NITROGENOUS CONSTITUENTS IN PLANTS

I. Free Amino Acids in Leaves and Leguminous Seeds

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Summary

A survey of the free amino acids in the leaves of various plant species has been made.

Large quantities of free proline in the leaves of the citrus group have been detected. The majority of the leaves are comparatively rich in asparagine, glycine, aspartic acid, glutamic acid and alanine although they are deficient in some of the essential amino acids.

An analysis of the amino acids of leguminous seeds with particular reference to *P. radiatus* has been carried out.

The free amino acids of *P. radiatus* at various stages of germination have been determined. Germination increases the amounts of free amino acids to many times their values in the dormant seed.

Several unknown ninhydrin-positive compounds including peptides have been detected.

The importance ascribed to the specific nutritive role played by certain amino acids for growth, reproduction, lactation, maintenance, etc., indicate the need for knowledge concerning the amino acid composition of foods, as they are commonly consumed by man and animals. It has been shown that about 15 per cent. of the organically combined nitrogen of fresh green leaves is in the form of low molecular weight compounds comprising mainly of free amino acids. Since leaves constitute an important source of food for the ruminant animal and human beings, it is necessary to have a better knowledge of the nature and occurrence of free amino acids in leaves. In addition, information concerning the amino acid composition of leaves will be useful in elucidating the specific role played by these substances in plant physiology. Although the work of Schulze during the latter part of the 19th century on the nitrogenous constituents of plant materials was of some significance, it was only at the beginning of this century that attempts were made to study the non-protein nitrogenous constituents in plant tissues. Investigations carried out by Vickery and co-workers (1924, 1925) on the nitrogenous constituents of alfalfa and those of Neuberger and Sanger (1942) on potato constituents may be considered as the starting point for further studies on this subject

During the last decade, the interest of plant biochemists has apparently shifted to the study of free amino acids which have been shown to be widely distributed in plants. This shift in emphasis has been brought about by the ever-increasing use of new techniques especially of paper chromatography.

In spite of the absence of substantial evidence the recent trend is to implicate free amino acids as substrates for enzymatic trigger action, they thus taking part in the regulation and control of protein synthesis (Steward and Thompson, 1954).

Dent *et al.* (1947) using two-dimensional paper chromatography, were able to detect in a cold alcoholic extract of potato tuber tissue asparagine, glutamine and 18 amino acids usually found in protein hydrolysates in addition to a number of unidentified ninhydrin-positive compounds.

Allsopp (1948) investigated the non-protein nitrogen fraction of growing points in various plants. It was found that the combined amino acids of the apical meristems were not much different in nature or proportion from those present in mature tissues. There was a close relationship between the amounts of free amino acids and the activity of development tissue.

Similar work has been done on chlorella (Stepka *et al.*, 1948), on legume nodules and roots (Hunt, 1951), on tobacco (Roberts and Wood, 1951), on certain fruits (Joslyn and Stepka, 1949) and on hops and wort (Harris, 1952). The literature on this aspect has been reviewed by Steward and Thompson (1950).

The recent spurt of activity in chromatographic studies of plant tissues has also led to the discovery of new amino acids which occur in the free state in higher plants. Miettinen *et al.* (1953) have recently isolated homoserine from growing pea. Among the newly discovered amino compounds the following deserve special mention: γ-aminobutyric acid (Reed, 1950; Hulme and Arthington, 1950; Synge, 1951 and Thompson *et al.*, 1953), β-alanine (Hulme and Arthington, 1950), baikiain (King *et al.*, 1950), pipecolic acid (Zacharius *et al.*, 1952; Morrison, 1953; Hulme and Arthington, 1952), γ-methylene glutamic acid and its amide (Done and Fowden, 1952) and arginosuccinic acid (Walker, 1952).

That the discovery of these new amino acids will stimulate further research on their metabolic role in plants is certain. Even at the present day indications are not lacking to show the metabolic significance of some of the newly discovered compounds. For instance β-alanine and γ-aminobutyric acid could be considered to be decarboxylation products of aspartic and glutamic acids. β-Alanine was detected by Virtanen and Laine (1939) among the excretion products of legume root bacteria. Subsequently it has been shown to be of more widespread occurrence (Dent *et al.*, 1947; Hulme and Arthington, 1950).

Steensholt (1946) has detected ethanolamine, a decarboxylation product of serine, in etiolated wheat seedlings. This and the reported presence of N-methyltyramine in barley roots (Kirkwood and Marion, 1950) clearly indicate that decarboxylation of naturally occurring amino acids takes place to a significant extent in plants.

Very little is known regarding the importance of the new nitrogenous compounds in legumes, *viz.*, baikiain and pipecolic acid. Baikiain was isolated from the bark of Rhodesian teak, *Baikiæa plurijuga*, by King *et al.* (1950). Pipecolic acid which is closely related to baikiain in structure was isolated from the leaves of *Trifolium repens* by Morrison (1952, 1953) who established its identity by analysis, racemization and comparison with a synthetic preparation. Zacharius (1952) has shown that it is present in a variety of plants, *e.g.*, in potato tuber, mushroom, tulip, asparagus and parsnip. Isolation of pipecolic acid was also achieved by Zacharius *et al.* (1954) and Grobbelaar *et al.* (1954) who provided critical evidence for its identity. It has been suggested by various workers (Grobbelaar and Steward, 1953; Rothstein and Miller, 1953; Lowy, 1953) that pipecolic acid can be derived from lysine by cyclization and elimination of ammonia.

The question of the occurrence of a third amide in plants has often been mooted (Vickery, 1936; Damodaran *et al.*, 1946). Damodaran *et al.* (1946) studied the amide and amino acid changes in germinated seedlings of horsegram, black-gram, and bengal-gram. It was found that a large percentage of proteins in the cotyledons was 'solubilized' within 24 hours germination. Evidence was obtained to show the accumulation of a third amide in addition to the presence of asparagine and glutamine. It was also reported that there was a concomitant decrease in the concentration of arginine indicating that it might be an amide precursor. Subsequently evidence was furnished by Damodaran and Venkateshan (1948) to show that urea was the third amide in plants.

