CIRCULAR PAPER CHROMATOGRAPHY

Part VII. A Technique for the Separation and Estimation of "Overlapping Amino Acids"

(With three figures)

BY K. V. GIRI AND N. A. N. RAO

(Department of Biochemistry, Indian Institute of Science)

Received July 20, 1953

SUMMARY

A technique is described for the separation of amino acids which overlap when developed with *n*-butanol-acetic acid-water as solvent by circular paper chromatography employing other solvent mixtures.

The separation of the overlapping amino acids is accomplished by cutting out the bands relating to these amino acids which separate as single band in *n*-Butanol-acetic acid-water as solvent and using the cut strip as 'wick' for developing another chromatogram with pyridine-water, pyridine-amylacetate-water and pyridine-isoamylalcohol-water as solvents.

The following groups of amino acids have been separated by using the above solvents:—Leucine-isoleucine; Valine-methionine; γ-Amino-butyric acid—tryptophane; α-Aminobutyric acid—tyrosine; β-Alanine—proline; Glutamic acid—threonine; Glycine—serine—aspartic acid; Asparagine—arginine and Lysine—histidine.

The technique and apparatus used for simultaneous elution and spotting of the amino acids contained in the cut out strip are described.

A quantitative procedure for the estimation of these amino acids has been outlined. The method has been applied to the amino acid analysis of casein and edestin hydrolysates.

In principle the technique can also be applied to the analysis of other substances.

In a previous paper Giri *et al.* (1953) have described a method for quantitative analysis of amino acids by means of circular paper chromatography. The mixture of amino acids, which is obtained when proteins are

---

* The term 'overlapping amino acids' is used for those amino acids which overlap each other and separate as a single band on the chromatogram run with *n*-butanol-acetic acid water (40 : 10 : 50) as solvent mixture.

hydrolysed by acids, is difficult to separate all of them into individual bands for quantitative estimation, by using one single solvent mixture. The use of *n*-butanol-acetic acid-water as solvent mixture separates some of the amino acids present in the hydrolysate. The use of other solvents, which can separate other amino acids into individual bands, is required for evolving a method for the complete quantitative analysis of all the amino acids present in protein hydrolysates. A technique for the separation of such overlapping* amino acids is described here which can lead to the separation, identification and quantitative estimation of all the amino acids present in acid hydrolysates of proteins. The method described includes the use of *n*-butanol-acetic acid-water as the first solvent mixture to separate some of the amino acids into individual bands, which can be estimated quantitatively by the method described before (Giri *et al.*, 1953), followed by the technique of cutting out the bands relating to the amino acids which overlap each other and using the cut strip as ' wick ' for development of another chromatogram, using another solvent in which these overlapping amino acids separate as individual bands. A method for the quantitative estimation of the overlapping amino acids by the technique of cutting out the band and simultaneous elution and spotting of the amino acid mixture on a separate chromatogram run with a different solvent in which the amino acids separated into individual bands, is also described.

The basis of this method has been reported briefly elsewhere (Giri and Rao, 1953).

EXPERIMENTAL

The procedure adopted for running the chromatogram is exactly similar to that described before (Giri and Rao, 1952). All chromatograms for quantitative estimations are run on Whatman No. 1 paper of 24 or 36 cm. diameter.

The amino acids used in the present investigation are pure samples obtained from Messrs. Nutritional Biochemical Corporation.

Solvent mixtures for the separation of the overlapping amino acids

In previous publications *n*-butanol-acetic acid-water was used as solvent for the separation of amino acids present in protein hydrolysates. This solvent is very useful for the separation of many amino acids in the hydrolysates into individual bands. However, some of the amino acids overlap each other in this solvent and separate as single bands. Several solvents have been tried for the separation of the overlapping amino acids. The solvent mixtures employed successfully for the separation of the over-

lapping amino acids include pyridine-water (80 : 20), pyridine- iso-amyl-alcohol-water (10 : 10 : 7), and pyridine-amylacetate-water (10 : 5 : 5). Table I gives the position of the amino acids on the chromatogram in various solvents. The R_f values are only intended as a guide to the relative position of these amino acids, as these values vary slightly from time to time. For purposes of comparison the R_f values in *n*-butanol-acetic acid-water have been included (Tara Rao and Giri, 1952).

