CHAPTER VIII

FREE AMINO ACIDS IN LEGUMES IN RELATION TO HEALTH AND DISEASE.

Plants, in contrast to animals, achieve protein synthesis from the fewest and simplest of substrates (Steward and Pollard, 1957). Systems that have been used in the study of protein metabolism include germinated seedlings, the green leaf, nitrogen-rich fleshy storage organs, growing regions of shoot and root, while more recently particular interest has come to centre on the use of plant tissue cultures, for they readily furnish experimental material for the critical study of growth metabolism in rapidly proliferating tissues (Steward and Pollard, IV Internat. Congr. Biochem. Vol. VI).

Since the factors of environment have a great bearing on the external and internal variables which impinge closely on nitrogen metabolism (Wood, 1953), and protein synthesis in plants (Steward and Street, 1947; Steward, Bidwell and Yemm, 1956; Steward, Thompson and Pollard, 1958; Pollard and Steward, 1958) these have received widest attention (Steward _et al._, 1959).

Steward and Pollard (1957) have suggested that the different investigations on nitrogen compounds morphological regions such as leaf, fruit, seed, storage organs of root or shoot, tracheal or exuded saps and growing points need particular study.

In the present study the quantitative composition of the free amino acids was studied following the two-dimensioned chromatographic techniques outlined by Stepka (Methods in Enzymology Vol.III, 1957) in Arachis hypogaea and Dolichos lablab in relation to day-length in the former and virus infection in the latter. For, in a preceding section, the effects of virus infection were found to influence nodulation and since changes in free amino acids consequent on virus infection have received wide attention in recent years, this was of particular interest. A compilation of the various fractions of free amino acids in the total alcohol-soluble nitrogen of a few legume nodules was of topical interest.

A. THE EFFECTS DUE TO DAY-LENGTH ON THE ALCOHOL-SOLUBLE NITROGEN CONSTITUENTS IN ARACHIS HYPOGAEA.

The problem of the effects of the environment hase to be considered in relation to the alcohol-soluble constituents of plants (Steward and Thompson, 1954; Steward, Zaccharius and Pollard, 1955; Steward and Pollard, 1957).

Thus, studies on the mint plant have shown that day length and nutrition of the plant profoundly influence its nitrogen metabolism (Steward *et al.*, 1959).

In the preceding studies of this work, it was found that day-length influenced nodule formation, nitrogen fixation and haemoglobin synthesis in ground nut plants (*Arachis hypogaea*) when these were grown in symbiotic association with a specific *Rhizobium* strain. Thus, when the tropical day-length of 8-10 hours was reduced to one half, the nodule number, weight and volume decreased by more than 75 per cent while haemoglobin in root nodules and nitrogen fixed decreased by 50 and 40 percent respectively. It was, therefore, decided to study this problem more fully in relation to the alcohol-soluble nitrogen constituents of the plant in relation to day length.

PLANT MATERIAL AND TECHNIQUES:

Groundnut plants of the bunch variety (*Arachis hypogaea* var. TMV 2) was chosen for the study. The methods of growing plants in sand culture, inoculation with an effective *Rhizobium* strain (R_4), maintenance and care of plant assemblies, provision of water and nutrient solution are essentially those described in Chapter IV. However, here, only two day lengths, 4 and 10 hours, were studied for their influence on free amino acids in plants.

HARVESTING AND EXTRACTION:

Leaf and stem material from the shoots of *Rhizobium*-inoculated and uninoculated series from 4 and 10 hour treatments were removed at 45 days after sowing.

Solubility in 80% ethanol was used as the criterion for the presence of nitrogenous substances. Extracts were obtained by homogenising the shoot, root or nodule tissues (5 g. fresh weight) with 80% ethanol at room temperature (25°C - 28°C), followed by centrifugation for 10 minutes at 3000 r.p.m. The clear supernatant

Fig. 18 Standard map of amino acids

Fig. 19 Standard map of amino acids showing approximate R_f values was concentrated to dryness in a vacuum desiccator, taken up in 1 ml. of 10 percent isopropanol and stored at 0°C until chromatographic analysis.

CHROMATOGRAPHIC PROCEDURES:

The amino acids in the alcohol soluble fraction of groundnut plants were separated, identified and quantitatively estimated employing a combination of procedures adopted from Stepka (1957) and Porter (1957). Amino acids in plant materials were identified with reference to figs.18 and 19. The Rf values of various amino acids are presented in Table 13.

TABLE 13.

Rf Values of amino acids on Whatman No.1 Filter Paper (10" x 15") at room temperature (28±) .

	PHENOL, H_2O (100:39 W/V).	n-Butanol, Acetic acid, H_2O (100 : 22; 50, V).
ALANINE	0.565	0.504
ARGININE	0.510	0.210
ASPARAGINE	0.402	0.204
ASPARTIC ACID	0.155	0.304
α-AMINO BUTRYIC ACID	0.650	0.471
γ-AMINO BUTRYIC ACID	0.700	0.502
CITRULLINE	0.600	0.207
CYSTINE	0.201	0.104
DJENKOLIC ACID	0.280	0.103
GLUTAMIC ACID	0.270	0.400
GLUTAMINE	0.580	0.301
GLYCINE	0.390	0.304
HISTIDINE	0.601	0.201
ISOLEUCINE	0.800	0.609
LEUCINE	0.802	0.701
LYSINE	0.420	0.106
METHIONINE	0.800	0.507
S-METHYL CYSTEINE	0.710	0.405
NORLEUCINE	0.804	0.705
ORNITHINE	0.209	0.106
PHENYLALANINE	0.802	0.608
PIPECOLIC ACID	0.850	0.502
PROLINE	0.808	0.400
SERLINE	0.340	0.304
TRYPTOPHANE	0.700	0.602
TYROSINE	0.601	0.500
THEREONINE	0.445	0.309
VALINE	0.705	0.506

