INTRODUCTION

The study of atmospheric nitrogen fixation which began with the classical researches of Bousingault[6] (1837), Lawes, Gilbert and Pugh (1857), Hellriegel, Willfarth and Beijerinck (1888), has taken several avenues of expression in the present century and encyclopaedic summaries have been written by Fred, Baldwin and McCoy (1932), Wilson (1940), Virtanen (1947), Allen and Allen (1950), Vincent (1954), Nutman (1956), Norris (1956), Hallsworth (1958) and Raggio and Raggio (1962).

The principal features of nodule formation, growth and symbiotic nitrogen fixation vary a great deal within different species of legumes (Nutman, 1958; Van Schreven, 1958; Hallsworth, 1958). The formation of nodules on legume roots and the fixation of nitrogen in them involve a complex array of variables of both the host and the bacteria. The environment acting through the physiology of the host plant further influences the extent and progress of symbiotic relationship between a Rhizobium and a leguminous plant (Nutman, 1956). A number of leguminous species are even known to lack nodulating ability (Allen and Allen, 1947; Allen and Baldwin, 1954). When nodules are formed, these may again be effective or ineffective in nitrogen fixation,

depending on whether or not the rhizobia themselves are effective or ineffective (Parker and Allen, 1952; Vincent, 1954; Erdman and Means, 1953; Jordan and Garard, 1951; Purchase et al., 1951). The ecological adaptation of Rhizobium strains to particular legume hosts presents another facet of the problem of the intricate relationship between the leguminous plants and the rhizobia (Nutman, 1952). Studies on species and varietal relationships and hybridization experiments have suggested that the symbiotic phenotype may be a heritable expression of plant and bacterial components (Wilson, 1937; Bjalfve, 1935; Nutman, 1946, 1947). The spontaneous or bacteriophage-induced loss of effectiveness of the bacteria might, again, have an impress on nodule nitrogen fixation (Jordan, 1952; Kleczkowska, 1950), although the biochemical characteristics of rhizobia by themselves cannot be correlated with their capacity for symbiotic nitrogen fixation (Allen and Allen, 1958).

Thus, against a background of evidence (Allen and Allen, 1939; McKnight, 1949; Andrew and Bryan, 1955; Bryan and Andrew, 1955; Andrew, 1958; Bowen, 1956 a, 1956 b; Masefield, 1952, 1957, 1958; Norris, 1956, 1958;

Manil, 1958; Corby, 1959; Bonnier, 1957 b, 1960; Brockwell and Holy, 1961; Joffee et al., 1961; Lange, 1961; Harris, 1961), it was clear that Rhizobia-Legume relationships in the tropics need to be studied in relation to:

(a) the presence of rhizobia in the soils;
(b) the varied susceptibility of legume hosts to infection with rhizobia;
(c) the variation in the effectiveness of strains in nitrogen fixation;
(d) the number, volume and weight of nodules in various species which reflect the amounts of nitrogen fixing tissue;
(e) the cross inoculation promiscuity;
(f) the effects of deficiency in bases and of essential soil nutrients;
(g) the acidity and the low humus content of soils; and
(h) the effects of low land relief, short day length, varying soil moisture content, high rainfall and high soil temperature on nodule nitrogen fixation.

It is, therefore, germane to state that nodule formation in the leguminous crops of the tropics and the associated aspects of effectiveness or ineffectiveness of Rhizobium strains, host plant and varietal variations in symbiotic effectivensss, and the influence of edaphic and environmental factors on symbiotic expression are factors of prime importance.

The first approach made in the present study was the isolation and selection of effective strains of bacteria. There were three aspects that were considered important in this regard, viz.,

1. The ability of rhizobia for symbiotic nitrogen fixation cannot be correlated with their biochemical and physiological properties (Allen and Allen, 1958).

2. Considerations of amounts of nitrogen fixing tissue concern directly nodule size and abundance in legumes. The specific nodule volume is a function of the host and is not influenced by bacterial variation or virulence (Nutman, 1958).

3. The amount of nitrogen fixed by a root nodule was a function of the central tissue volume of a nodule and the time for which this tissue persisted without deterioration (Thornton, 1939; Chen and Thornton, 1940). The specific nodule volume may thus provide a means for comparison of the effectiveness in symbiotic nitrogen fixation, of various strains of root nodule bacteria (Nutman, 1959).

These aspects, in relation to the ideas that flow from them, have been fully taken into consideration in the present work in determining the effectiveness of Rhizobium strains and nodule function in various legumes.