TABLE I

R_f values of amino acids in various solvent mixtures

Diameter of paper used is 24 cm. Distance travelled by the solvent: 10 cm.

Sl. No.	Amino acids	*n*-Butanol acetic acid-water (40 : 10 : 50)	Pyridine-water (80 : 20)	Pyridine-iso-amyl alcohol-water (10 : 10 : 7)	Pyridine amyl-acetate water (10 : 5 : 5)
1	Cystine	0·20	0·28	0·24	0·20
2	Lysine	0·28	0·22	0·20	0·18
3	Histidine	0·28	0·42	0·40	0·31
4	Asparagine	0·32	0·31	0·36	0·31
5.	Arginine	0·32	0·25	0·22	0·20
6	Aspartic acid ..	0·37	0·31	0·30	0·22
7	Glycine	0·37	0·44	0·45	0·33
8	Serine	0·36	0·51	0·45	0·36
9	Hydroxy proline ..	..	0·56	0·55	0·40
10	Threonine	0·40	0·60	0·57	0·44
11	Glutamic acid ..	0·40	0·37	0·36	0·22
12	Alanine	0·45	0·58	0·57	0·40
13	Proline	0·48	0·61	0·61	0·45
14	β-Alanine	..	0·48	0·48	..
15	α-Amino butyric acid ..	0·57	0·66	0·66	0·54
16	Tyrosine	0·57	0·76	0·77	0·70
17	γ-Amino butyric acid ..	0·69	0·46	0·59	0·38
18	Tryptophan	0·69	0·79	0·79	0·71
19	Valine	0·72	0·74	0·71	0·64
20	Methionine	0·72	0·75	0·76	0·71
21	Phenyl alanine ..	0·75	0·81	0·79	0·75
22	Iso-leucine	0·79	0·80	0·80	0·75
23	Leucine	0·79	0·81	0·85	0·80

It can be seen from the R_f values of amino acids in the solvents investigated the overlapping amino acids can be separated into individual bands by using other solvent mixtures.

Preparation of solvent mixtures

(1) n-*Butanol-acetic acid-water* (40 : 10 : 50).—This solvent was prepared as described in Part I of the series.

(2) *Pyridine-water* (80 : 20).—Prepared by mixing 80 parts of pyridine with 20 parts of water. This is kept for 10 minutes at room temperature before use.

(3) *Pyridine-iso amyl alcohol-water* (10 : 10 : 7).—Prepared by mixing 10 parts of pyridine with 10 parts of iso amyl alcohol and 7 parts of water. It is shaken well and kept for 10 minutes at room temperature and used.

(4) *Pyridine-amyl acetate-water* (10 : 5 : 5).—Mixed in the above proportions, kept for 10 minutes and used.

Procedure for the separation and identification of the overlapping amino acids

On the circumference of a circle (about 4 cm. diameter) drawn at the centre of a filter paper (24 cm. diameter) six equi-distant points are marked with a pencil. At the alternate points the test samples are spotted, usually not more than 10 μl. of the protein hydrolysate. At the other points known mixture of overlapping amino acids are spotted, leaving a space of about 5 mm. between the spots. The chromatogram after development with *n*-butanol-acetic acid-water, is dried at room temperature, and divided into sectors (usually six) by drawing lines with a pencil, each sector enclosing the bands of the separated amino acids from the solution spotted on the sector (Fig. 1). The sectors (M) containing the mixture of known amino acids are cut out, without completely detaching them from the chromatogram. This can be achieved by leaving a short distance from the centre of the paper without cutting. These sectors are treated with ninhydrin and used as guide sectors for marking the position of the bands on the untreated sector (HY). The bands are then cut and used as ' wick ' for developing another chromatogram using the following solvents in which the overlapping amino acids separate into individual bands.

A typical chromatogram showing the separation of the five amino acids —aspartic acid, glutamic acid, glycine, serine and threonine into individual bands by the above method, using pyridine-water as the solvent is shown in Fig. 2. These amino acids separate into two bands, each containing glutamic acid-threonine and aspartic acid-serine-glycine respectively on the chromatogram run with *n*-butanol-acetic-acid. The two bands can be cut and used as ' wick ' together for development of another chromatogram.