An important contribution in this field was that of Done and Fowden (1952) who discovered the presence of the monoamide of γ-methylene glutamic acid in the peanut plant (*Arachis hypogea*). Zacharius (1952) has shown that this substance is also present in the tulip bulb.

From the foregoing it is clear that the application of the chromatographic technique has opened up a new field of research in plant biochemistry, which if pursued systematically shows great promise of providing a fund of information on amino acid and protein metabolism, our present knowledge of which is only rudimentary. So far chromatographic studies on the free amino acids in plants were confined to a qualitative examination of the ninhydrin-reactive compounds. Steward *et al.* (1954) have, however, struck a departure in investigating the nitrogen compounds of the shoot apical meristems of *Lupinus albus*, *Syringa vulgaris* and *Adiantum pedatum* by quantitative paper chromatography. In contrast to the results of Allsopp (1948) it was found by these workers that a wide range of amino acids and amides appeared as definite constituents of the meristem itself.

A programme of investigation of the free amino acids and peptides in leaves and Indian pulses was initiated in these laboratories, which culminated in the detection and subsequent isolation and characterization of *allo*-hydroxy-L-proline in *Santalum album* (Giri *et al.*, 1952; Radhakrishnan and Giri, 1954). In this article the results of investigations carried out on the free amino acids and peptides of the leaves of several plant species and certain leguminous seeds are presented.

EXPERIMENTAL

Extraction of amino acids

Leaves.—Fresh leaves were extracted with ethanol (1 : 6 w/v) in a Waring blendor, to a final concentration of 75% ethanol. A portion of the alcoholic extract was shaken well with chloroform (3 vol.) and centrifuged. The upper aqueous layer containing all the free amino acids was employed for paper chromatography. This procedure also enabled to remove chlorophyll which is taken up by the chloroform layer.

Seeds.—The seeds selected for the investigation were green-gram (*Phaseolus radiatus*), field bean (*Dolichos lablab*), bengal-gram (*Cicer arietinum*) and horse-gram (*Dolichos biflorus*). 5 g. of the seed powder (200 mesh) were extracted with 30 ml. of 75% ethanol in 10 ml. lots. The local variety was selected for the purpose.

The ripening seeds of *Dolichos lablab* were extracted with ethanol (1 : 6 w/v) to a final concentration of 75% ethanol.

Germination

The seeds were soaked in running water for 12 hours and germination allowed to proceed in Petri dishes on wet cotton pads for 24 hours. The germinated seeds were extracted with ethanol as in the case of the ripening seeds.

For quantitative studies with *Phaseolus radiatus*, 10 g. of the seeds were allowed to germinate in Petri dishes with sufficient amount of distilled water. After various periods of germination the seeds were dried between the folds of a filter-paper and the dry weight determined in a sample. The remaining seeds were extracted repeatedly with ethanol as before and made up to a known volume; known aliquots from this extract were used for the quantitative determination of amino acids.

Hydrolysis

To detect the presence of acid-labile compounds, the aqueous layer (2 ml.) from the alcoholic extract, obtained after treatment with chloroform, was hydrolysed with 3 ml. of 6 N HCl for 1 hour in an autoclave at 15 lb. pressure. The hydrolysate after evaporation to dryness *in vacuo* was taken up with distilled water (2 ml.) and after treatment with silver oxide to remove the last traces of HCl was filtered. The filtrate was used for chromatography. Similar aliquots from the aqueous layer and the hydrolysate could be compared on the same chromatogram to indicate the appearance or disappearance of bands and also the intensification of some of the original bands.

Chromatographic procedure

The procedure employed for the qualitative analysis of amino acids was the same as described by Giri and Rao (1952 *a*, *b*).

For quantitative studies with *Phaseolus radiatus* the following solvent systems were employed (Rao and Wadhwani, 1954) to determine the amino acids which separated as distinct bands.

Solvent I.—*n*-Butanol saturated with water at 20° C.—three runs.

Solvent II.—*n*-Butanol-acetic acid-water (40 : 5 : 14)—one run.

Solvent III.—*n*-Butanol-acetic acid-water (40 : 15 : 5)—three runs.

Paper

Whatman No. 1 paper chromatographically washed first with N/100 HCl and then twice with distilled water, was employed.

Quantitative procedure.—The subsequent procedure for the estimation of the amino acids was the same as described by Giri *et al.* (1952, 1953).

Abbreviations employed

α-Alanine (α-Al.); γ-Aminobutyric acid (γ-AB.); β-Alanine (β-Al.); Arginine (Arg.); Asparagine (Asp.); Aspartic acid (AA.); Cystine (Cy.); Glutamic acid (Glu.); Glycine (Gly.); Histidine (Hi.); Hydroxyproline (HPr.); Isoleucine (IL.); Leucine (L.); Lysine (Ly.); Methionine (Me.); Ornithine (Orn.); Phenylalanine (PhAl.); Proline (Pr.); Serine (Se.); Threonine (Th.); Tryptophan (Try.); Tyrosine (Ty.); Valine (Val.).

VP = Very prominent; + = fairly prominent; — = absent; T = traces only; Aq = Aqueous layer used for chromatography; Hyd = Aqueous layer hydrolysed with acid; ++ = Intensification of the band; ± = Partial disappearance of the band on acid hydrolysis.

RESULTS AND DISCUSSION

The variation in the 'free amino acid spectrum' of the leaves of various plant species is presented in Table I and the chromatograms relating to these are shown in Figs. 1, 2 and 3.