-ooOoo-

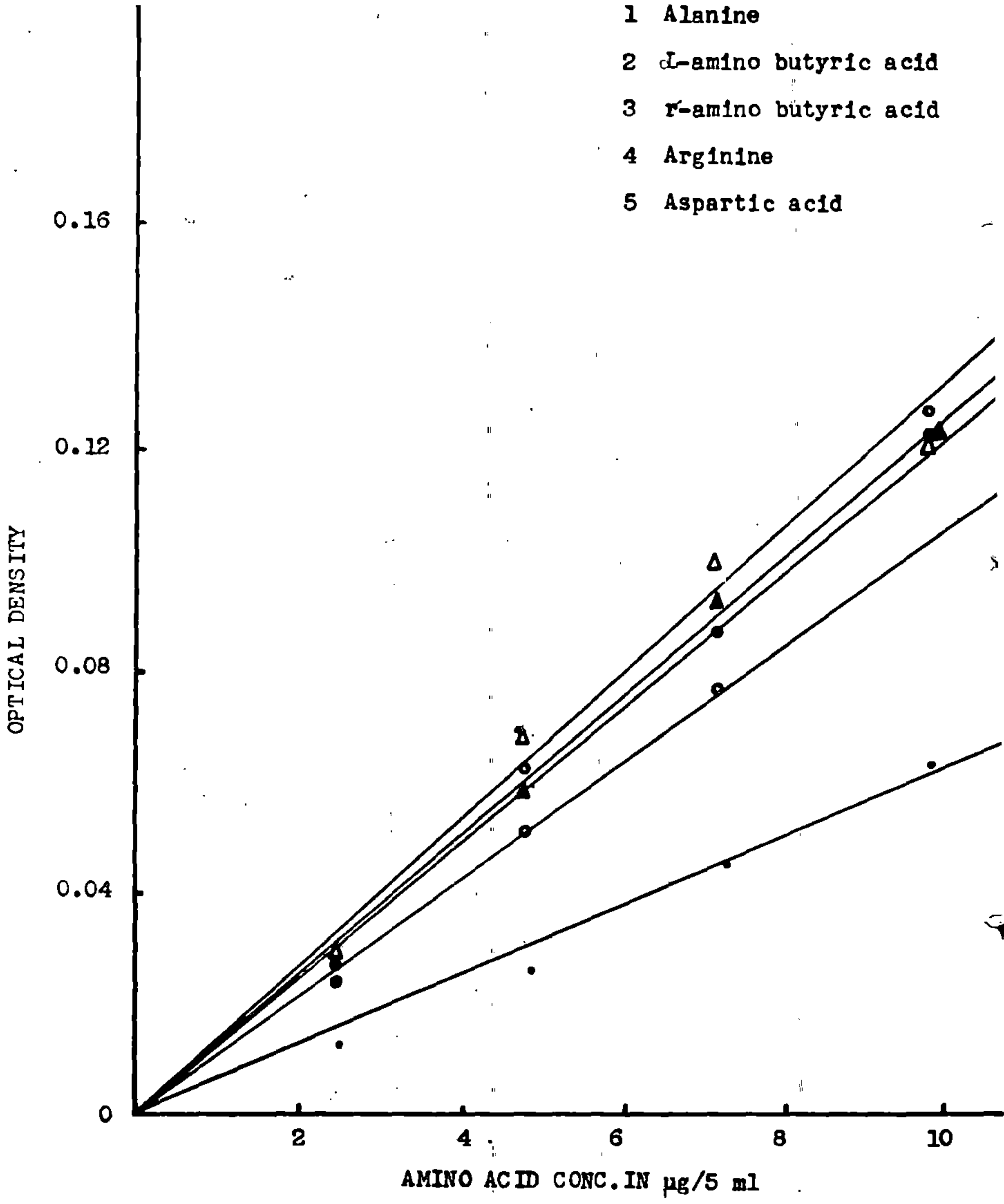


Fig.20. AMINO ACID STANDARD CALIBRATION CURVES

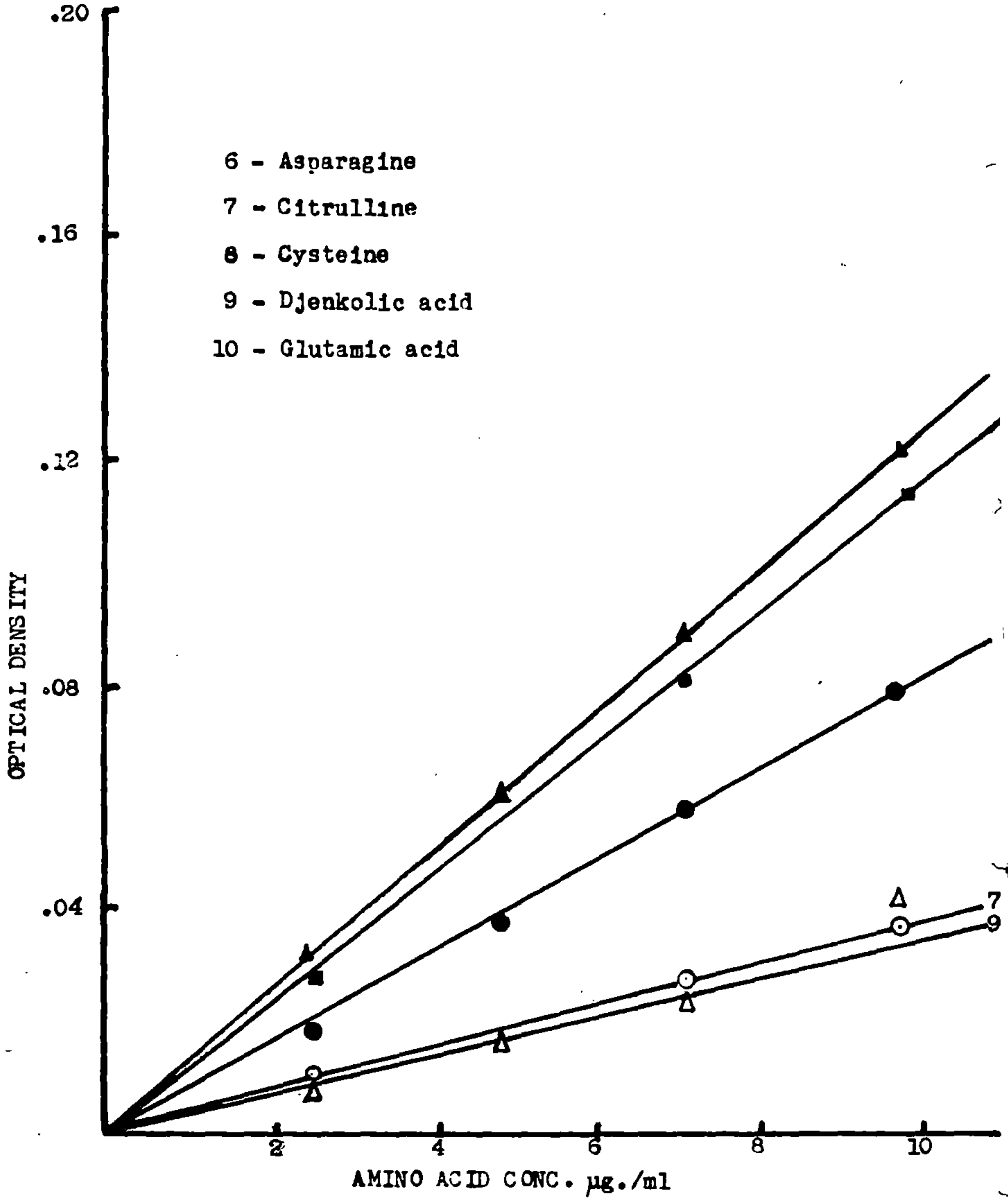


Fig. 21. AMINO ACID STANDARD CALIBRATION CURVES

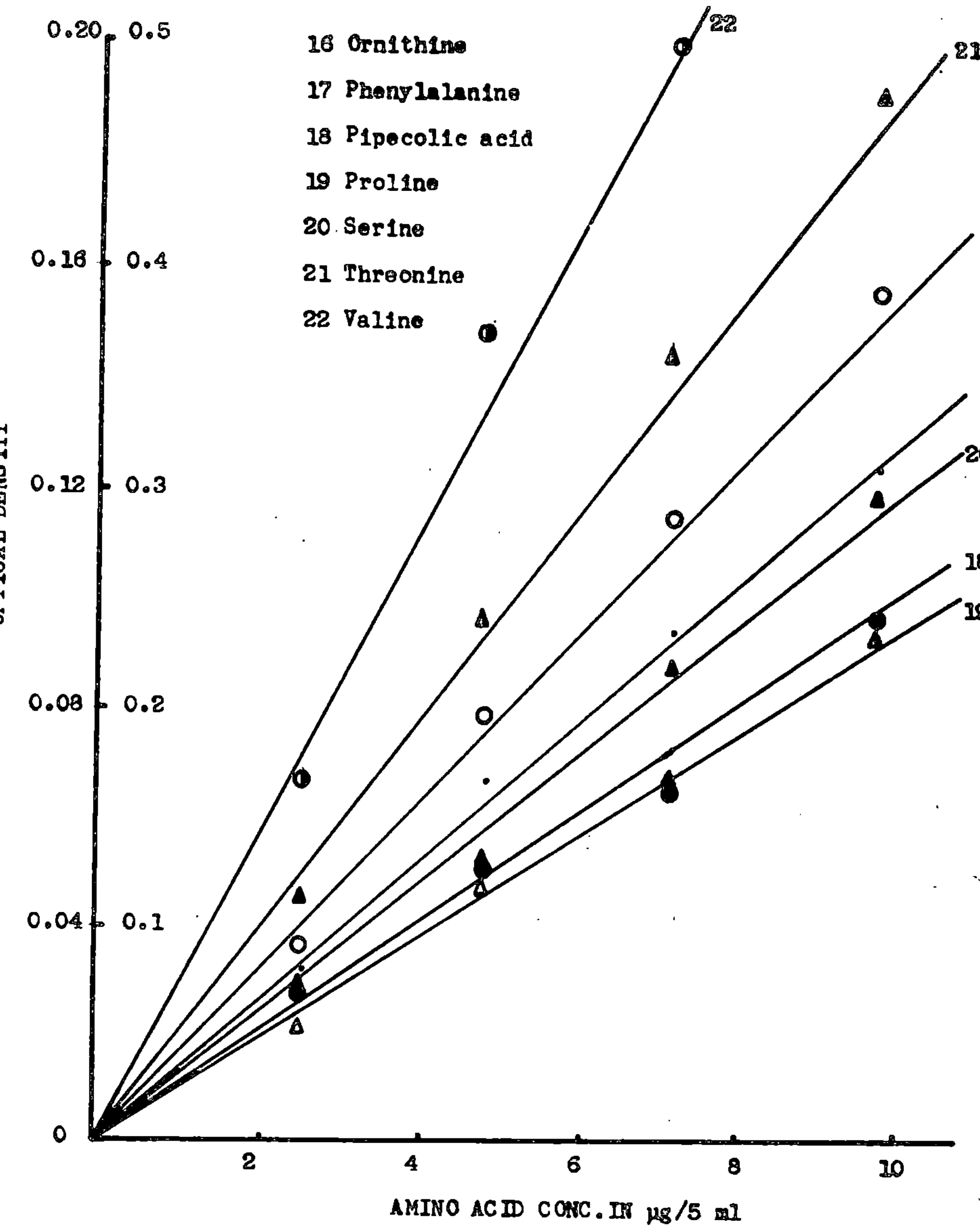


Fig. 23. AMINO ACID STANDARD CALIBRATION CURVES
(inner scale refers to serine)