Pyridine-water (80 : 20)

Group	Amino acids	Colour
1	Lysine	Violet
	Histidine	Light brown
2	Arginine	Violet
	Asparagine	Brown
3	Aspartic acid	Green
	Glycine	Tan
	Serine	Pink
4	Glutamic acid	Bluish violet
	Threonine	Violet
5	Proline	Yellow
	β-Alanine	Green
6	Tyrosine	Bluish violet
	α-Amino butyric acid ..	Bluish violet
7	Tryptophane	Bluish violet
	γ-amino butyric acid ..	Violet

Pyridine-Isoamylalcohol-water (10 : 10 : 7)
or
Pyridine amylacetate-Water (10 : 5 : 5)

8	Methionine	Violet
	Valine	Violet
9	Leucine	Violet
	Iso-leucine	Bluish violet

Quantitative estimation of the overlapping amino acids

For quantitative estimation of the overlapping amino acids, the following procedure can be adopted:

A chromatogram is run according to the procedure described above for the separation and identification of the overlapping amino acids. The bands relating to the overlapping amino acids are located, marked and cut into strips. The cut strips are then trimmed at one end into a pointed shape. The amino acids present in the strips are eluted according to the procedure described by Consden *et al.* (1947) with the following modification for simultaneous elution and spotting on a separate circular filter paper.

Method of eluting the material from the cut strips and spotting on the paper

A schematic diagram of the apparatus used for eluting the material from the cut strips and spotting on another circular paper is shown in Fig. 3.