From a consideration of the foregoing account, it follows that nodule volume and its central tissue are factors for prime consideration in the determination of effectiveness or ineffectiveness.

This central tissue, which is composed of enlarged host cells (Bergersen, 1961) contains the pigment haemoglobin. Its presence in root nodules was

shown to be a pre-requisite for nitrogen fixation (Keilin and Wang, 1945; Virtanen, 1945). Nodules formed by ineffective strains of rhizobia do not contain haemoglobin, and evidence for a close positive correlation between the haemoglobin content of root nodules and the intensity of nitrogen fixation appears rather conclusive (Smith, 1949 a; Virtanen, Erkama and Linkola, 1947; Virtanen et al., 1947; Virtanen, 1955; Jordan and Garard, 1951; Falk et al., 1959). The recent emphasis by Bergersen (1961) on the correlation between the effective central tissue volume of root nodules and the haematin concentration in terms of weight concentration, nodule content and tissue content, has further clarified that nitrogen fixation is a function of the haemoglobin-containing cells of the central nodule tissue.

Knowledge on these aspects on haemoglobin formation and its relation to the nitrogen fixation potential of root nodules has been fully utilized in the present work, in an effort to understand the effectiveness of locally isolated strains of rhizobia, as well as the symbiotic performance of a variety of legume host species.

A necessary corrolary to this study, which closely followed was gaining information on the synchronization between host and symbiotic development, and the influence of the environment on symbiotic expression, for, recently, considerable emphasis was laid on the influence of the tropical environment on the functioning of legume nodules (Norris, 1958; Masefield, 1958).

Such studies formed the basis for an understanding of the integrated development of host and legume bacteria in the symbiotic state, and nodule formation was construed in terms of the development of haemoglobin in root nodules, chlorophyll in leaves and associated with this their impact on nitrogen fixation. It is pertinent here to refer to recent work which points to a physiological connection between the two pigments, haemoglobin and chlorophyll (Hill and Hartree, 1953; Granick, 1950; R Rimington, 1957).

Effectiveness in symbiosis in a few species studied in terms of nodule weight and haemoglobin concentration per unit nodule volume have further stressed on the remarkable uniformity of nitrogen fixing systems of these legumes, albeit the inherent variations of nodule size, structure and abundance between one legume and another.

Since there was a consensus of opinion on the basic importance of the host plant in its genetic and physiological behaviour in determining the extent of symbiotic development (Nutman, 1958), the effects that accrue from pathologically altered host protein metabolism such as occur in virus infections (Bawden and Pirie, 1956) were studied in relation to nodule development.

Since every problem of nitrogen metabolism culminates with the problem of protein synthesis and its regulation in tissue systems, the soluble nitrogen constituents in legume nodules merited study in the present work.

ISOLATION OF ROOT NODULE BACTERIA:

a) Culture medium: Yeast mannitol agar of the following composition was used for isolation and maintenance of stock cultures:

Mannitol 1.0%; K_2HPO_4 0.05%; $MgSO_4.7H_2O$ 0.02%; NaCl 0.02%; $CaSO_4$ 0.01%; $CaCO_3$ 3.0%; Yeast extract (Difco) 0.2%; Agar 1.5%. pH 7.0. The medium was sterilized at 15 lb. for 15 minutes.

b) Temperature and period of incubation during isolation of cultures from root nodules and tests for freedom from contamination:

The media in Petri dishes were incubated in the dark for a period of ten days at 25°C - 28°C and tested for the presence of Agrobacterium radiobacter by the tests outlined by Hofer (1941).

c) Smear and Gram stain: Smears prepared from yeast mannitol agar medium were tested for freedom from Gram positive cells. All the isolates examined were tested for the gram negative character.

d) Maintenance of stock cultures: The isolated strains of root nodule bacteria were labelled ~~(R$_1$-R$_9$)~~

and maintained on yeast mannitol agar slants in pyrex test tubes (plugged with cottonwool) at a temperature of 20 ± 1°C in an airconditioned room. The pure cultures were periodically sub-cultured and examined for contaminants.