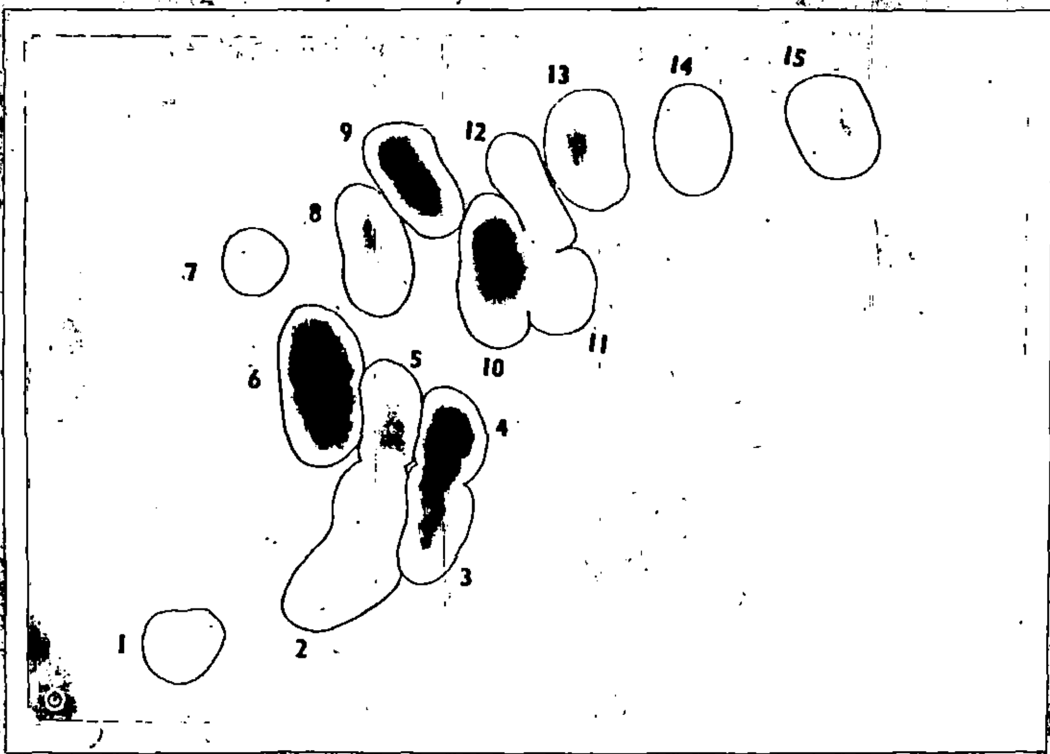


Fig.24

Fig.24 A paper chromatogram of Arachis hypogaea illustrating the relative positions of amino acids

1. Cystine
2. Aspartic acid
3. Glutamic acid
4. Glycine
5. Serine
6. Asparagine
7. Arginine
8. Histidine
9. γ- Methylene glutamic acid
10. Glutamine
11. Unknown
12. Threonine
13. Alanine
14. Valine
15. Leucine

The quantitative estimation of amino acids was carried out with reference to standard calibration curves which are represented in figs. 20 (Ala-Asp); 21 ($AspNH_2$ - Glu); 22 ($GluNH_2$ - Lcys) and 23 (orn-val). The precautions outlined by Stepka (1957) and Steward (1956) were observed in chromatography. The procedural details are previously described (vide Materials and Methods).

Two-dimensional chromatograms of the alcoholic extracts of root nodules of *Arachis hypogaea* showed the presence of fifteen different amino acids. These were identified as ornithine, aspartic acid, glutamic acid, glycine, serine, asparagine, arginine, histidine, γ methylene glutamic acid, glutamine, threonine, alanine, valine and leucine. One of the amino acids could not be identified. These are illustrated in fig. 24.

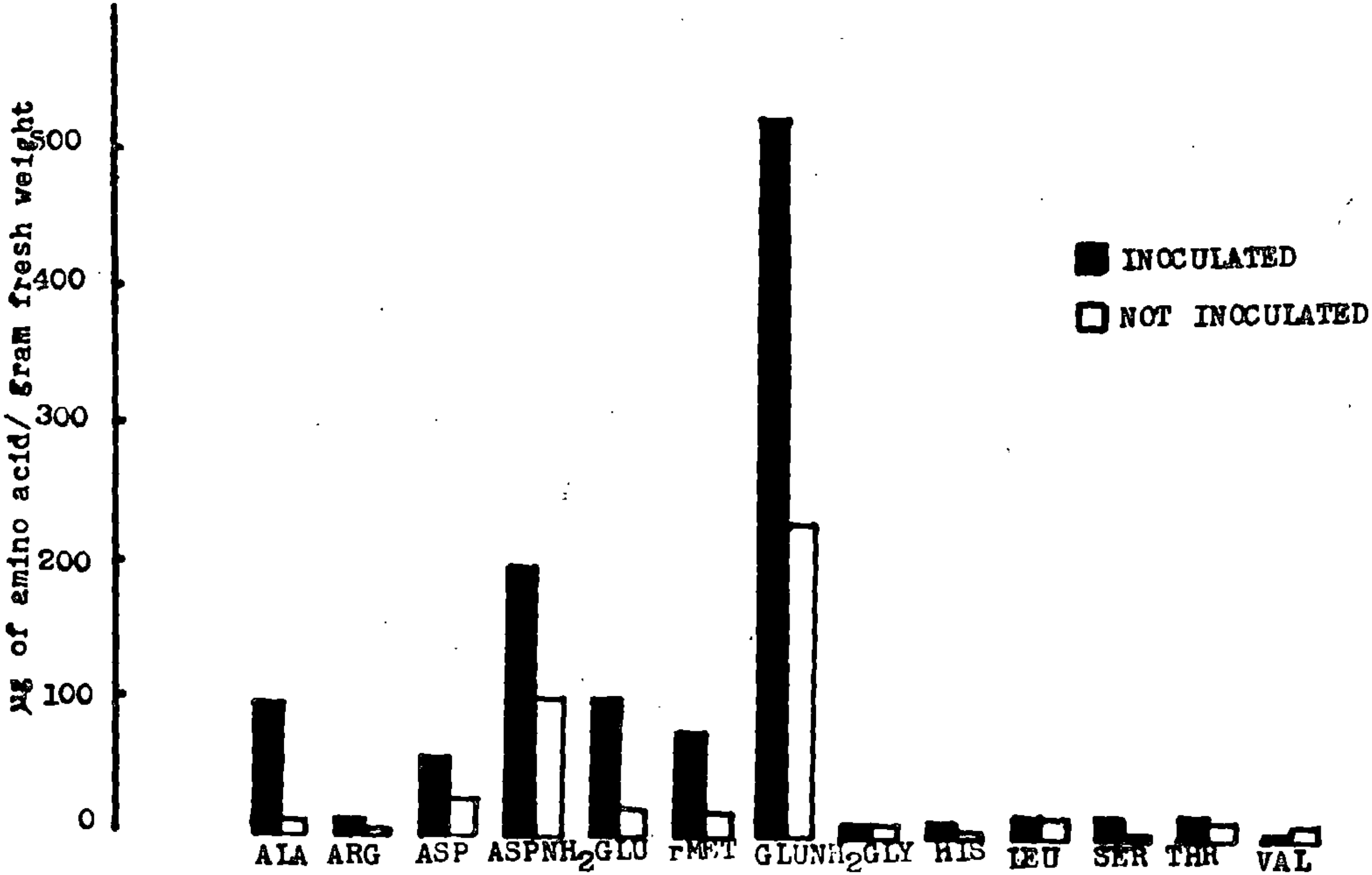


Fig.25. THE EFFECT OF RHIZOBIUM INOCULATION ON THE BALANCE OF FREE AMINO ACIDS IN THE SHOOT SYSTEM OF ARACHIS HYPOGAEA(45 days after sowing)IN STERILE SAND CULTURES.