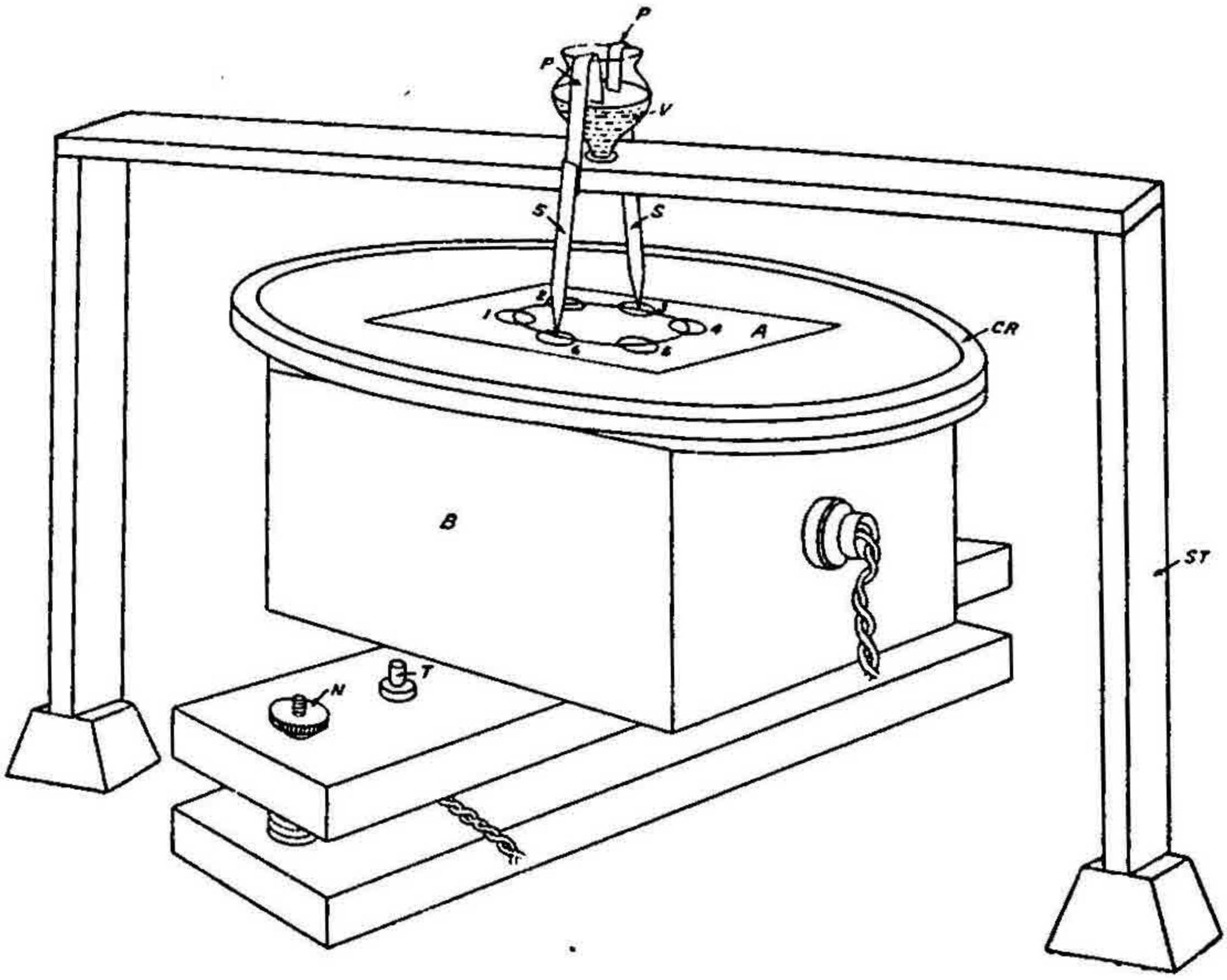

FIG. 3

A container with water (V) is kept on a stand (St) made of perspex. A strip of paper (P) is cut to the same width as the cut strip from the chromatogram and long enough (about 2″) to accommodate the cut strip in such a way that the pointed end of the strip is just above the paper on which the material is to be spotted. One end of this strip is immersed in water in the container, the other end hanging outside the container. To elute the cut strip (S) the end opposite to the pointed end of the strip is allowed to overlap the hanging edge of the wet strip immersed in the water container as indicated in Fig. 3. The water creeps down the cut strip slowly carrying the amino acids with it. The level of water in the container is so adjusted as to avoid rapid capillary siphoning and at the same time to facilitate steady but slow movement of water down the strip. After some time (about 30 minutes) when the amino acids are carried down and concentrated at the pointed tip of the strip, the latter is allowed to touch a point on the circumference of a circle drawn at the centre of a circular filter paper

(A) resting on a circular platform (CR) over a box (B) which is lighted with a 40 watt. electric bulb. The amino acids are slowly transferred and spotted on the paper. When the spot is about ½ cm. in diameter the platform (CR) is slowly lowered by turning the knob (N), thereby breaking the contact between the strip and the paper. After the spot is dried at room temperature, the process is repeated two or three times depending on the concentration of the amino acids to be eluted. Finally it is desirable to allow small amount of water to creep down the edge of the paper strip by means of capillary pipette, to facilitate complete transfer of the amino acids on the paper. About 1–2 hours are more than sufficient for the elution and spotting. In order to ensure that the amino acids are completely eluted from the strip, the latter is dried and treated with ninhydrin reagent. The lighted box is mainly intended to observe and control the spreading of the spot. Two cut strips can be used at a time and spotted at points diametrically opposite to each other as indicated in Fig. 3.

Instead of raising and lowering the platform for spotting, it will be more convenient to have a pinion arrangement for the stand supporting the water container to move the latter up and down, for spotting the materia on the paper, keeping the platform stationary.

After the elution and spotting are completed, standard mixtures of known amino acids are spotted on the chromatogram and developed with the solvents in which the amino acids separate into individual bands. The procedure for quantitative estimation of the amino acids separated on the paper by measuring the colour intensity of the bands on the chromatogram treated with ninhydrin is exactly similar to that described by Giri *et al.* (1953).

Analysis of casein and edestin

To demonstrate the applicability of this technique to the analysis of proteins, casein and edestin have been analysed. 100 mg. of protein are weighed into a small tube with 3 c.c. of 6 normal HCl. The tube is sealed and placed in an autoclave for about 15 hours. After the hydrolysis is completed the tube is opened and the HCl is removed by evaporation in vacuum and dried in a desiccator. Finally, water is added to make up the volume to 10 c.c. 10 μl. containing about 15·7 microgram of nitrogen is used for spotting. Standard mixtures of amino acids are prepared and spotted along with the protein hydrolysate on the same paper. The chromatogram is run first with *n*-butanol-acetic acid-water and the amino acids—cystine, lysine, histidine, arginine, alanine, proline, tyrosine, phenyl-alanine, which separate into individual bands are estimated (Giri *et al.*, 1953). The other amino acids which overlap and separate as one band in

the solvent are separated by the cutting out technique and estimated by running another chromatogram using the other solvent mixtures mentioned before.