It is found that leaves belonging to the citrus group are particularly rich in free proline, the presence of which was confirmed by the isatin test (Acher *et al.*, 1950). The analysis of leaves from healthy and diseased (frenching) trees showed considerable differences in their amino acid content. While aspartic and glutamic acids were very prominent in healthy leaves, only traces of these amino acids could be detected in the diseased leaf. This observation is significant especially because aspartic and glutamic acids are known to be important parent substances for a number of other amino acids. The full implications of this observation can be clarified only by an extensive study of mineral nutrition of the plants and also the metabolic lesions brought about by the onset of the disease.

One of the characteristic features of most of the leaves analysed was the relatively high concentration of asparagine, glycine, aspartic acid, glutamic acid and alanine. The majority of the edible leaves appear to be deficient in the essential

TABLE I

Free amino acids in leaves (*Solvent:* n-*butanol-acetic acid-water*. 4 : 1 : 5)

Family	Cy.	Orn. Ly.	Asp.	Hi.	Arg.	Gly-Se.-AA.	HPr.	Glu. Th.	α-Al.	Pr.	Ty.	Try.-γ-AB.	Me.-Val.	Ph. Al.	L.-IL.
1. *Citrus decumana*	+	T	T	+	+	VP	−	+	+	VP	+	+	−	−	−
2. *C. medica*	+	+	+	+	+	+	−	+	+	+	T	+	T	−	T
3. *C. acida*	T	+	+	+	+	VP	−	T	VP	+	T	+	+	−	T
4. *C. aurantium*															
(*a*) Healthy	T	−	VP	+	+	VP	−	VP	+	VP	−	+	T	+	+
(*b*) Frenched	−	−	+	+	+	+	−	T	+	VP	−	+	T	+	+
5. *Atriplex hortensis* ..	+	+	+	+	+	VP	−	VP	VP	T	+	−	+	+	VP
6. *Amaranthus gangeticus* ..															
(*a*) Root	+	−	−	−	T	+	−	+	+	−	+	+	−	−	+
(*b*) Stem	+	−	−	−	T	+	−	+	+	−	+	T	−	−	+
(*c*) Leaf	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+

Remarks: 1. *Citrus decumana*: Val. and L. appeared on hyds. No. of acid-labile bands 3.
2. *C. medica*: Try. and γ-AB. present; Glu. intensified on hyds.; No. of acid-labile bands 2 other than Asp.
3. *C. acida*: γ-AB. present; No. of acid-labile bands 3 other than Asp.
4. *C. aurantium*: Asp. and Glu. prominent in healthy but traces only in diseased.
5. *A. hortensis*: AA. and Glu. intensified on hyds.; No. of acid-labile bands 1.
6. *A. gangeticus*:

Root and Stem: Very few amino acids: a prominent blue band (acid-labile) between Cy. and AA.; No. of acid-labile bands 2; on hyds. 3 new bands appear.

TABLE I (*Continued*)

Family	Cy.	Orn.-Ly.	Asp.	Hi.	Arg.	Gly.-Se.-A.	HPr.	Glu.-Th.	α-Al.	Pr.	Ty.	Try.-γ-AB.	Me. Val.	Ph.-I.	L.-IL.
7. *Seshania grandiflora* ..	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
8. *Trigonella Fœnum-græcum*	T	T	+	T	T	+	−	+	+	−	T	VP	+	−	+
9. *Murraya kœnigii* ..	−	T	+	T	T	+	−	+	+	+	+	+	−	+	+
10. *Coriandrum sativum* ..	+	+	+	T	T	+	−	+	+	+	+	+	+	−	+
11. *Piper betel*	T	T	VP	T	T	+	−	+	+	+	T	+	+	+	+
12. *Mentha piperita* ..	−	−	+	−	−	+	−	+	+	+	−	+	+	−	+

Remarks: *S. grandiflora*: A new band between Glu. and AA. on hyds.; No. of acid-labile bands 3 other than Asp.

T. Fœnum-græcum: Unidentified yellow band above Hi.; No. of acid-labile bands 3. other than Asp. one in Try.; position being very prominent.

M. kœnigii: Unidentified band above Try.: Two new bands appear on acid hyds.; No. of acid-labile bands 1 other than Asp. and Try.

C. sativum Unidentified band between Glu. and Al.; No. of acid-labile bands 3 other than Asp.

P. Betel β-Al. and γ-AB. present; No. of acid-labile bands 2 other than Asp. and Try.

M. piperita: No. of acid-labile bands 1 other than Asp.

amino acids tyrosine, typtophan, methionine, valine and phenylalanine (fast running amino acids).

Only a few amino acids could be detected in the roots and stems of *Amaranthus*. There was an intense blue band occupying the position between cystine and aspartic acid in the chromatograms. This was later traced to the presence of a peptide which on acid hydrolysis intensified the bands of glycine and glutamic acid.

Although asparagine is found to be of common occurrence in leaves, *P. Betel* and healthy orange leaves were conspicuous in that they contained unusually large concentrations of this important amide.

Of the edible leaves *Amaranthus, Sesbania* and *Coriandrum* contained the full complement of essential amino acids and can be recommended as supplements to a 'poor South Indian diet'.

It should be mentioned, however, that in addition to the widely known amino acids, there were several unidentified ninhydrin-positive compounds, some of them presumably peptides (or new amides ?) in almost all the leaves studied. As some of the unknown compounds occur in very high concentrations it is likely that they may be metabolically active. The prominent unidentified acid-labile bands in *Trigonella* and in *P. radiatus* are worthy of notice.

The free amino acid pattern of various leguminous seeds is given in Table II and the chromatograms of *D. lablab* and *P. radiatus* are shown in Figs. 4, 5 and 6. On germination of the seeds there was a rapid increase in the concentration of all the free amino acids.

The unusual increase in the concentration of α-alanine in *Dolichos lablab* on germination is striking. In the ripening seed fast-running amino acids like leucine, methionine, tryptophan and α-alanine occur only in traces whereas the glutamic acid band is conspicuous both in the resting and the ripening seeds. There is also a remarkable increase in the concentration of arginine in the germinated seeds of field bean, green gram, horse-gram and bengal-gram. It has been reported by Vaidyanathan and Giri (1953) that arginase activity of field beans was enhanced by nearly 50% on germination. With the increase in concentration of arginine and the simultaneous enhancement of arginase activity, the germinated seed would indeed be a seat of vigorous metabolic activity.