B. EFFECT OF RHIZOBIUM INOCULATION ON THE SOLUBLE NITROGEN OF GROUNDNUT PLANTS.

The composition of the free amino acids in *Rhizobium* inoculated and uninoculated plants (shoots) of groundnut are shown in fig. 25. The results show that alanine, asparatic acid, glutamic acid, γ methylene glutamic acid, asparagine and glutamine represent a larger percentage of the soluble nitrogen of inoculated plants in comparison with uninoculated plants. The percentage increase in the various amino acids in the inoculated plants, calculated from the mean values of three replicate chromatograms of inoculated and uninoculated plants ranged from 92 to 503. The values showed an increase of 137.5 percent in the total free amino acid as a consequence of *Rhizobium* inoculation and symbiotic growth in comparison with uninoculated control plants.

C. THE FREE AMINO ACID COMPOSITION OF THE ROOT NODULES.

The soluble nitrogen constitutents of root nodules of *Rhizobium* inoculated plants of *Arachis hypogaea* are represented in fig. 26. It was observed that a relatively greater

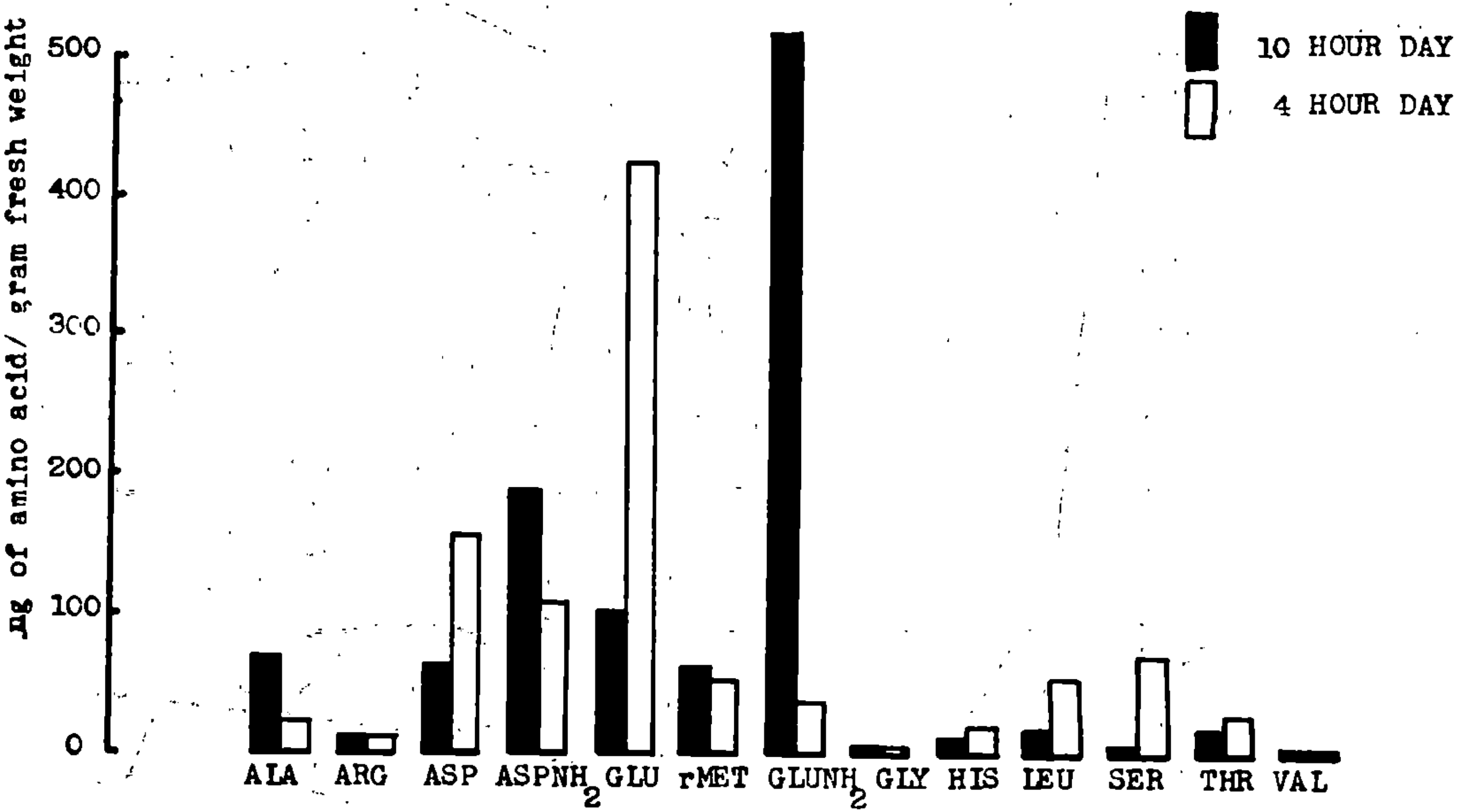


Fig.27. THE EFFECT OF DAY LENGTH ON THE BALANCE OF FREE AMINO ACIDS IN THE SHOOT SYSTEM OF <u>ARACHIS HYPOGAEA</u>(45 days following sowingin sterile sand culture and inoculant <u>Rhizobium</u> R4 strain used.)

proportion (44.4%) of the soluble nitrogen occured in the amides, asparagine and glutamine than in any other single amino acid in root nodules.

D. THE COMPOSITION OF THE SOLUBLE NITROGEN OF GROUNDNUT PLANTS IN RELATION TO DAY LENGTH - THE SHOOT SYSTEM:

The data for the soluble nitrogen compounds of the shoot system of groundnut plants (var. TMV 2) grown under long-day (10 hrs) and short-day (4 hrs) conditions are represented in fig. 27.

The long and short-day growth habit not only results in differences in the size and chlorophyll content of leaves, length of internodes and floral initiation, but accompanies striking metabolic differences. Fig. 27 shows that the shoots of the long-day plants contained an abundance of glutamine and asparagine, whereas the short-day shoots contained less total amide. The short-day plants contained a relatively larger amount of aspartic and glutamic acids than the long-day shoots. Although the long-day plants showed slight increases in the quantity of γ methylene glutamic acid, this was not very significant in comparison with the short-day shoots. Again, alanine was found in conspicuously