In Tables II and III are presented the results of the analysis of casein and edestin and compared with published data.

TABLE II

Percentage composition of the amino acids in casein hydrolysate

Sl. No.	Amino acids	Values cited by Gordon	Values obtained by Gordon	Values obtained by Thompson and Steward, 1951	Values compiled by Block and Bolling, 1951	Values obtained by circular paper chromatography
1	Cystine ..	0·34	0·34	..	0·36	0·85
2	Aspartic acid	7·1	7·2	6·9	6·3	6·9
3	Glutamic acid	22·4	22·0	22·6	22·8	22·9
4	Serine ..	6·3	5·9	5·4	7·5	5·76
5	Glycine ..	2·7	1·9	2·3	0·5	2·9
6	Threonine ..	4·9	4·6	4·4	3·9	4·46
7	Alanine ..	3·0	3·5	3·1	5·6	3·1
8	Lysine ..	8·2	8·3	8·2	6·9	7·6
9	Arginine ..	4·1	4·0	3·8	4·1	4·0
10	Histidine ..	3·1	3·2	..	2·5	3·1
11	Proline ..	11·3	11·6	12·9	8·2	11·73*
12	Valine ..	7·2	7·2	6·9	7·0	6·65
13	Methionine ..	2·8	3·1	2·7	3·5	2·6
14	Phenyl alanine	5·0	5·5	6·8	5·2	7·0
15	Tryptophan ..	1·2	1·2	..	1·8	..
16	Tyrosine ..	6·3	6·2	5·7	6·4	6·4
17	Leucine ..				12·1	12·44
18	Iso-leucine ..	..	..	..	6·5	5·6
	(Leucines) ..	15·3	17·9	15·2	18·6	18·02
TOTAL NITROGEN ..		111·24	113·54	106·0	115·6	111·47

* Value for proline was taken from the unpublished work of K. V. Giri and Nagabhushanam. The proline was determined by 1 : 2 Naphthoquinone : 4 : sulphonate reagent.

TABLE III

Percentage composition of the amino acids in edestin hydrolysate

Sl. No.	Amino acids	Values obtained by					
		Block and Bolling (1951)	Tristram 1949	Henderson and Snell (1948)	Cohn and Edsall (1943)	Thompson and Steward (1952)	Circular paper chromatography Giri and Rao (1953)
1	Cystine ..	1·3	1·4	..	1·4	0·8	2·6
2	Aspartic acid	12·0	12·0	13·4	12·0	13·2	12·98
3	Glutamic acid	20·7	20·7	19·4	20·7	23·2	15·9
4	Serine ..	6·3	6·3	..	6·3	5·8	5·51
5	Glycine ..	5·1	..	..	3·8	5·1	4·55
6	Threonine ..	3·8	3·8	3·7	..	3·9	5·46
7	Alanine ..	5·5	4·3	..	3·6	5·4	3·5
8	Histidine ..	2·9	2·9	2·6	2·4	..	2·8
9	Lysine ..	2·7	2·4	2·1	2·4	2·0	2·8
10	Arginine ..	16·6	16·7	17·4	16·8	14·8	14·4
11	Methionine ..	2·3	2·4	4·6	4·1	2·9	2·2
12	Proline ..	4·6	4·2	2·1	2·4	1·0	..
13	Valine ..	6·3	5·7	6·6	5·6	5·5	5·34
14	Phenylalanine	5·8	5·4	5·2	3·1	4·7	4·6
15	Tryptophan ..	1·5	1·5	0·9	1·5	..	..
16	Tyrosine ..	4·5	4·3	3·7	4·5	4·7	3·64
17	Leucine ..	7·7	4·7	7·5	..	..	6·45
18	Iso-leucine ..	4·7	7·5	6·5	..	..	5·54
	(Leucines) ..	12·4	12·2	14·0	20·9	14·0	12·8
	TOTAL NITROGEN ..	114·3	108·5	95·7	107·5	107·0	111·77

The percentage recovery of the overlapping amino acids added to the protein hydrolysates ranges between 96 to 106. In the case of methionine, however, somewhat lower recovery (86%) was obtained.