The leguminous seeds are in general deficient in the sulphur-containing amino acids (methionine and cystine). This is particularly true in the case of green-gram.

The results of the detailed investigations on the free amino acids and amides in green-gram and their quantitative determination are presented next.

Two-dimensional chromatography (Table III) of the alcoholic extract of the resting seeds of green-gram revealed the presence of lysine, aspartic acid, glutamic acid, glycine, serine, β-alanine, α-alanine, tyrosine, valine and leucine. Circular paper chromatography on large size papers (35 cm. diameter) confirmed that the

TABLE II

Free amino acids in Indian pulses

Family	Cy.	Orn.-Ly.	Asp.	Hi.	Arg.	Gly.-Se.-AA.	HPr.	Glu.-Th.	α-Al.	Pr.	Ty.	Try.-γ-AB.	Me.-Val.	Ph.Al.	L.-IL.
1. *Dolichos lablab* (Field-bean)															
(*a*) Ripening	–	+	T	+	+	T	–	VP	T	–	T	T	T	–	T
(*b*) Resting	–	+	T	+	+	+	–	VP	+	–	+	+	+	–	+
(*c*) Germinated (24 hrs.)	+	+	T	+	+	+	–	+	VP	T	+	+	+	T	+
2. *Dolichos biflorus* (Horse-gram)															
Germinated (24 hrs.) ..	–	–	T	+	+	+	–	+	VP	–	+	+	+	+	+
3. *P. radiatus* (Green-gram)															
Germinated (24 hrs.) ..	–	T	T	+	+	+	–	+	VP	T	+	VP	+	+	+
4. *Cicer arietinum* (Bengal-gram)															
(*a*) Resting	T	T	T	+	+	VP	–	VP	T	T	T	VP	T	–	T
(*b*) Germinated (24 hrs.)	T	+	–	+	VP	VP	–	+	VP	T	T	VP	+	T	+

TABLE III

Free amino acids of Phaseolus radiatus *at various stages of germination*
(*Two-dimensional chromatography*; *solvents*: n-*butanol-acetic acid-water*, 4 : 1 : 5 *and Pyridine-water* 8 : 2)

	Lys.	AA.	Glu.	Gly.	Se.	β-Al.	α-Al.	Pr.	Tyr.	Val.	L+Ph. Al.	Asp.	γ-AB	Unidentified I	II	III	IV
(*a*) 0 *Hour* (Resting seeds)																	
Aq.	+	+	+	+	+	+	+	+	+	+	+			+	−	+	−
Hyd.	±	+	++	+	++	++	+	+	+	+	+			−	+	−	−
(*b*) 6 *Hours*																	
Aq.	+	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+	+
Hyd.	±	++	++	+	+	+	++	+	+	+	+	−	+	−	+	−	−
(*c*) 12 *Hours*																	
Aq.	+	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+	+
Hyd.	±	+	++	+	+	+	+	+	+	+	+	−	+	+	+	−	−
(*d*) 24 *Hours*																	
Aq.	+	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+	+
Hyd.	±	++	++	+	+	+	+	+	+	+	+	−	+	+	+	−	−
(*e*) 48 *Hours*																	
Aq.	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	−
Hyd.	±	++	++	+	+	+	++	+	+	+	+	−	+	−	+	−	−
(*f*) 72 *Hours*																	
Aq.	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	−
Hyd.	±	++	++	+	+	+	+	+	+	+	+	−	+	−	+	−	−
(*g*) 96 *Hours*																	
Aq.	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	−
Hyd.	±	++	++	+	+	+	++	+	+	+	+	−	+	−	+	−	−

Remarks: Unidentified.
I. Peptide overlapping with Arg. in butanol solvent; it is practically absent after 96 hours germination. No. 4 in Fig. 5
II. 'Yellow spot' (No. 16 in Fig. 6) appearing on hydrolysis of the alcoholic extract.
III. Peptide overlapping with Lys. in two-dimensional chromatograms (No. 1 in Fig. 6).
IV. Peptide overlapping with AA. in two-dimensional chromatograms.

resting seeds of green-gram contain a surprisingly full range of free-amino acids (Radhakrishnan and Vaidyanathan, 1954). This is, however, at variance with the findings of Ganguli (1954) who could detect only five amino acids. Traces of γ-aminobutyric acid also could be detected in the dormant seed extract. This is in conformity with the observation of several workers with respect to its ubiquitous distribution in the plant kingdom (Steward and Thompson, 1954). Evidence was also obtained to show the presence of two peptides in fairly high concentrations. On hydrolysis of the alcoholic extract, glutamic acid and β-alanine bands were intensified. On the chromatogram of the hydrolysate, a new yellow spot was clearly visible.

There was a marked increase in the concentration of γ-aminobutyric acid after 6 hours germination.

Upto 24 hours of germination there was no perceptible change in the qualitative pattern of amino acids.

Asparagine made its appearance only in the 48-hour old seedlings. As the germination of the seed was carried out in the diffused light, this observation bears out the view of Damodaran *et al.* (1946) that the formation of asparagine is a normal process in the germinating seedling and not the result of etiolation. One of the peptides was considerably reduced in concentration after 72 hours germination and was practically absent in the 96-hour old seedlings. The other peptide overlapping with lysine was, however, present throughout. The preferential destruction of only one of the peptides during germination cannot be explained.