METHODS OF GREEN HOUSE AND FIELD CULTIVATION:

a) Seed Sterilization and Sowing: In all sterile sand culture experiments, the seeds were washed thoroughly and surface sterlized by immersion for three minutes in 80% ethanol followed by three minutes in 0.2% mercuric chloride, and washed in sterile water until the washings no longer contained chloride. The sterilized seeds were planted aseptically with sterile forceps just below the surface of the moist sterilised sand. Five seeds were sown in each jar and aseptically thinned out after germination to three plants per jar.

b) Preparation of plant assemblies: All the legumes grown in sterile sand were kept under aseptic conditions in the green house at a temperature of 22°C-27°C during the winter and 29°-32°C during the summer. In

Preparing plant assemblies, coarse river sand was thoroughly washed with tap water followed by distilled water, dried in the sun on clean brown paper, sieved through holes 3 mm. in diameter and used to fill glazed jars of one litre capacity. The assemblies were sterilized in an autoclave for two hours at 20 lb. pressure. This time and pressure were found suitable to prepare completely sterile assemblies.

c) Inoculation with rhizobia: Inoculation was carried out at the time of germination of the seeds with a sterile water suspension of the specific Rhizobium strain, prepared from 72-hour agar slant cultures. Ten millilitres of the suspension were used to inoculate each plant assembly by placing the fluid around the base of the germinated seedlings.

d) Provision of sterile water and nutrients: Glass distilled water and tap water, sterilized at 15 lb. for 15 minutes were used whenever necessary. In sand culture experiments, the sand was dressed prior to sterilization with 0.05 percent of a nitrogen-free Crone-Bryan's salt mixture of the following composition.

KCl 10.0 g; $CaSO_4$ 3.5 g; $MgSO_47H_2O$ 2.5 g;
$Ca_3(PO_4)_2$ 2.5 g; $FePO_4$ 2.5 g; $MnSO_4$ 1 mg;
Borax 1 mg; $CuSO_4$ 1 mg; Na_2MoO_3 1 mg; $ZnSO_4$ 1 mg.

e) General green house and field procedures: After inoculation, the plant assemblies were maintained in a green house under conditions of broad day light. Plants were grown under dust-free conditions and the space for growing plants was kept clean, and occasionaly disinfected by pouring a solution of a commercial disinfectant over the side walks. Field plots adjoining the green house were used for field trials with some Rhizobium strains.

f) Legumes used in study: Healthy and viable seeds of Arachis hypogaea varieties TMV 2, 3, 5 and HG 1 (The abbreviation TMV refers to the origin of seed material, namely Tindivanam); Dolichos lablab var DL 231; Crotalaria juncea L; Cyamopsis tetragonoloba (L) Taub; Dolichos biflorus L; Phaseolus mungo L; Vigna catiang Walp and Cajanus cajan (L) Millsp var. N.P.80.were used. The seeds were obtained from the Agricultural Experiment Station, Tindivanam.

NITROGEN DETERMINATIONS:

a) Dry weight determination: Plant materials were dried in an oven held at a temperature of 100°C until the weights remained constant.

b) Estimation of nitrogen: The dried plant material was ground in a clean porcelain mortar with a pestle by hand and the quantity of nitrogen was determined by the Kjeldahl method. The procedure followed was that described by Humphries (1956) and is as follows:

> 50 mg. of dried and finely powdered plant material were weighed and transferred to a micro-Kjeldahl flask. 0.06 g. of the catalyst (copper sulphate 1.0 g; potassium sulphate 8.0 g. selenium dioxide 1.0 g. were ground separately in a mortar and then mixed together), and 0.5 mg. of nitrogen free sulphuric acid were introduced into the Kjeldahl flask. The flasks were gently heated on a digestion stand until fumes of sulphuric acid evolved and then were strongly heated until the digests in the flasks turned an apple green colour. Blanks were run alongside at the time of every determination of nitrogen. During digestion, care was exercised to avoid particles of carbon from sticking to the sides of the Kjeldahl tubes.
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> The digests were cooled, made up to 50 ml. with glass distilled water using a volumetric flask. Using a suitable aliquot of this, to which 15.0 ml. of 30% sodium hydroxide were added, nitrogen was

estimated as the amount of ammonia evolved during steam distillation of the digest. The liberated ammonia was collected over a solution of 2% boric acid to which two drops of the Conway indicator were added. Distillation was continued till about 25.0 ml. of distillate was collected. After distillation, the boric acid was titrated against N/28 HCl. to a faint pink colour. One millilitre of N/28 HCl was taken as equivalent to 0.5 mg. of nitrogen, (Humphries, loc. cit).

ESTIMATION OF CHLOROPHYLL:

The method for extraction and determination of chlorophyll described in the Journal of Association of Agricultural Chemists (A.O.A.C. 1950) was followed.