The standard curves of the amino acids after chromatography in the three solvents—pyridine-water; pyridine-iso-amyl alcohol-water, pyridine-

amyl-acetate water show linearity between the concentration of the amino acids and the optical density in the range 2 μ g.–15 μ g.

The reproducibility of the results has been tested by carrying out estimations on different days. The agreement between the replicates of three determinations of the amino acids has been found to be very satisfactory, the percentage error being within 5%.

DISCUSSION

It can be seen from the results that the separation of all the amino acids present in protein hydrolysates can be accomplished by the application of circular paper chromatographic method, using different solvents, without recourse to the more elaborate two-dimensional technique. Pyridine-water has proved to be a very useful solvent for the separation of many of the pairs of amino acids which cannot be separated by *n*-butanol-acetic-acid water.

From the R_f values given in Table I, it can be seen that the amino acids with close or similar R_f values in *n*-butanol-acetic acid-water, show wide variations in their R_f values in other solvents. These differences in R_f values of the amino acids in the solvents investigated provide the basis for the separation of all the amino acids present in protein hydrolysates. Furthermore, the solvent mixtures—*n*-butanol-acetic acid-water and pyridine-water has been found to be very useful in obtaining clear separation of all the amino acids by two-dimensional technique, especially for the detection of γ-amino butyric acid and β-alanine with their characteristic positions on the chromatogram. But, for quantitative estimations, circular paper chromatographic method is simpler and more convenient than the two-dimensional technique.

Certain general comments may be made regarding the values obtained for the amino acid composition of casein and edestin by this technique compared to other published data. It may be seen that the values obtained for most of the amino acids are in good agreement with other published values. The values obtained for cystine in both casein and edestin are higher than those reported by others. The glutamic acid content of edestin is found to be lower than the reported values.

The results obtained on the analysis of casein and edestin show that the technique can be applied to the quantitative amino acid analysis of proteins as well as to the quantitative determination of amino acids in plant and animal tissues. It is apparent that complete analysis of proteins can be made by circular paper chromatographic technique which can also be successfully applied to the quantitative determination of other substances.