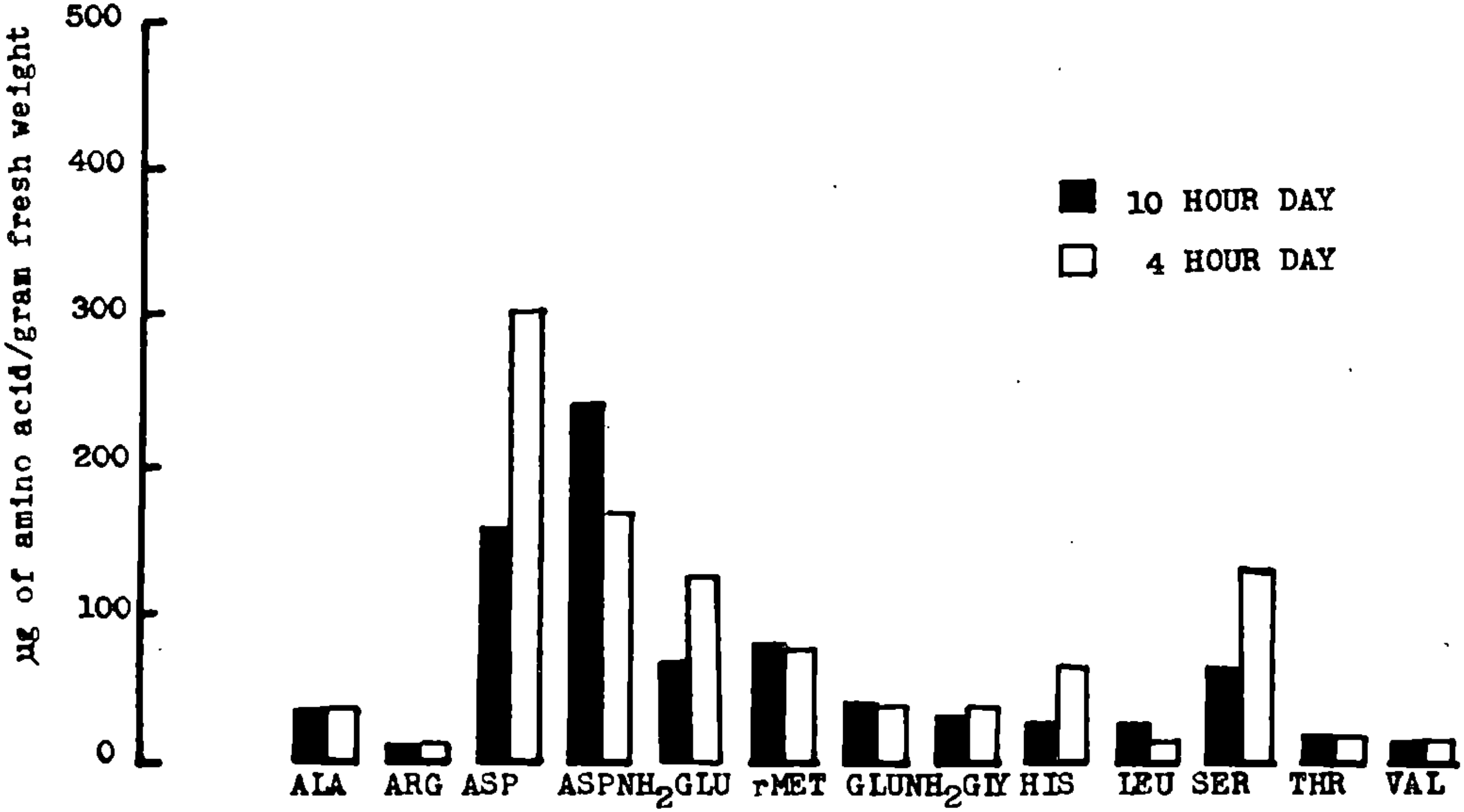


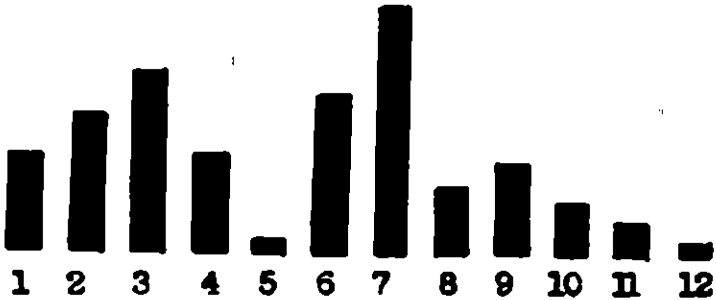
Fig. 28. THE EFFECT OF DAY LENGTH ON THE BALANCE OF FREE AMINO ACIDS IN THE ROOT SYSTEM OF ARACHIS HYPOGAEA (45 days following sowing in sterile sand culture and inoculant *Rhizobium* R_4 strain used.)

larger amounts in long-day plants in contrast to short-day plants. The amino acids histidine, serine and threonine were quantitatively conspicuous in the short-day shoots in comparison with long day shoots. There was no change in the quantitative composition of arginine, glycine and valine in relation to long and short-day conditions. The significance of these changes in the soluble nitrogen constituents in relation to day length are discussed subsequently (*vide* discussion).

E. THE COMPOSITION OF THE SOLUBLE NITROGEN OF GROUNDNUT PLANTS IN RELATION TO DAY-LENGTH - THE ROOT SYSTEM.

The data for the soluble nitrogen constitutents of the roots of groundnut plants (var. TMV 2) grown in long-day (10 hrs) and short-day (4 hrs) conditions are represented in fig. 28.

This shows that the relative composition of the amide glutamine is less affected by length of day. The short-day roots showed significant increases in the quantity of aspartic and glutamic acids when compared to plant roots which grew under long-day conditions. Asparagine, however, occured in greater quantity in the roots of plants cultivated under long-days than in short-days. Further, the roots of these plants grown under short-days showed an accu-



THE FREE AMINO ACID COMPOSITION OF THE ROOT NODULES OF *VIGNA CATJANG*

ne 2.L-Amino butyric acid 3.Arginine 4.Aspartic
Cysteine 6.Glutamic acid 7.Glutamine 8.Glycine
dine 10.Leucine 11.Serine 12.Valine

mulation of histidine and serine and a reduction in leucine. Alanine, arginine, glycine, threonine and valine were comparatively unaffected by length of day.

Thus, in comparison with the changes that follow long and short day conditions on the composition of the soluble nitrogen of shoots, the roots seem to be relatively less affected. The significance of these changes in the soluble nitrogen constitutents in shoots and roots in relation to day-length are discussed subsequently (*vide* discussion).

F. THE COMPARATIVE COMPOSITION OF THE FREE AMINO ACID AND AMIDE FRACTIONS OF LEGUME ROOT NODULES.

The study of the percentage composition of the free amino acid and amide fraction in the total soluble nitrogen of a few legume nodules harvested prior to the time of flowering led to the following observations. These are represented in Table 14 and Figs. 26, 29, 30, 31, and 32.

