthy seeds of the variety DL 231 of Dolichos lablab were sown in sterilized sand in glazed containers. The plants were inoculated with an effective strain of Rhizobium (R_4) at the time of germination of the seedling, as was the practice in previous work. Inoculation with the virus (DEMV) was done by rubbing the primary leaves, 10 days after germination with an extract prepared from young leaves of Dolichos lablab showing severe symptoms of mosaic from systemically infected plants (vide Materials and Methods).

The green house sterile sand culture procedures consisted of the maintenance of the following four series of pots.

A. Dolichos lablab plants (DL 231) alone.

B. DL 231 + inoculation with rhizobia (R_4)

C. DL 231 + R_4 + inoculation with virus (DEMV)

D. DL 231 + DEMV.

Each pot contained three plants and the four series replicated five times were maintained in the green house. The temperature of the green house ranged from 22°C to 27°C.

Field cultivation of plants for the study of the effects of virus infection on nodulation were done alongside green house sand culture work. In the field two plots, 20' x 10', were used, one each for growing healthy and virus infected plants. The two plots were spaced apart from one another and received separate routes of water supply from a single source. Although the plants meant for the infected series were inoculated with virus, the rhizobial inoculum was not given in order to study the impact of virus infection under natural conditions of field growth where nodulation of plants depended on the dual factors of (a) the number of rhizobia in the soil and (b)the changes of infection as occur under natural conditions.

Unsterile pot cultivation was again resorted to in order to study the influence of virus infection under conditions where soil factors could perforce be made more favourable by provision of an abundance of rhizobial inoculum and creating optimum conditions for the spread of the rhizobia in the rhizosphere.

Whilst in the field 50 plants were grown widely spaced apart in soil well sifted, in pots under unsterile pot cultivation the healthy and infected series included 30 pots equally divided among the healthy and infected treatments, with five plants in each pot.

While the green house grown plants received sterile tap water regularly and nutrient solution free from combined nitrogen once in 15 days, the field grown and unsterile pot bound plants outside the green house received water supply regularly at the full complement of field saturation capacity without however undue waterlogging. Thus, care was exercised in growing the plants under green house, field and pot culture conditions, in order to minimise factors that might introduce variation in uniformity of experimental conditions.

The methods of virus and rhizobial inoculation are as described earlier (vide Materials and Methods). Field experiments were repeated twice on two different occasions. The plants were harvested at the end of 80 days following sowing from the field, whilst a period of 100 days were allowed to elapse prior to harvesting the plants from sand cultures in the green house. These times were chosen since they corresponded to a time just prior to the flowering of the host plant.

At the time of harvest the root nodules from a batch of 20 plants each from the field, green house, sand culture and unsterile pot culture series were carefully removed from healthy and infected plants. Nodules were carefully removed and the soil around plant roots were sifted thoroughly to collect any nodule that might have got detached.

The progress of virus infection under green house and field conditions of plant growth was determined serologically (vide Materials and Methods) and is represented in Table 10.

TABLE 10.

The progress of virus infection under green house and field conditions of plant growth in Dolichos lablab (DL 231). Temperature: 22°-27°C (November/January).

:::::

Days after virus inoculation.	Relative Serological Virus Titre (~~Dilution end points~~).			
	Green House		Field.	
	RH + V	V	H	V
30	1/25	1/5	0	1/5
35	1/25	1/25	0	1/25
50	1/25	1/25	0	1/25
60	1/25	1/5	0	1/25
70	1/25	-	0	1/25

RH + V: Rhizobium inoculated, virus (DEMV) infected.

V: Virus infected.

H: Healthy

V: Virus infected.

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PLATE VIII

Fig.a

Fig.b

PLATE IX

Fig.a

Fig.b

PLATE VIII

The figures represents root and nodule development in relation to:

a) Healthy plants

b) Virus infected plants

PLATE IX

The figures illustrate the effects of virus infection (DEMV) on plant growth in Dolichos lablab infected with Dolichos enation mosaic virus.

Fig. a. shows the treatments:

A. Dolichos lablab. DL 231 alone.
B. DL 231 + inoculation with rhizobia (R_4).
C. DL 231 + R_4 + inoculation with virus (DEMV).
D. DL 231 + DEMV.

b. An infected leaf of Dolichos lablab showing cup-like laminar outgrowths and filiform enations.