It was found that the hydrolysis of the alcoholic extract of the seed during any stage of germination invariably gave rise to β-alanine and the 'yellow spot'. There were only traces of β-alanine in the unhydrolysed extracts. No attempt was made to estimate its concentration. The origin of β-alanine is, however, not clear. It is usually suggested that it may be a decarboxylation product of aspartic acid (Steward and Thompson, 1950). This is unlikely since aspartic acid decarboxylase has so far not been detected in plants. The fact that there is no increase in the free β-alanine content on germination rules out the possibility that it arises from the peptide which disappears after 96-hours germination. The observation that the concentration of β-alanine increases on hydrolysis of the alcoholic extract suggests that it may form part of a peptide molecule. This must be confirmed by the isolation and characterization of this peptide. It is also probable that this amino acid may be formed by the hydrolysis of pantothenic acid which might be present in the alcoholic extract.

The identity of the 'yellow spot' which appeared on hydrolysis is not yet established. Although proline, hydroxyproline and presumably other imino acids react with ninhydrin to give a red or yellow colour, no definite conclusion can be drawn regarding the nature of the unknown compound merely on the basis of the yellowish-brown colour produced with ninhydrin (Steward and Thompson, 1950).

TABLE IV

Distribution of free amino acids in the roots, stem, cotyledons and leaves of Phaseolus radiatus
(*Period of germination*: 120 *hours*)

	Lys. + Pep.	AA.	Glu.	Gly.	Se.	β-Al.	α-Al.	Pr.	Tyr.	Val.	L+Ph. Al.	Asp.	γ-AB	Unidentified I	II	III
(*a*) *Roots*																
Aq.	+	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+
Hyd.	±	++	++	+	+	+	++	+	+	+	+	+	−	+	−	−
(*b*) *Stem*																
Aq.	+	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+
Hyd.	±	++	+	+	+	+	+	+	+	+	+	+	−	+	−	−
(*c*) *Cotyledons*																
Aq.	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Hyd.	±	++	++	+	+	++	+	+	+	+	+	+	−	+	−	−
(*d*) *Leaves*																
Aq.	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−
Hyd.	−	++	++	+	+	++	++	+	+	+	+	+	−	+	−	−

Ramarks: Unidentified.
I. Peptide overlapping with Arg. in butanol solvent (No. 4 in Fig. 5).
II. 'Yellow spot' (No. 16 in Fig. 6) appearing on acid hydrolysis.
III. Peptide overlapping with Lys.

The distribution of amino acids in the roots, stem, cotyledons and leaves of 120-hour old seedlings was studied (Table IV). The roots and stem contained very high concentrations of asparagine. γ-Aminobutyric acid was present in the roots, cotyledons and leaves but only traces were detected in the stem. While the cotyledons and leaves contained free β-alanine this was absent in the roots and stem.

The cotyledons at 120 hours germination contained lysine, aspartic acid, glutamic acid, asparagine, glycine, serine, α-alanine, β-alanine, γ-aminobutyric acid, tyrosine, valine, leucine and probably phenylalanine. The recent statement of Ganguli (1955), *viz.*, "but after 72 hours, except aspartic acid and glutamic acids all the other remaining amino acids have disappeared and practically no amino acid is detectable in the cotyledons with further progress in germination" is not in conformity with our observation.

Except in *Santalum album* (Radhakrishnan and Giri, 1954), hydroxyproline has not been detected in the free state in any of the plant species examined.

The results of the quantitative estimation of amino acids in green-gram during various stages of germination are given in Tables V and VI. Asparagine and arginine were not estimated as separation of the two could not be achieved owing

TABLE V

Free amino acid composition of Phaseolus radiatus *(milligram amino acid per gram dry weight of the seeds)*

Amino acid	Period of germination (hours) 0	24	48	96
1. α-Alanine	0·39	1·06	2·02	3·06
2. γ-Aminobutyric acid ..	..	2·89	2·83	3·47
3. Aspartic acid ..	1·04	1·14	4·05	5·56
4. Glutamic acid ..	0·99	1·56	4·37	2·92
5. Glycine	0·29	0·76	..	..
6. Histidine	..	..	3·57	3·61
7. Isoleucine	0·24	0·53	1·30	5·14
8. Leucine	0·23	0·53	1·13	3·20
9. Phenylalanine ..	0·44	1·22	2·27	5·98
10. Serine	0·26	0·65	2·27	3·75
11. Threonine	0·49	0·82	1·54	2·78
12. Tryptophan	0·68	1·14	..	..
13. Tyrosine	0·51	0·87	1·70	2·47
14. Valine	0·38	0·72	1·78	6·95
Nitrogen in aqueous layer employed for chromatography (mg./ml. Total Soluble N)	0·36	0·46	0·89	1·48

TABLE VI

Free amino acid composition of Phaseolus radiatus (*amino acid nitrogen as percentage of the total soluble nitrogen*)

Amino acid	Period of germination (hours) 0	24	48	96
1. α-Alanine	2·85	4·78	4·38	2·36
2. γ-Aminobutyric acid ..	..	11·19	5·39	2·30
3. Aspartic acid ..	5·07	3·48	5·96	2·91
4. Glutamic acid ..	4·38	4·24	5·73	1·35
5. Glycine	2·50	4·02	..	..
6. Histidine	..	..	1·35	4·73
7. Isoleucine	1·18	1·63	1·91	2·70
8. Leucine	1·11	1·63	1·69	1·68
9. Phenylalanine ..	1·74	2·94	2·70	2·40
10. Serine	1·60	2·50	4·16	2·43
11. Threonine ..	2·71	2·83	2·47	1·62
12. Tryptophan	4·31	4·46	..	..
13. Tyrosine	1·81	1·96	1·80	1·28
14. Valine	2·08	2·50	2·92	4·05
Nitrogen in aqueous layer employed for chromatography (mg./ml. Total Soluble N) ..	0·36	0·46	0·89	1·48

to the exceedingly high concentrations of the former. Lysine also could not be determined because of the overlapping of the lysine band with that of an unidentified peptide.