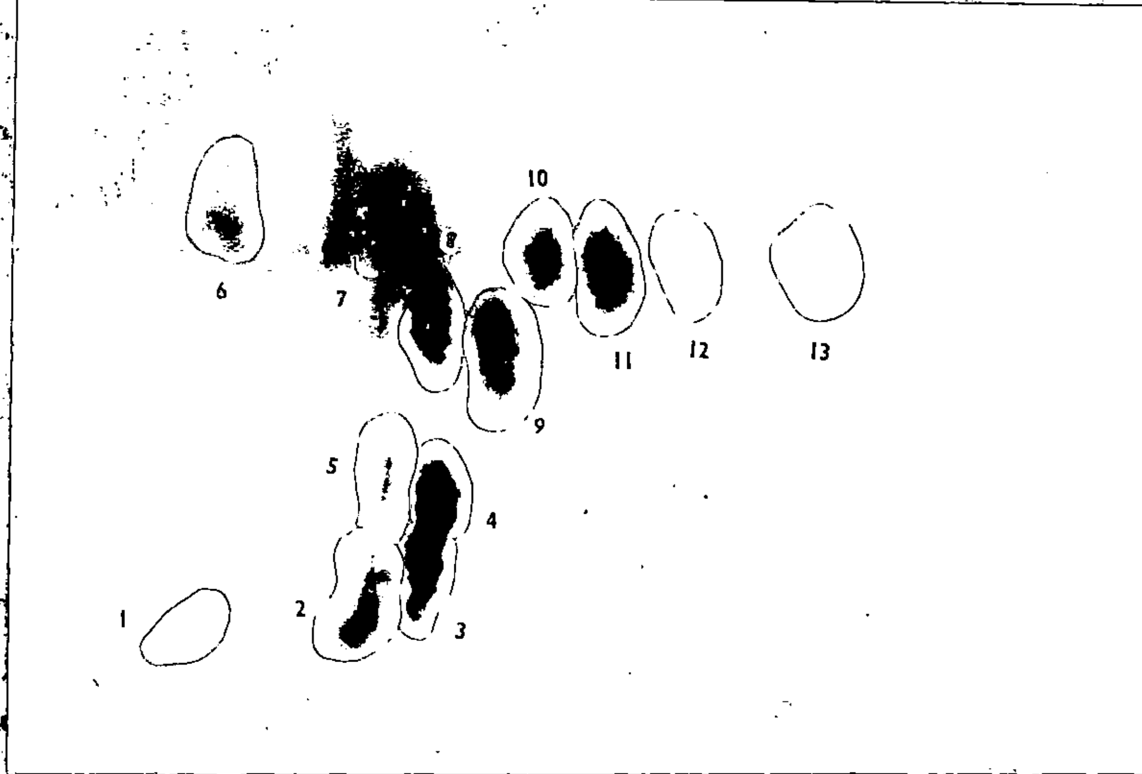


Fig. 29

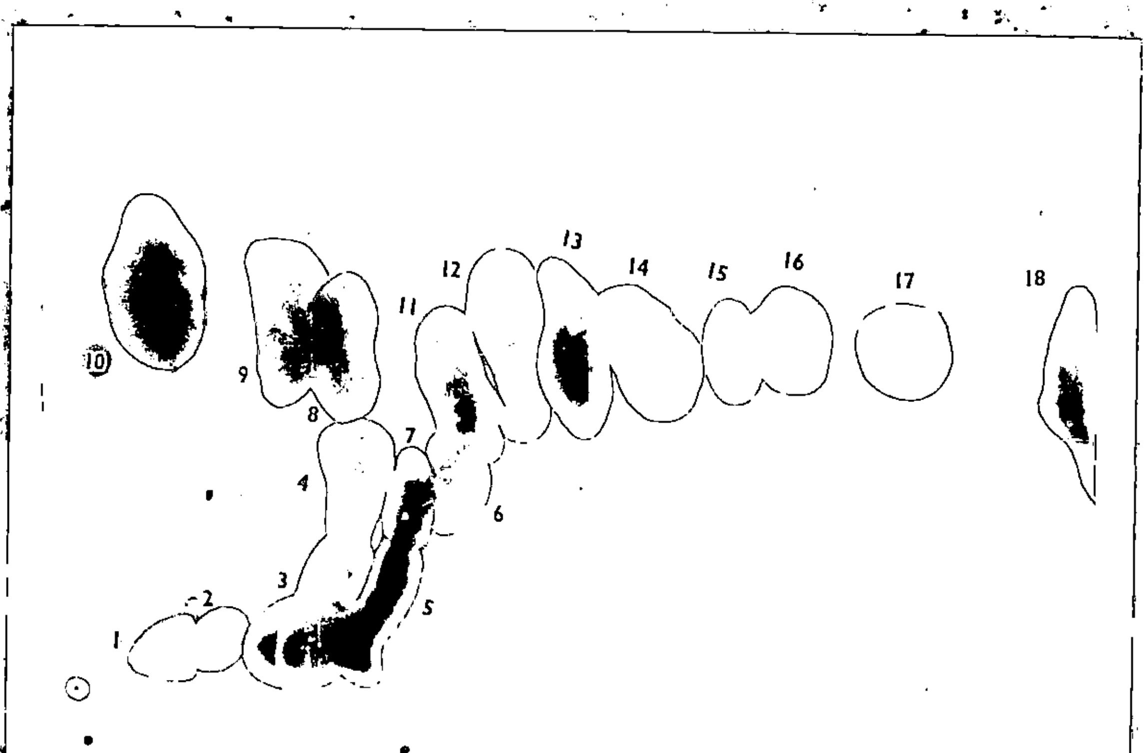


Fig. 30

Fig.29 A paper chromatogram of amino acids from root nodules of Vigna catiang.

1. Cystine 2. Aspartic acid 3. Glutamic acid 4. Glycine 5. Serine 6. Unknown 7. Arginine 8. Histidine 9. Glutamine 10. γ - amino butyric acid 11. Alanine 12. Valine 13. Leucine

Fig. 30 A paper chromatogram of amino acids from root nodules of Dolichos biflorus

1. Cystine 2. Ornithine 3. Aspartic acid 4. Serine 5. Glutamine acid 6. Glutamine 7. Glycine 8. Arginine 9. Histidine 10. Unknown 11. γ - amino butyric acid 12. Phenylalanine 13. Alanine 14. Valine 15. Leucine 16, 17, and 18 Unknown.

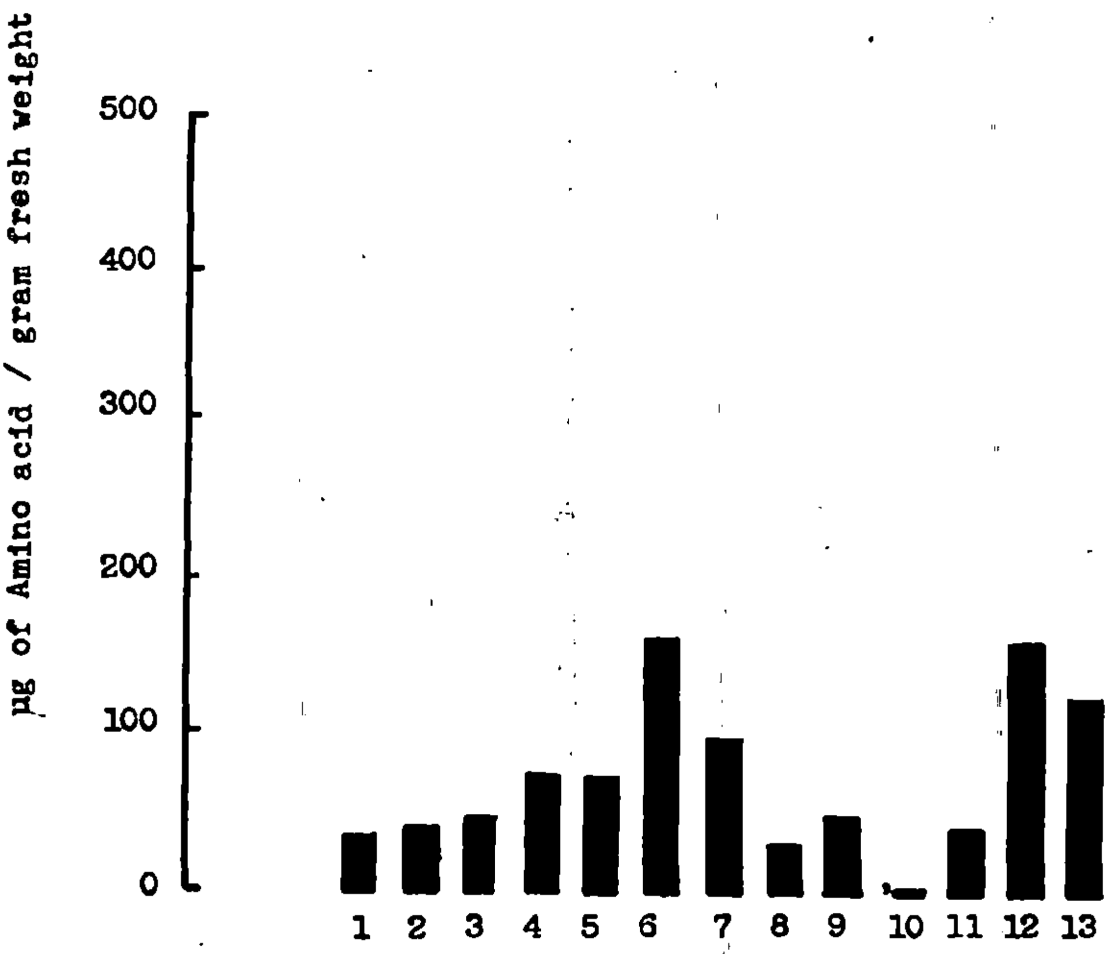


Fig.31. THE FREE AMINO ACID COMPOSITION OF THE ROOT NODULES OF <u>PHASEOLUS MUNGO</u>

1.Alanine 2.α-amino butyric acid 3. γ-amino butyric acid
4.Arginine 5.Aspartic acid 6. Glutamic acid 7.Glutamine
8.Glycine 9. Histidine 10. Leucine 11.Pipecolic acid
12.Serine 13.Valine