The Dolichos Enation Mosaic Virus was serologically demonstrable in the infected plants of Dolichos lablab both under conditions of inoculation with Rhizobium as well as without it in the plants grown in the green house in sand cultures (RH + V and V). In those which were infected with the virus, but which did not receive Rhizobium inoculation, the serological virus titre showed a decrease after 60 days of growth in the host plant. In the field, the serological virus titre remained at a constant tissue level for a further period of time.

The methods adopted for nodule excision, determination of their number, weight and volume as well as haemoglobin in root nodules were the same as those described previously.

The observed effects of virus infection on nodulation in Dolichos lablab are presented in plates VIII & IX and Tables 11 and 12 and relate to

1. The increase in nodule number, weight and volume in virus infected plants in contrast to healthy plants.
2. The increase in root nodule haemoglobin consequent on systemic virus infection.

TABLE 11.

t of virus infection by Dolichos Enation Mosaic virus on Nodulation aemoglobin content in Dolichos lablab, grown under field conditions ll as under sterilised sand culture conditions (free from supply of ned nitrogen) and inoculated with an effective strain of Rhizobium(R_4).

	Green house sand culture.			Field cultivation.		
	Healthy.	Virus infected.	% increase on infection.	Healthy.	Virus infected.	% increase on infection.
ge nodule me cc/nodule	1.1	1.0	--	4.1	7.0	70.7
ge nodule ht mg/nodule	10.50	10.20	--	30.91	70.41	131.6
globin odule	2.10	5.30	152.3	19.10	23.61	23.0
globin/unit le volume	0.109	0.409	266.1	0.009	0.020	122.2

---ooOoo---

The average nodule volume and weight do not exhibit any significant difference between the healthy and virus-infected plants grown in the green house in sand cultures with a specific Rhizobium inoculant. However, virus infection under field cultivation of the host plant resulted in an increase of 70.7 percent in mean nodule volume, whilst the mean nodule weight increased by 131.6 per cent. There was an increase of 152.3 percent in haemoglobin content per nodule in infected plant nodules over healthy ones. The increase in haemoglobin per unit nodule volume as a result of virus infection was 266.1 per cent in the green house grown plants. In the field this percentage was 122.2.

The increase in root nodule haemoglobin on a tissue weight basis (µg/g fresh weight of nodules) following virus infection of the host plant ranged from 16.7 to 36.2 as is shown in Table 12.

TABLE 12.

EFFECT OF VIRUS INFECTION BY DOLICHOS ENATION MOSAIC VIRUS ON ROOT NODULE HAEMOGLOBIN CONTENT IN DOLICHOS LABLAB.

Host plant.	Root nodule haemoglobin content (µg/g fresh weight of nodules).			
	Sterile sand cultures +	Pot culture unsterile soil.+	Field Soil I trial*	Field Soil II trial.*
Healthy	260	275	400	560
Virus-infected	330	340	545	655
% increase in heaemoglobin consequent on virus infection	26.9	23.6	36.2	16.7

+A pure culture of Rhizobium (R_4) was used as an inoculant.

*Inoculation with rhizobia was not done. Nodulation was a result of infection by the soil population of rhizobia.