5.0 g. of fresh and clean leaf material were ground in a glass mortar. 10 ml. of 85% acetone (B.D.H) was added to the leaf pulp. Extraction of the leaf was carried out with acetone until all the colour of the leaf tissue was extracted into the solvent. The extracts were centrifuged at 3000 r.p.m. for 10 minutes. The clear supernatant was transferred to a volumetric flask and made upto 50 ml. with 85% acetone.

In estimations of leaves of varieties of field grown plants which involved a large number of samples, the acetone extracts were directly used for spectro photo-

metric estimations utilizing 85% acetone as blank solutions. For the estimation of chlorophyll in leaves of plants grown with diverse bacterial strains, the acetone extracts of leaf material were purified by passage through ether in a separatory funnel for the selective elimination of water. The ether extracts of the pigment made up to a volume of 25 ml. in a volumetric flask were used for the estimation of chlorophyll.

The optical density of the chlorophyll extracts was determined in a Uvispek photoelectric spectro photometer (Hilger) in the infra red, at 660 mμ and 642.5 mμ against pure ether as blank. The chlorophylls were calculated from the formulae given in methods of the A.O.A.C.(1950).

Total chlorophyll (mg./l).

7.72 x O.D. at 660 mμ + 16.8 x O.D. at 642.5 mμ

Chlorophyll a (mg./l).

9.93 x O.D. at 660 mμ + 0.777 x O.D. at 642.5 mμ.

Chlorophyll b (mg./l) by subtraction of the chlorophyll a from the value for total chlorophyll.

PREPARATION OF CRYSTALLINE HAEMIN FROM OX BLOOD.

The method followed was in strict accordance with the procedures outlined by Hartree (1955).

Blood of ox was collected in sterile saline bottles and defibrinated by shaking with glass beads. The coagulated fibrin was strained off by filtering through muslin. The blood was then dispensed into pyrex glass cylinders of 250 ml. capacity. 200 ml. of defibrinated blood was left standing in the glass cylinders at 5°C for 24 hours. The upper layer of serum was then sucked off carefully. The resulting red cell suspension was used for isolation of haemin.

A mixture of 360 ml. glacial acetic acid, 20 ml. saturated potassium chloride solution and 20 ml. water in an one-litre pyrex conical flask was quickly heated on the direct heat of a Bunsen burner without a gauze and the contents were brought to a vigorously boiling stage. The flask was removed and the contents were kept swirling while 80 ml. red cell suspension were added in a thin stream from a separatory funnel within a period of 30-40 seconds. Care was exercised to see that the blood fell directly

into the hot acid and not touch the inside wall of the flask. This mixture was then rapidly heated to a boiling point within two minutes and set aside to cool. Separate batches of red cell suspensions were worked up in this way, and when the heated mixtures have cooled, they were poured into glass cylinders and allowed to stand for three hours. After this period the supernatant was removed. The sedimented bluish black crystalline haemin was collected and the crystals were suspended in 200 ml. 10 N acetic acid and transferred to centrifuge tubes. Following centrifugation at 3000 r.p.m., for ten minutes, the supernatant along with the flocculated material at the surface was sucked off. The haemin was washed twice with distilled water and sedimented under gravity. After the final washing, the haemin was transferred by means of absolute alcohol to a sintered glass funnel and filtered under reduced pressure. The final product was washed with pure ether dried on the pump and stored in stoppered vials at 5°C.

PREPARATION OF NODULE HAEMOGLOBIN:

The methods described by Virtanen et al., (1947) and those described by Green and Hughes (1955) on protein fractionation by solubility were employed.

Nodules from Rhizobium - inoculated plants grown in the ~~laboratory~~ field were utilized for the purpose. Nodules were detached from plant roots just prior to the time of flowering. Initially, 100 grams of nodules were used for the purification of nodule haemoglobin. The nodules were thoroughly washed to free them of adhering soil and were crushed at 0°C in a porcelain mortar kept in a freezing mixture. The crushed nodules were extracted with distilled water, centrifuged at 3000 r.p.m. for ten minutes and solid ammonium sulphate was added to the clarified extracts to an initial 58% saturation. The preparation was neutralized and left standing overnight at 0°C. The following day, the precipitate was removed by centrifugation and the clear red supernatant was collected. The concentration of ammonium sulphate in this solution was raised to 75% by addition of solid ammonium sulphate. After a further standing overnight in the cold at 0°C, precipitate was separated, dissolved in a small quantity of water and dialyzed against distilled water in a collodion membrane until freed from ammonium sulphate. The extinction of this preparation was determined as specific extinction co-efficient in the study of haemoglobin from the root nodules of healthy and virus-infected plants.~~using the haemoglobin of blood as control.~~

ESTIMATION OF HAEMOGLOBIN AS PYRIDINE HAEMOCHROMOGEN.