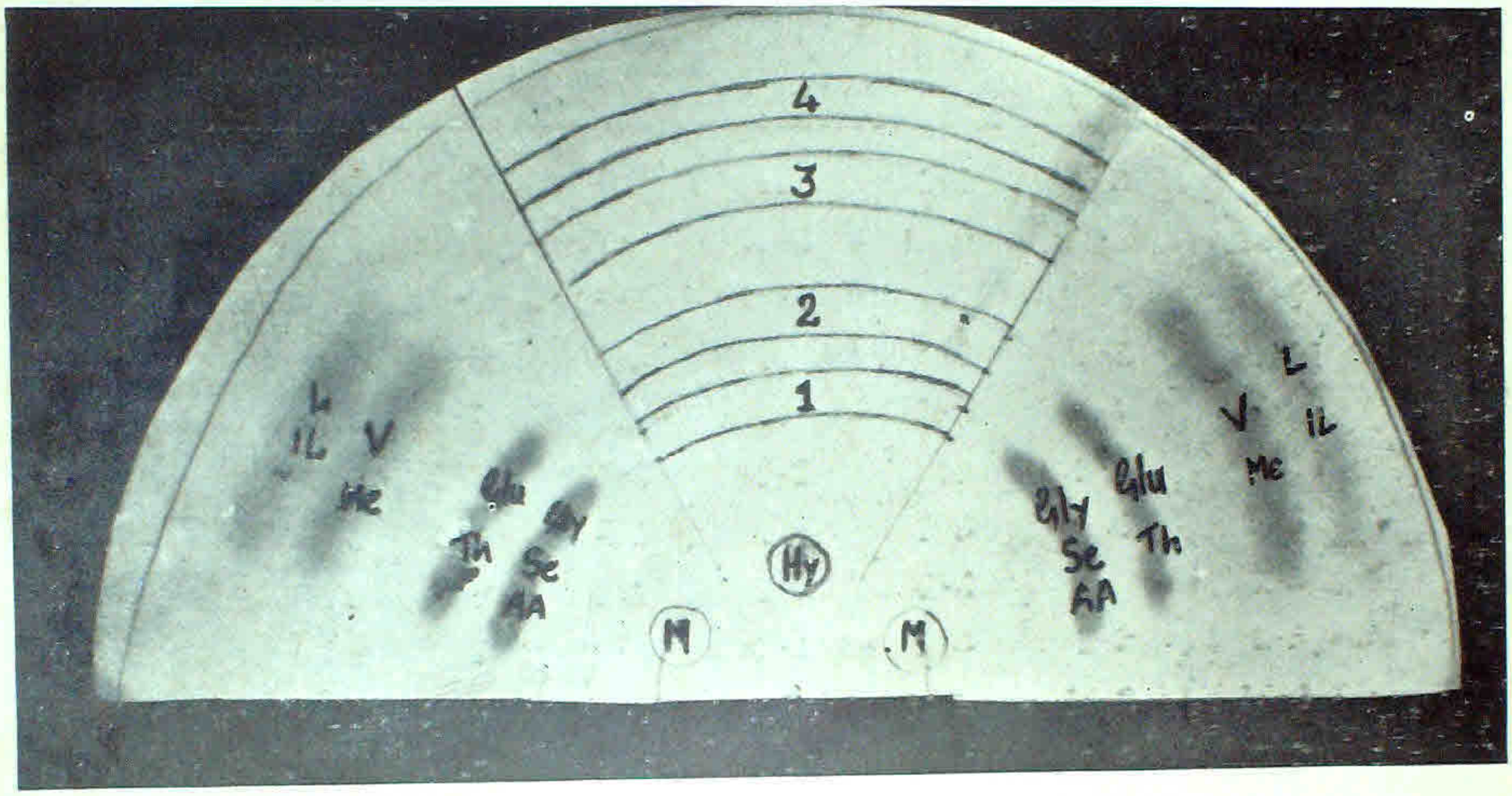

Fig. 1. Diagram showing method of cutting of the chromatogram for the separation and identification of the overlapping amino acids

Hy—Casein hydrolysate; M—Mixture of known amino acids; 1, 2, 3, 4—The bands relating to the overlapping amino acids