In Table V, the concentration of each amino acid is expressed as milligrams per gram weight of the seeds. It was found that there was a rapid increase in the concentration of most of the free amino acids on germination. Histidine which occurred only in traces in the resting seed showed an abrupt increase after 48 hours of germination. Glycine and tryptophan tended to increase upto 24 hours after which their concentration was considerably decreased. The concentration of glutamic acid showed a downward trend after 48 hours. This was not unexpected especially because of the well-established fact that it acts as a precursor of other amino acids.

Although γ-aminobutyric acid was present only in traces in the resting seed, its concentration rose to a significant level after 24 hours germination. The origin of γ-aminobutyric acid in plants is not clear. As it occurs in fairly high concentrations in the growing tissues, it is natural to surmise that it may be an active intermediate in the nitrogen metabolism of the plant. It is generally presumed that it is a decarboxylation product of glutamic acid. Thompson *et al.* (1953) have, however, pointed out that there is no correlation between the glutamic acid

decarboxylase activity of a plant tissue (potato tuber) and its γ-aminobutyric acid content. A simultaneous recarboxylation of γ-aminobutyric acid was therefore suggested by these authors.

In order to assess the relative concentration of each amino acid in the seed extract, the nitrogen in each compound is expressed as a percentage of the total N (*i.e.*, non-protein N of the seedling) present in the aqueous layer for chromatography (Table VI). It was observed that the percentage of most of the amino acids decreased sharply after 48 hours germination. This is due to the phenomenal increase in the amide N (mostly asparagine). Histidine and valine, however, registered an increase in the percentage.

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Explanation of Plates

Fig. 1. Chromatogram showing the free amino acid composition of the leaves of: *Atriplex hortensis* (A), *Mentha piperita* (B) and *Trigonella Fœnum-græcum* (C)—Refer Table I (suffix Hy. refers to the extracts after acid hydrolysis).

Fig. 2. Chromatogram showing the free amino acid composition of: the leaf (A), stem (B) and roots (C) of *Amaranthus gangeticus* and the leaves of *Murraya kœnigii* (D)—Refer Table I (suffix Hy. refers to the extract after acid hydrolysis).

FIG. 3. Chromatogram showing the free amino acid composition of: the leaves of *Piper betel* (1, 2), *Coriandrum sativum* (3, 4) and *Sesbania grandiflora* (5, 6)—Refer Table I (2, 4 and 6 refer to the extracts after acid hydrolysis).

FIG. 4. Circular Paper Chromatogram (mixed) showing the free amino acid composition of the germinated seeds of *Dolichos lablab* (field bean)—Refer Table II (Paper dia. 35 cm., solvent: *n*-butanol-acetic acid-water, 4 : 1 : 5 ; multiple development).

FIG. 5. Two-dimensional chromatogram (10" × 10" showing the free amino acid composition of the resting seeds of *Phaseolus radiatus* (green gram)—Refer Table III.

(1) Lys. + peptide, (2) AA., (3) Glu., (4) Peptide, (5) Gly., (6) Se., (7) β-Al., (8) α–Al., (9) Pr., (10) Tyr., (11) Val., (12) L.+PhAl.,

FIG. 6. Two-dimensional chromatogram of the hydrolysate of the alcoholic extract of cotyledons of green-gram at 120 hours germination—Refer Table IV.

(1) Lys., (2) AA., (3) Glu., (4) Unidentified. (5) Asp. (present in alcoholic extract), (6) Gly., (7) Unidentified (present in alcoholic extract), (8) Se., (9) α-Al., (10) β-Al., (11) γ-AB., (12) Tyr., (13) Unidentified, (14) Val., (15) L+PhAl., (16) Yellow spot, (17) Unidentified.