Ox blood haemoglobin was used as the standard for comparison and the haemoglobin of ox blood and root nodules were converted to their haemochromogens by addition of sodium hydroxide followed by pyridine and sodium dithionite. Spectrophotometric comparison of root nodule haemoglobin and blood haemoglobin enabled the quantitative estimation of the former in relation to the latter pigment. The details of the procedure followed and the related observations are presented in Chapter ~~II~~ I.

TWO DIMENSIONAL CHROMATOGRAPHY AND SPECTROPHOTOMETRIC ESTIMATION OF AMINO ACIDS.

The amino acids in the alcohol soluble fraction of plant materials were separated, identified and quantitatively estimated by ~~employing~~ following procedures outlined by Stepka (1957); Porter (1957) and Shaw and Colotelo (1961).

An ascending two dimensional chromatographic technique was used employing Whatman No.1 (11.5 x 16.5 in, with an assay area of 10 x 15 in.) paper of chromatographic grade, using Petridishes for holding the solvents under bell jars (18 x 10 in.) at 25 $\pm$ 1°C.

Phenol water (100:39 W/V) was used as the first; and n-butanol, acetic acid, water (100:22:50 V) as the second irrigating solvents. Mixed in the given proportions, the components of each solvent system formed a single phase at room temperature. Development in the first solvent run with phenol-water required 10-12 hours, while with the second solvent, n-butanol-acetic acid-water, it was 30-32 hours.

Freshly distilled phenol stored at -20°C and butanol marked 'for chromatography' (Merck) as well as acetic acid (A.R) were employed in the preparation of solvents with thrice glass distilled water.

Following development in the first solvent the papers were thoroughly dried at room temperature for a 24 hour period prior to transfer into the second solvent. After development in the second solvent, the papers were dried overnight. The ninhydrin positive spots (sprayed with 0.4% solution of ninhydrin in ethanol) were developed at 60°C for 30 minutes, the spots were cut out after identification and further cut into small pieces approximately 1 cm^2 in area. The colour from these was eluted with 50% ethanol. The optical densities of these eluates as well as the approximate blank solutions were measured within a period of two hours in a 'Uvispek' photoelectric

spectrophotometer (Hilger) using a 10 mm. light path. The absorbance of all the amino acids were read at 570 mμ except for proline at 430 mμ and asparagine at 360 mμ. Standards with pure amino acids were subjected to the above procedure. The approximate Rf values of various amino acids in each solvent system were determined separately and their relative positions on a two dimensional chromatogram were plotted as standard co-ordinates. Concomitantly, the chromatographic separation of an applied aliquot containing all the amino acids helped to determine the relative position of each amino acid on a two dimensional chromatogram. The amino acids in plant extracts were identified with reference to these standard maps (figs. 18 and 19).
16, 17 and Table 9)

Amino acids in plant materials from an applied aliquot equivalent to 10 - 25 mg. fresh weight of tissue were quantitatively estimated with reference to standard curves prepared from chromatographing known amounts of pure amino acid solutions at a number of known concentrations (2 to 10 ug). The amount of a particular amino acid in a tissue extract

was then determined by reference to a standard curve relating absorbance of the ninhydrin derivative to weight of the pure amino acid chromatographed and quantitatively estimated under the same conditions (figs. 20, 21, 22 and 23).
18 19 20 and 21

VIRUS INOCULATION AND SEROLOGICAL ASSAY OF VIRUS IN PLANT TISSUES:

The host plants of Dolichos lablab were inoculated ten days after germination, with a standard extract of the virus (Dolichos enation mosaic virus - DEMV) prepared from severely infected plant leaves of Dolichos lablab. One gram of infected leaves ground with 'celite' in 1 ml. of distilled water provided the material for the preparation of the virus extract for purposes of sap transmission, by gentle rubbing of the leaf surface using the forefinger dipped in the inoculant. The serological assay of the virus was ~~determined~~ carried out by the precipitin end-point method (Matthews, 1957). Antiserum to DEMV was prepared following the procedure of Badami (1959). The procedure consisted in the preparation of the antigen from infected leaves, immunization of the rabbits, preparation of the antiserum and study of precipitin reaction at 37°C using 0.85% saline for all dilutions.