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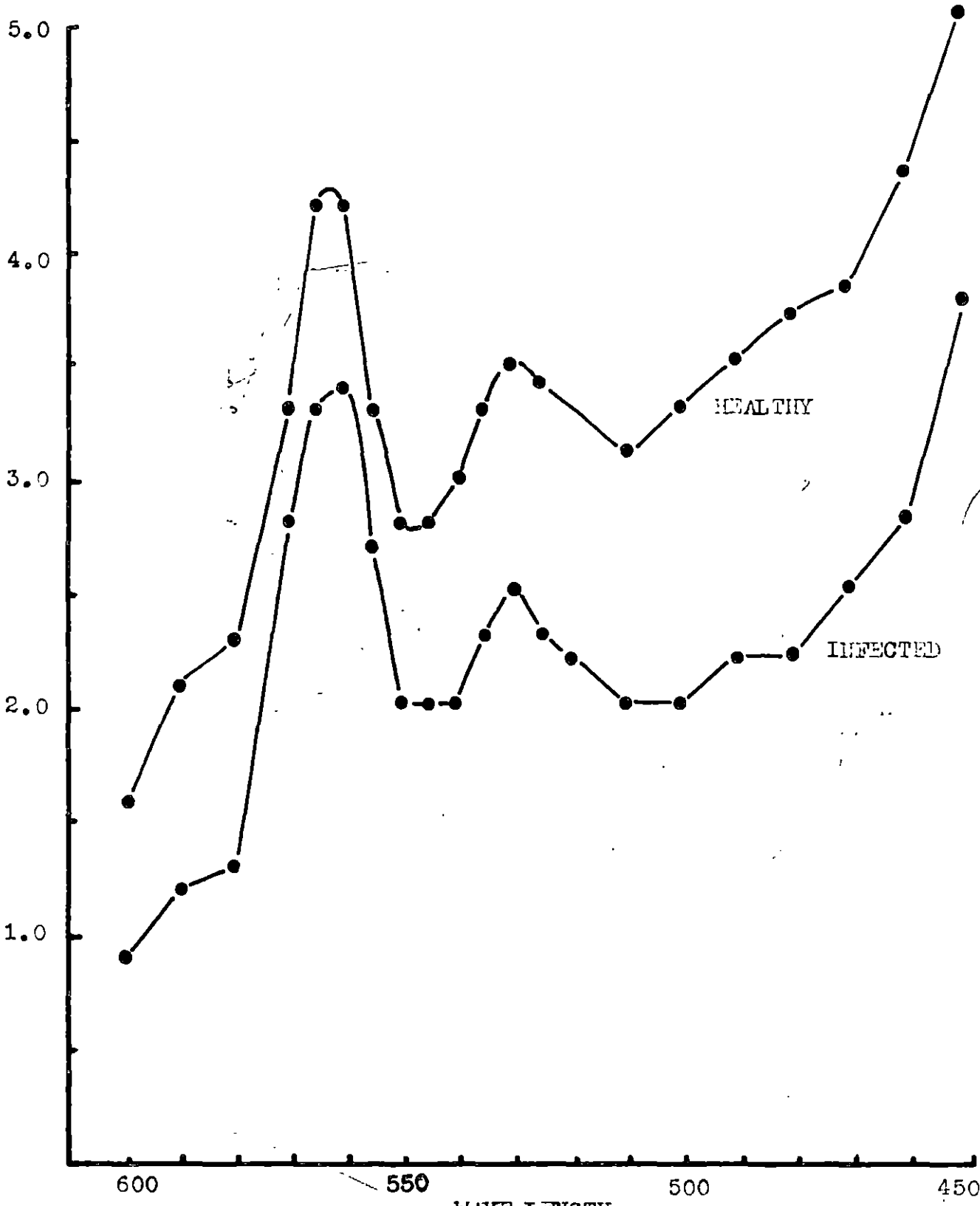

Fig. 17. SPECTRA OF PYRIDINE HAEMOCHROMOGENS OF PURIFIED HAEMOGLOBIN FROM THE ROOT NODULES OF HEALTHY AND VIRUS INFECTED PLANTS

$$\text{SP.EX}^{\circ}.K = \frac{\text{Optical Density}}{\text{mM Concentration of Haematin} \times \text{Optical Depth}}$$

Thus, an increase in root nodule haemoglobin content was associated with virus infection in Dolichos lablab.

The pigments contained in the healthy and virus-infected plant nodules were extracted as described previously and their absorption spectra were studied after conversion of the haemoglobin into solutions of pyridine haemochromogen. The absorption spectrum of the pigment (fig. 16) from the healthy plant nodules differed from that of the infected plant nodules in the presence of a band at 610 mμ in the former and its absence in the latter. Subsequently, the haemoglobin from the healthy and virus-infected plant nodules were obtained in a purified state through ammonium sulphate precipitation (vide Materials and Methods). The purified pigments from the healthy and infected plant nodules were converted to solutions of pyridine haemochromogen and their absorption spectra were studied. The specific extinction against wave lengths of the two pigments are presented in fig. 17. The spectra show that the pigments obtained on purification from healthy and virus infected plant nodules were essentially identical.