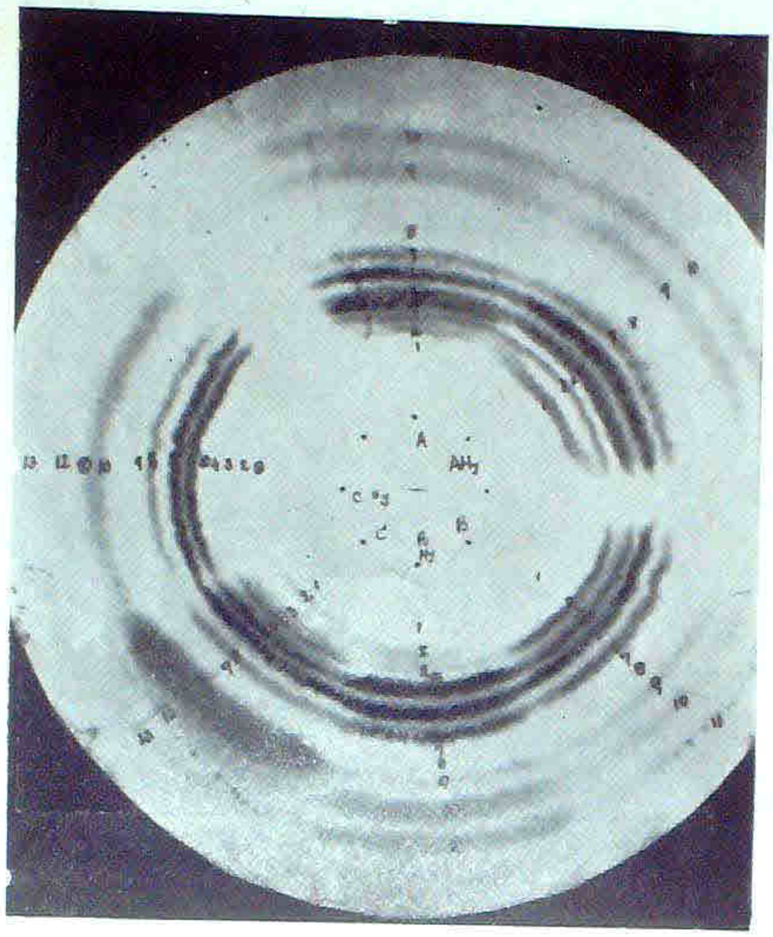

FIG. 1

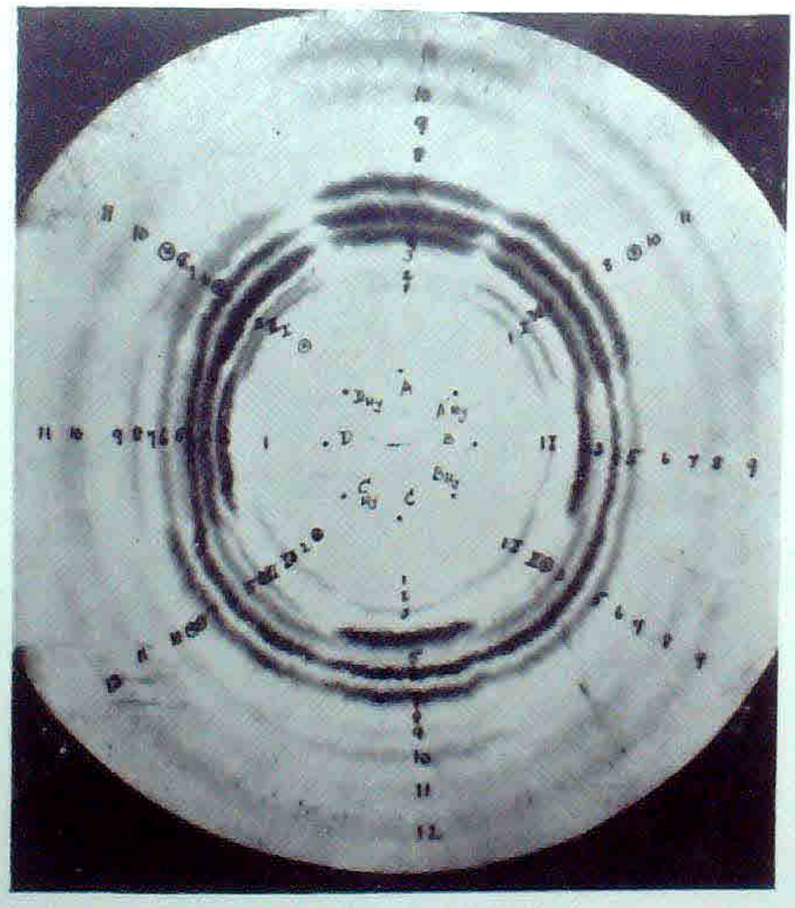

FIG. 2

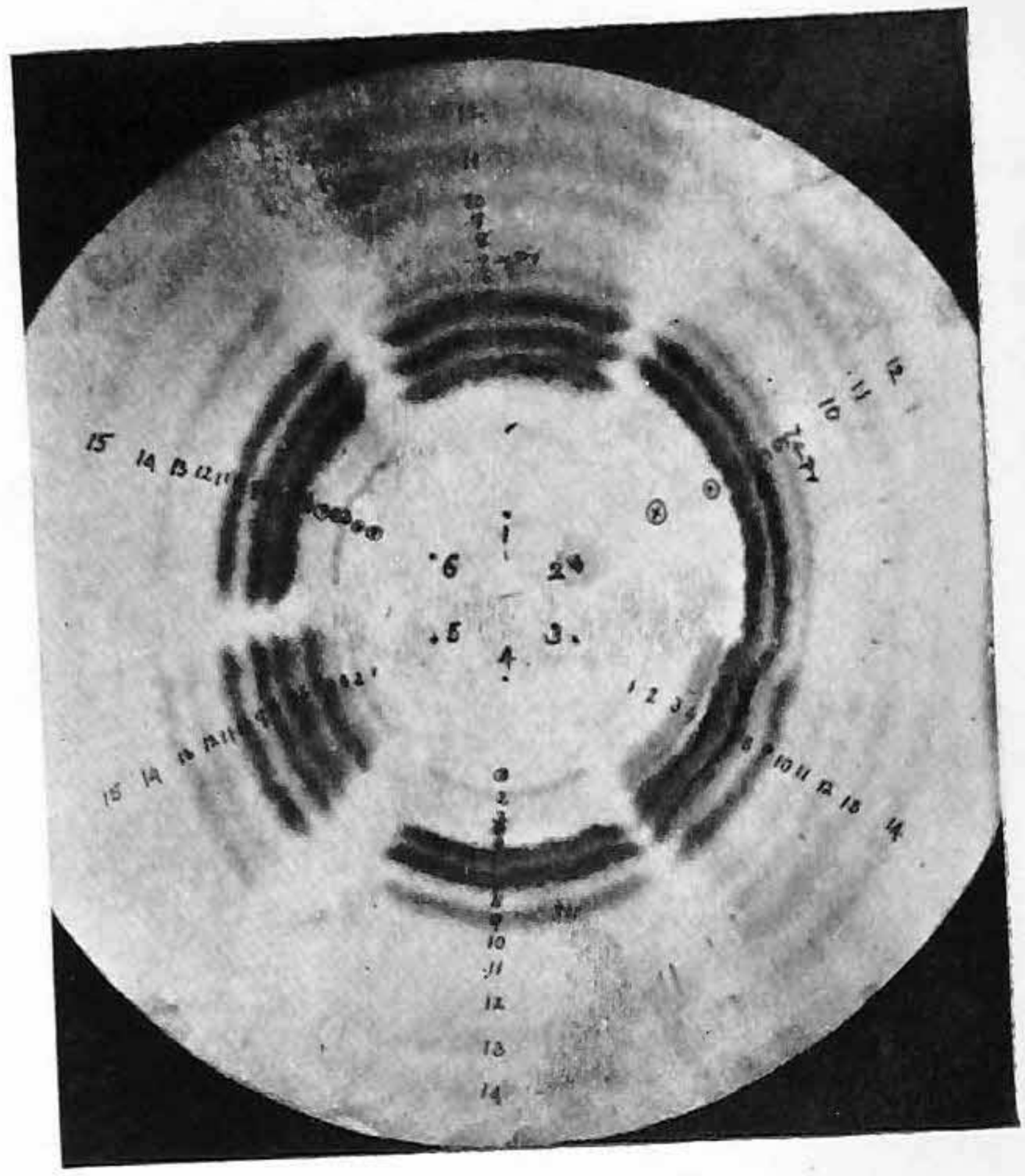

FIG. 3

FIG. 4

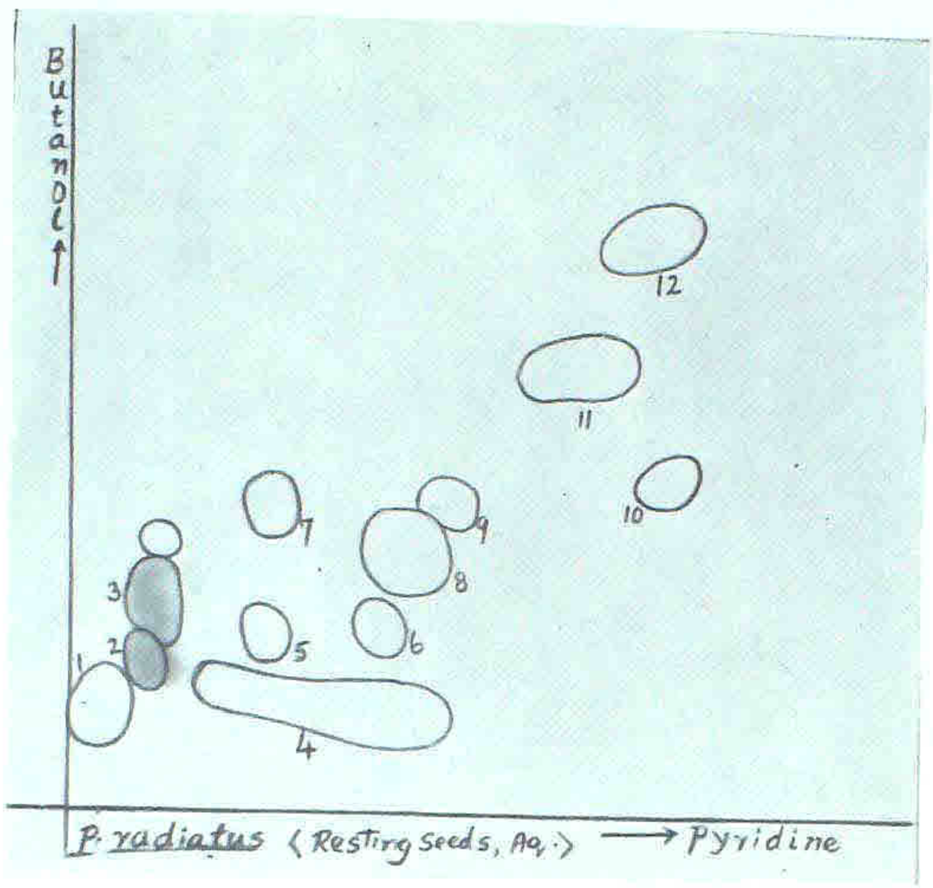
Butanol
12
11