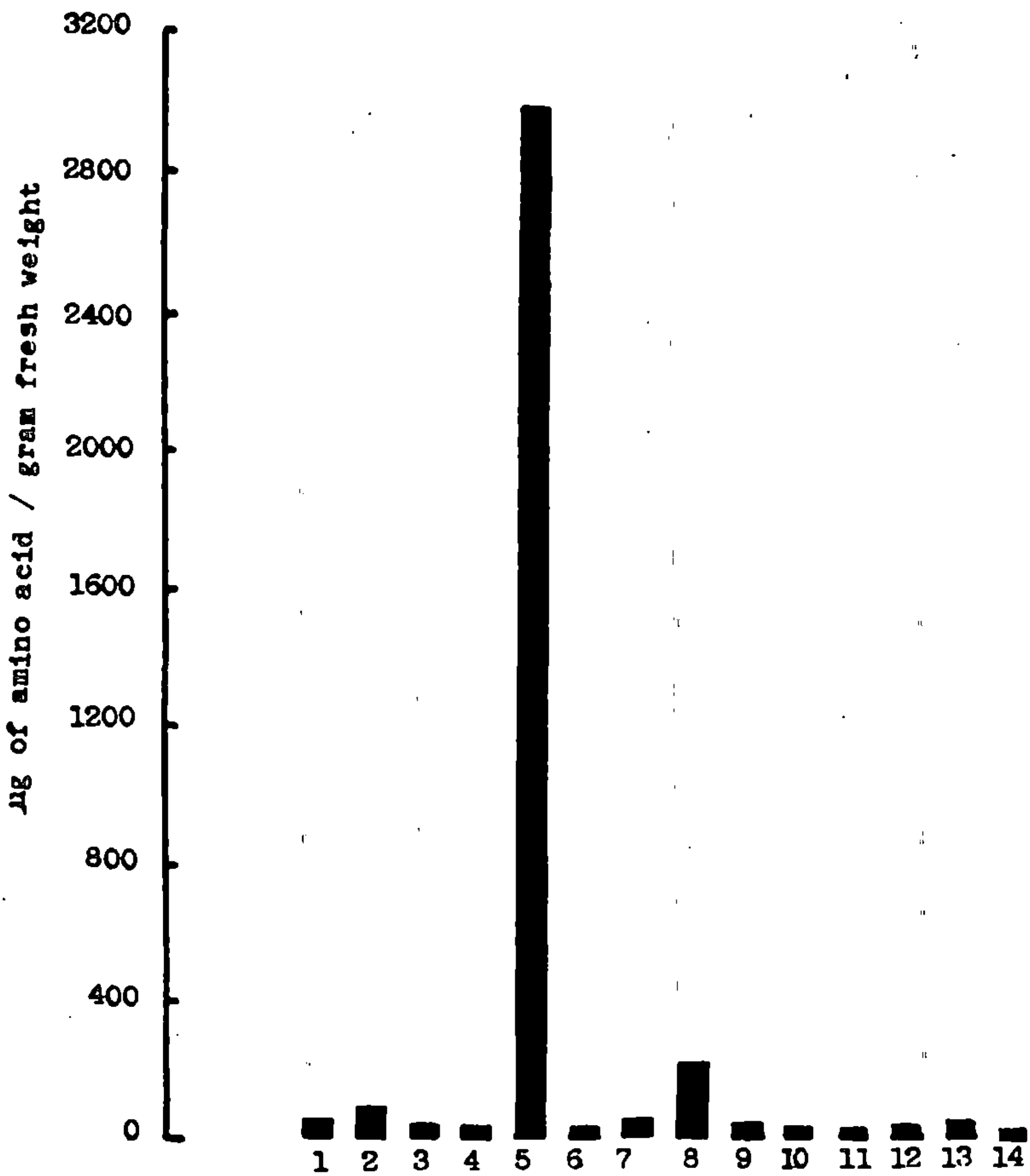


Fig.32. THE FREE AMINO ACID COMPOSITION OF THE ROOT NODULES OF <u>CYAMOPSIS TETRAGONOLOBA</u>.

1.Alanine 2. γ-amino butyric acid 3.Arginine 4.Aspartic ac
5.Asparagine 6.Cysteine 7.Glutamic acid 8.Glutamine 9.Glyc
10.Histidine 11.Leucine 12.Ornithine 13.Serine

TABLE 14.

COMPOSITION OF THE FREE AMINO ACID AND AMIDE FRACTIONS AS PERCENTAGE OF THE TOTAL SOLUBLE NITROGEN OF LEGUME ROOT NODULES.

...es and ...o acids.	Arachis hypogaea	Phaseolus mungo	Vigna catiang	Cyamopsis tetragonoloba.	Dolichos biflorus.	Dolichos lablab Healthy
...s	44.0	9.9	21.0	88.8	20.0	34.3
... acids:						
...c	17.5	25.0	21.5	2.3	20.4	14.6
...	19.8	13.3	23.0	2.0	16.0	14.3
...c Imino	-	4.5	-	-	-	-
...tic	-	-	-	-	1.3	1.6
...ur	-	-	1.1	0.5	1.2	4.9
...xy	2.7	17.7	3.0	0.6	4.9	2.8
...al	16.0	29.6	30.4	5.8	36.2	27.5

... (ASP. NH_2, GLU.NH_2); Acidic (ASP, GLU, γ-MET.GLU); Basic (ARG, ORN, ... Cyclic Imino (PRO, PIP); Aromatic (PHE, TRY, TYR); Sulphur (CYS, MET, ... CYS); Hydroxy (SER,THR); Neutral(GLY,ALA,VAL,LEU,γ-AMB, α-AMB).

The two plant amides asparagine and glutamine constituted a greater percentage of the total soluble nitrogen, followed by the neutral amino acids (glycine, alanine, valine, leucine, α - amino butyric acid and γ amino butyric acid), the acidic (asparatic acid, glutamic acid, γ methylene glutamic acid) and basic (arginine, ornithine and cystine) amino acids. It was interesting to observe that in Cyamopsis tetragonoloba (ref: Fig.32 and plate) the amide fraction alone comprised 88.8 percent of the total soluble nitrogen. In the nodules of this plant, unlike in other legume nodules studied, the acidic and the basic amino acids formed a comparatively lesser quantity in relation to the total soluble nitrogen.

Of the hydroxy amino acids, serine formed a characteristic component in Phaseolus mungo. Phenyl alanine, which was the only one of the three aromatic acids (phenyl alanine, tryptophane and tyrosine) observed was found to represent a small percentage (1.3 to 1.6) in the soluble fraction of the root nodules of Dolichos lablab. Pipecolic acid, a characteristic imino acid observed in Phaseolus mungo comprised about 4.5 percent of the soluble fraction.

Thus, the acidic amino acids and the amides (ranging from 34.9% to 91.1%), the basic and neutral acids (ranging from 7.8% to 53.4%), the hydroxy acids (ranging from 0.6% to 17.7%) with the sulphur, aromatic and the cyclic imino acids comprising a smaller proportion were observed in the present study on the amino acid distribution in a few legume nodules.

G. THE EFFECT OF VIRUS INFECTION ON THE SOLUBLE NITROGEN CONSTITUTENTS OF THE ROOT NODULES OF DOLICHOS LABLAB.

Virus diseases are regarded essentially as a change in the protein metabolism of host cells (Bawden and Pirie, 1956). The synthesis of virus proteins by plant tissues almost certainly involve changes in the free amino acids (Porter, 1961), albeit the fact that these changes may be transitory or small (Selman *et al.*, 1961). The changes in the free amino acids consequent on virus infection have taken several avenues of expression (Andrae and Thompson, 1950; Allison, 1953; Diener and Dekker, 1954; Diener, 1960; Fife, 1956; Lalaroya *et al.*, 1956; Porter and Weinstein, 1957, 1960; Porter, 1959; Miećzynski, 1959; Selman and Milne, 1961; Selman *et al.*, 1961).

Since ion-accumulation and respiration which are linked to protein synthesis and breakdown (Steward and Milner, 1954) are affected in virus infected plants, the study of the alcohol-soluble nitrogen in these plants continues to deserve emphasis.

In the preceding work virus infection was found to have marked effects on nodule formation and haemoglobin synthesis. This was expressed as an increased formation of nodules in terms of weight and volume as well as an increase in the root nodule haemoglobin content in infected plants in comparison to healthy plants. In order to study these effects more fully, a comparison was made between the nodules of healthy and virus-infected plants in terms of their soluble nitrogen constituents.

METHODS:

The methods followed in the cultivation of plants (Dolichos lablab), rhizobial and virus inoculation are described in 'Materials and Methods'. The procedural details of harvesting nodules, extraction and quantitative estimation of amino acids are the same as described in an earlier part of this section. The study was made with nodules harvested from plants growing in unsterile soil in glazed containers. The plants were grown in broad day light outside the green house and were inoculated with a heavy suspension of rhizobia at the time of germination. The virus was inoculated on to the leaves on the 10th day following the sowing. The two series, infected and healthy were kept apart from one another and the nodules were harvested on the 25th day following inoculation with Dolichos Enation Mosaic Virus.

OBSERVATIONS:

Two dimensional chromatograms of the alcoholic extracts of nodules from healthy and virus-infected plants showed the presence of sixteen different amino acids. These were identified as alanine, γ amino butyric acid, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, leucine, phenyl alanine and serine.

Two of the amino acids could not be identified and are shown in figs. 33 and 34.