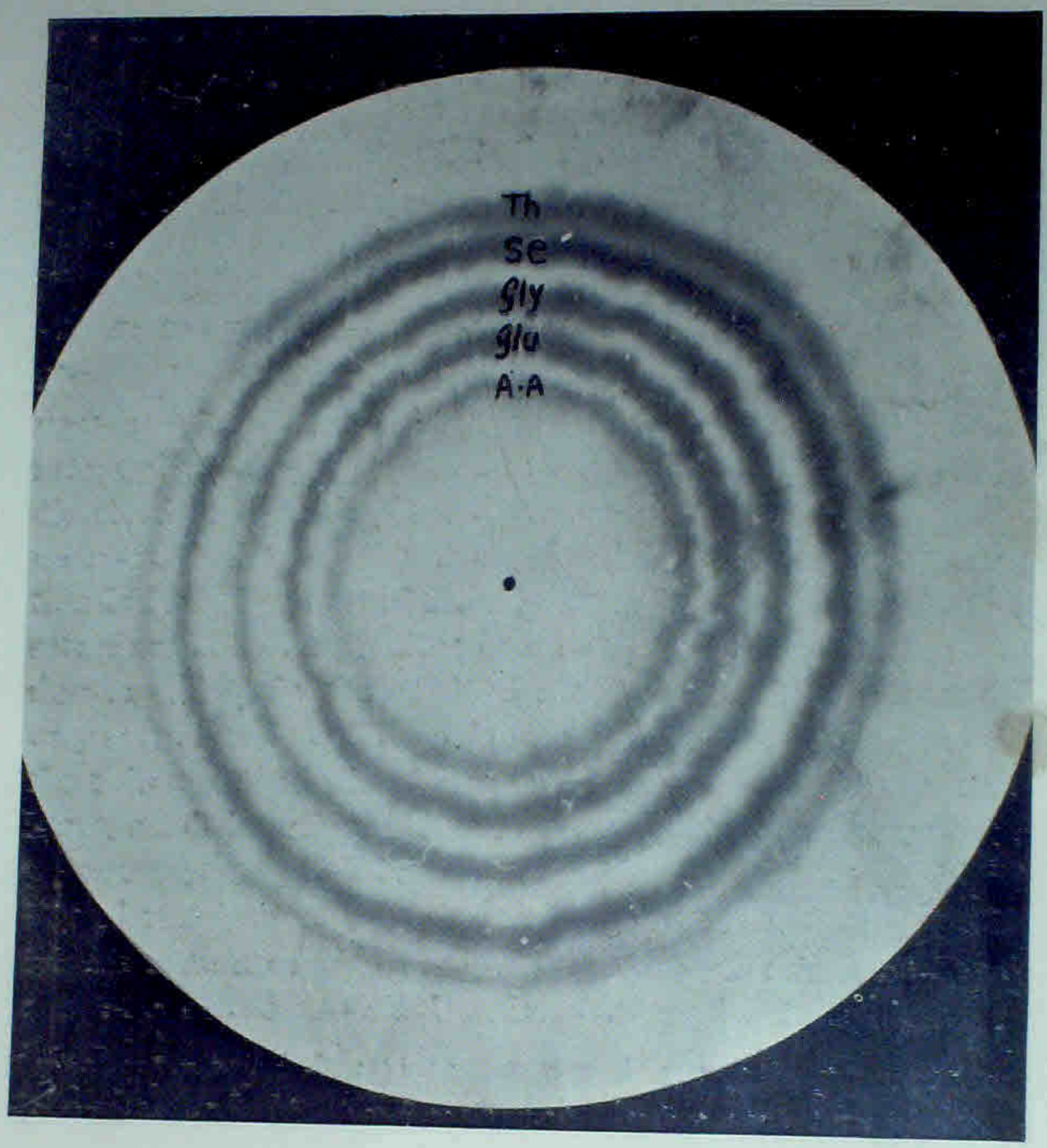

FIG. 2. Chromatogram showing the separation of overlapping amino acids (Nos 1 and 2, Fig. 1) by cutting out technique

A.A—Aspartic acid
Glu—Glutamic acid
Gly—Glycine
Se—Serine
Th—Threonine

} Single band separation

Note added in proof :—

While correcting this proof, a note by A. M. Moore and J. B. Boylen (*Science*, 1953, **118**, 19) appeared in which the authors describe a simple method for making transfers in paper chromatography, which is somewhat similar to the technique described in this paper and reported earlier in *Current Science* (1953). The authors, however, improved a devise for carrying out elution in a chamber, so that the air inside the chamber is nearly saturated with water vapour and for evaporating the elute on the paper as rapidly as it flows by directing a stream of air against the underside of the paper on which the solution is spotted. They have also suggested a method to determine the time necessary to complete the elution by using a marker.

REFERENCES

1. Block, R. J. and Bolling, D. .. *The Amino Acid Composition of Proteins and Food.* C. C. Thomas, Spring Field, Illinois, Second Edition, 1951, First Printing.
2. Cohn, E. J. and Edsall, J. T. .. *Protein, Amino Acids and Peptides*, Reinhold Publication Corporation, New York, 1943, p. 358.
3. Consden, R., Gordon A. H. and Martin A. J. P. *Biochem. J.*, 1947, **41**, 590.
4. Giri, K. V. and Rao, N. A. N. .. *J. Ind. Inst. Sci.*, 1952, **34**, No. 2, 95; *Nature*, 1952, **169**, 923.
5. ———, Radhakrishnan, A. N. and Vaidyanathan, C. S. *Ibid.*, 1953, **35**, No. 2, 145; *Anal. Chem.*, 1952, **24**, 1677.
6. ———, and Rao N. A. N. .. *Curr. Sci.*, 1953, **22**, 114.
7. ———, and Nagabhushanam, A. .. (Unpublished paper).
8. Gordon, W. G. Semmett, W. F., Cable, R. S. and Morries, M. *J. Amer. Chem. Soc.*, 1949, **71**, 3293.
9. Henderson, L. M. and Snell, E. F. .. *J. Biol. Chem.*, 1948, **172**, 15–27.
10. (Miss) Tara Rao and Giri, K. V. .. *J. Ind. Inst. Sci.*, 1953, **35**, No. 1, 77.
11. Thompson, J. F. and Steward, F. C. *J. Expt. Botany*, 1952, **3**, No. 8, 170.
12. ———— .. *Plant Physiol.*, 1951, **26**, 421–40.
13. Tristram. G. R. .. *Advances in Protein Chemistry*, 1949, **5**, 83–153.