10
7
9
8
3
2
5
6
1
4
P. radiatus (Resting seeds, Aq.) → Pyridine

FIG. 5

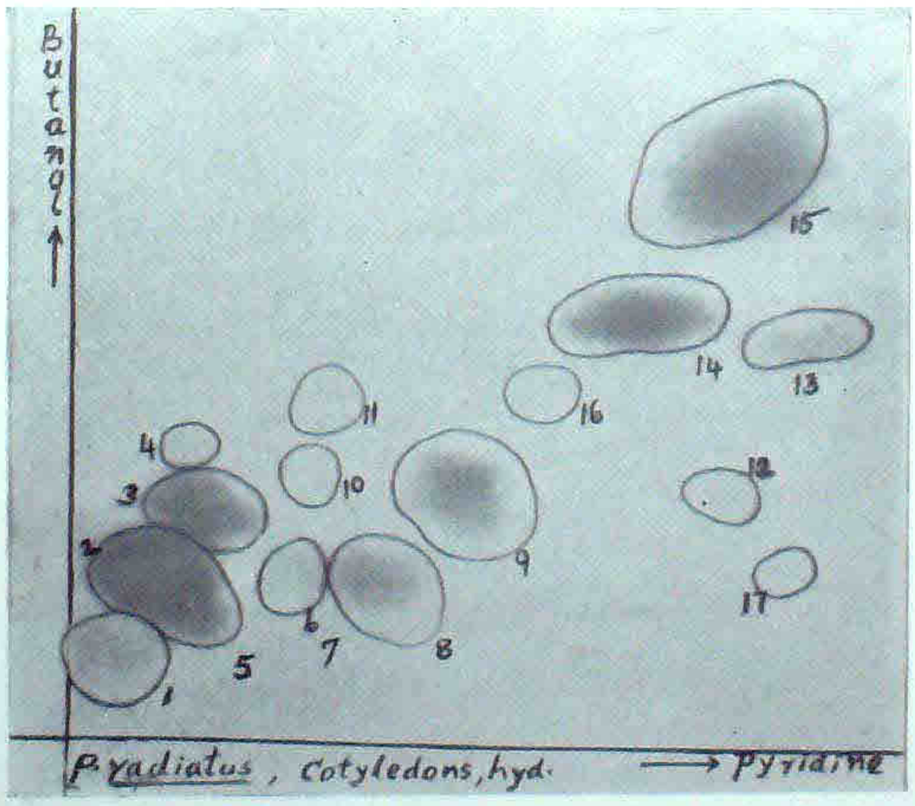
Butanol
15
14
13
16
11
4
10
12
3
9
2
17
6
7
8
5
1
P. radiatus, Cotyledons, hyd. → Pyridine

FIG. 6