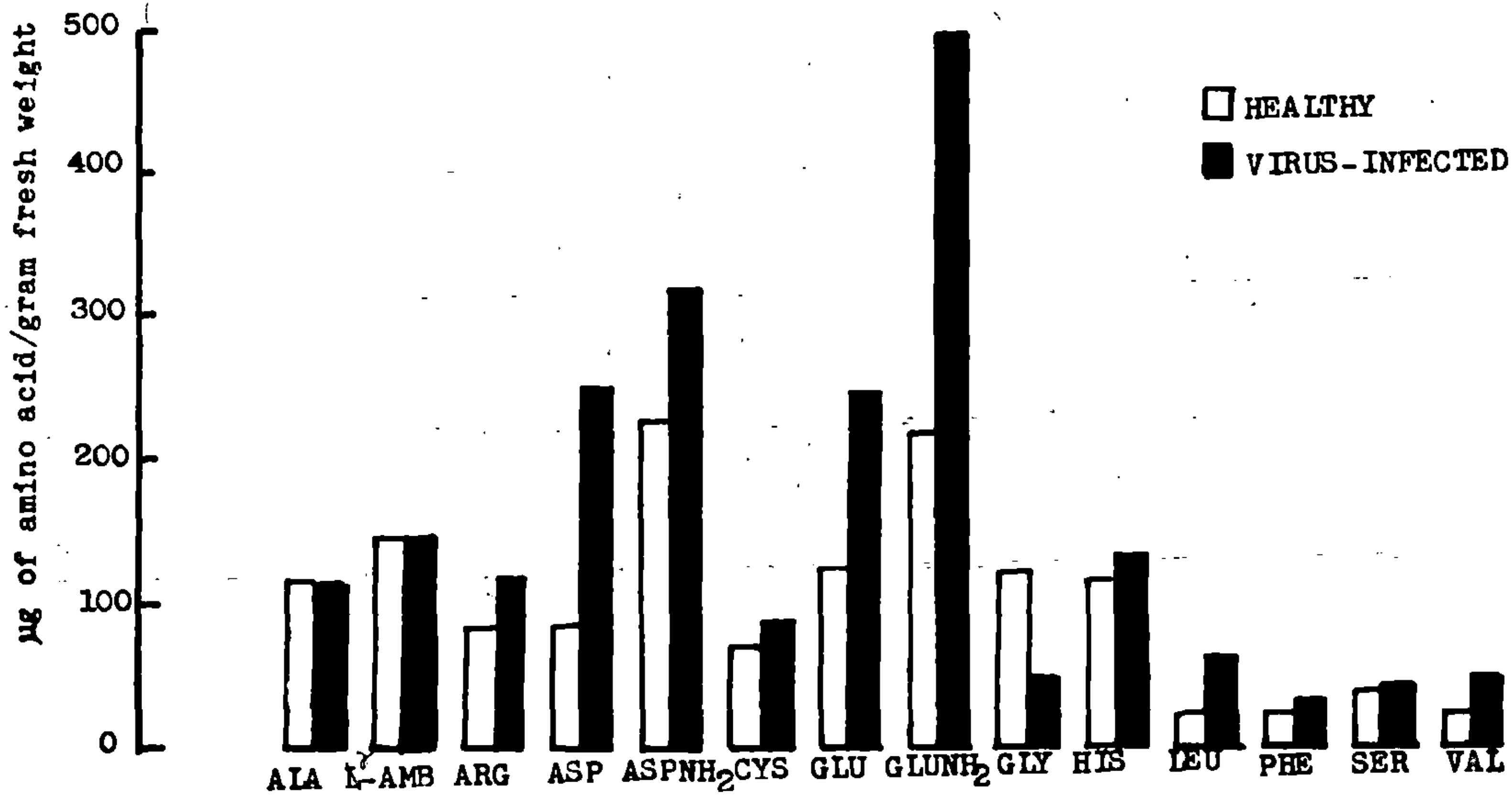


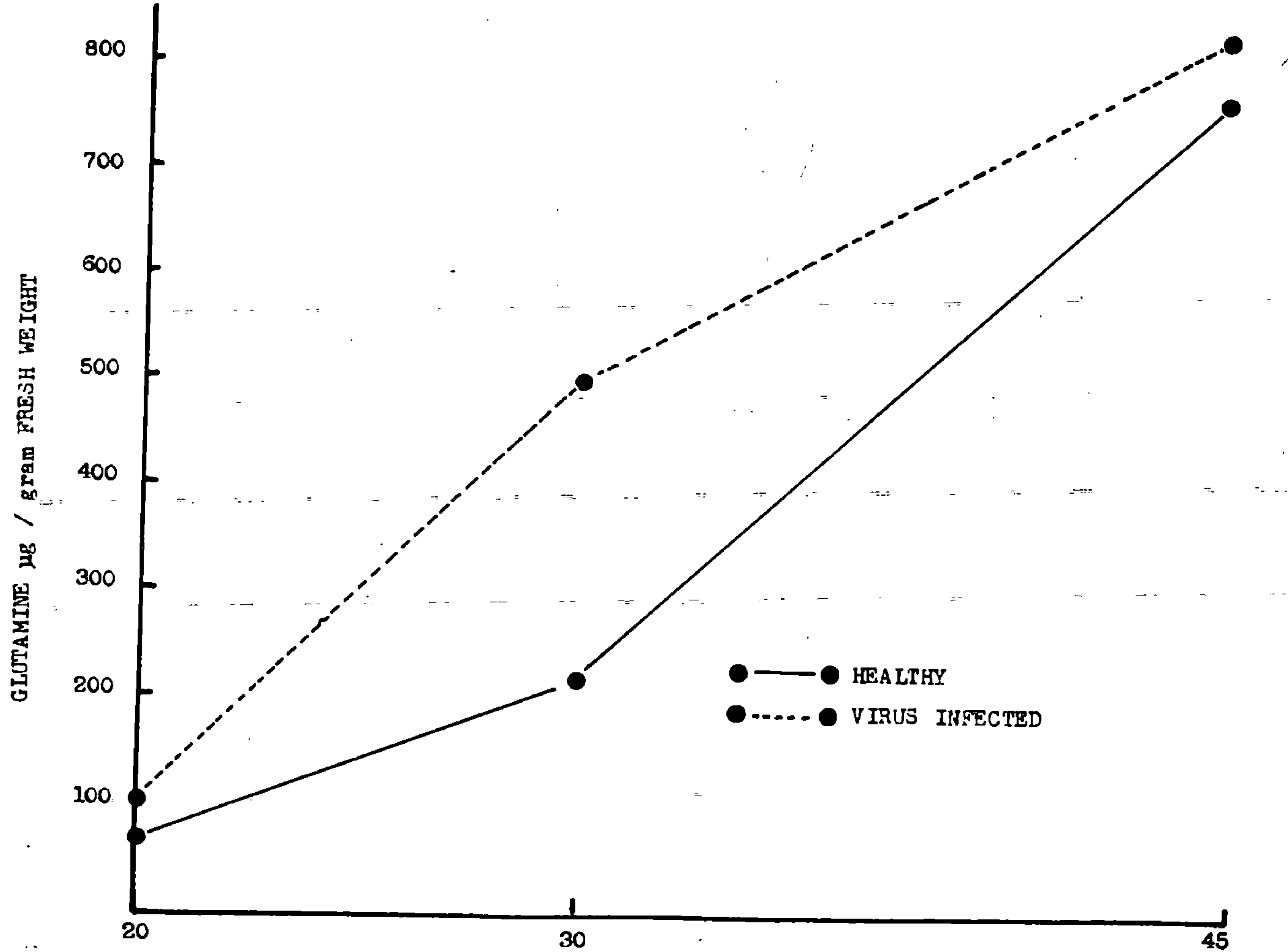
Fig . 35. THE EFFECT OF VIRUS INFECTION ON THE BALANCE OF FREE AMINO ACIDS IN HEALTHY AND VIRUS INFECTED PLANT NODULES OF <u>DOLICHOS LABLAB</u>. (35 days after sowing and 25 days after inoculation with virus)

H. THE QUANTITATIVE COMPOSITION OF THE SOLUBLE NITROGEN CONSTITUENTS OF THE ROOT NODULES OF HEALTHY AND VIRUS-INFECTED PLANTS.

The relative quantities of the free amino acids in the nodules of healthy and virus (Dolichos Enation Mosaic virus) infected plants are illustrated in fig.35. This shows that the nodules of virus-infected plants contained significantly larger quantities of most of the amino acids observed, in comparison with healthy plant nodules. The total free amino acid in the nodules of infected plants showed an increase of 55.9 percent over that of the healthy plant nodules. A greater proportion of this soluble fraction was composed of aspartic and glutamic acids and in their amides, asparagine and glutamine. There was a significant increase in arginine in the nodules of infected plants in contrast to healthy ones. The amino acids cysteine, histidine, leucine, γ amino butyric acid and serine remained relatively unaffected by virus infection. Glycine, however, showed a quantitative reduction in the infected plant nodules.

I. The soluble nitrogen fraction of the root nodules of healthy and virus-infected plants in relation to plant age.

Since the pronounced effects of virus infection presumably through a derangement of host protein metabolism were associated


GLUTAMINE µg / gram FRESH WEIGHT
800
700
600
500
400
300
200
100
20
30
45
HEALTHY
VIRUS INFECTED

with a distinct accumulation of aspartic and glutamic acids and their amides, a study of these was made in relation to plant age at 20, 30 and 45 days after inoculation with the virus (DEMV). The results are presented in Figs. 36, 37, 38 and 39. These show that aspartic and glutamic acids and the amide glutamine show a steady increase in the nodules of virus infected plants in comparison to the nodules of healthy plants from 20 to 45 days, while the levels of asparagine tend to decrease after 30 days from the time of virus inoculation.