CIRCULAR PAPER CHROMATOGRAPHY

Part II. Amino Acid Analysis of Proteins

By K. V. Giri, K. Krishnamurthy and T. A. Venkitasubramanyan
(Department of Biochemistry, Indian Institute of Science, Bangalore-3)

Summary

Circular paper chromatographic technique has been applied to the study of the amino acid composition of proteins.

Casein, gelatin, zein, silk fibroin, egg albumin, rice and cambu proteins have been subjected to chromatographic analysis and the chromatographic patterns showing the amino acid composition of these proteins have been given.

By the application of this technique it is shown that proteins can be easily characterised and differentiated with respect to their amino acid composition.

The possible application of this technique to the study of other allied problems is discussed.

In Part I (Giri and Rao, 1952), a rapid and simple method for the separation and identification of amino acids by circular paper chromatographic technique has been described. In this part of the investigation, the application of this technique to the qualitative analysis of the amino acids of protein hydrolysates will be considered. A brief description of the technique and its application for separation and identification of amino acids from casein hydrolysate has been reported in an earlier publication (Giri, *et al.*, 1952). It is the purpose of this paper to give further details concerning the separation and identification of the amino acids in a number of proteins using the above technique, to show how proteins can be readily characterised by even visual comparison of the chromatograms of the protein hydrolysates, to present chromatographic patterns of some protein hydrolysates and to discuss the possible application of this technique to other allied problems. It is shown that by this technique proteins can be characterised sharply enough to provide an adequate method for detecting chemical differences between proteins from different sources.

Experimental

The basic procedure involved in the separation of amino acids from the protein hydrolysates by circular paper chromatographic technique was

exactly the same as described by Giri and Rao (1952), which has proved its value in the course of routine amino acid analysis in this laboratory.

Whatman No. 1 Circular filter-papers (18 to 24 cm. diameter) and *n*-butanol-acetic acid-water as solvent were used throughout this investigation.

The following proteins were used for the chromatographic studies:—

1. *Casein* (according to Hammersten).

2. *Gelatin* (B.D.H.).

3. *Silk fibroin.*—The pure Japanese silk cocoons (Japanese multivoltine, n 112) were broken open and the silkworms were removed. The empty cocoons were boiled in water for nearly half an hour several times until the water was no more coloured. Thus sericin was removed. The pure silk fibroin was filtered and washed free from the coloured solution. The fibroin was dried in an oven at 40° C. Silk fibroin, manufactured by Nutritional Biochemical Corporation Inc., was used for obtaining the complete circular chromatogram (Fig. 7). A sample of fibroin from a cross-bred (Mysore × *C. nichii*) was also used in this investigation.

4. *Zein* (Corn protein).—(Merck).

5. *Rice protein* (Glutenin).—The method of preparation was the same as that described by Tadokoro (1926).

6. *Egg albumin.*—(B.D.H.).

7. *Whole egg protein.*—Fresh eggs were broken and stirred into five times its volume of acetone. The mixture was shaken for half an hour and filtered off. The same operation was carried out four times. The residue was washed thrice with five times its weight of ether and filtered off. The residue was dried in a desicator at low pressure.

8. *Protein from cambu* (Pennisetum typhoideum).—Prepared in the same way as the protein from rice.

HYDROLYSIS OF PROTEINS FOR AMINO ACID ANALYSIS

Hydrolysis of the proteins was accomplished with 6N HCl (25 c.c. per gram of sample) autoclaved at 15 lb. pressure for 6 hours. After removing the acid in the usual manner, the solution was diluted to a suitable volume. A portion of this solution was removed for total nitrogen determination by the micro-Kjeldahl procedure. The volume of the final solution in all cases was so adjusted that it contained 5 mg. nitrogen per c.c.

Procedure for Carrying out Chromatographic Analysis

The technique used was essentially the same as that described before. The hydrolysate was spotted at the centre of a circular filter-paper or on a circumference of a circle drawn from the centre as the case may be and developed using *n*-butanol-acetic acid-water as solvent. After drying, the paper was sprayed with 0·1% ninhydrin solution in acetone and dried at 60° C. for 15–20 minutes.

Typical chromatograms of casein, gelatin, silk fibroin, zein, egg albumin and whole egg protein are shown in Figs. 1 to 8.

The abbreviations given in the chromatograms indicating the bands relating to the amino acids are as follows: Cy, Cystine; Ly, Lysine; Hi, Histidine; Ar, Arginine; Gly, Glycine; Se, Serine; H.Pr, Hydroxy Proline; Glu, Glutamic acid; Th, Threonine; Al, Alanine; Pr, Proline; Ty, Tyrosine; Me, Methionine; V, Valine; Ph, Phenylalanine; L, Leucine; I-L, Iso Leucine.

Mixed Chromatograms.—Mixed chromatogram of the hydrolysates of the proteins, casein, gelatin, egg albumin, silk fibroin, zein, and cambu protein is shown in Fig. 8. A comparison of the amino acid patterns of these hydrolysates shows the difference in amino acid composition of the proteins. The procedure adopted for obtaining the mixed chromatograms was similar to the one described in Part I of this series of publications.

Identification of Amino Acid Bands

The amino acid bands in the chromatograms can be identified by their position and by specific tests. Cystine, lysine, histidine, arginine, glycine-serine band, alanine, proline, hydroxyproline, tyrosine, tryptophan (absent in acid-hydrolysis), phenylalanine, valine-methionine band, leucine-isoleucine band can all be unequivocally identified when *n*-butanol-acetic acid-water is used as solvent. The identification of the overlapping amino acids such as methionine, threonine, serine, histidine and arginine can be confirmed by specific colour tests and reactions. The following is the order in which the amino acids present in the protein hydrolysates separate into distinct bands, starting first with outermost band (leucine-isoleucine). The methods used for the identification of the bands by means of specific colour tests and reactions are given.

1. *Leucine-isoleucine Band.*—These two amino acids do not resolve into two distinct bands and they are not, therefore, differentiated. These amino acids are present in very low concentration in gelatin and silk fibroin

and in high concentration in zein, egg albumin and c sein, as can be seen from the mixed chromatogram (Fig. 8).

2. *Phenylalanine.*—This amino acid is separated into a distinct band immediately below leucine-isoleucine band. This band can also be distinguished from the adjoining bands by its bluish-violet colour, while the adjoining bands are coloured violet. This amino acid is present in all the proteins. It is present in zein and egg albumin in highest concentration, while in silk fibroin it occurs in somewhat lower concentration. This is clearly shown in the chromatograms.

3. *Valine-methionine.*—These two amino acids always overlap each other and take the position immediately below the phenylalanine band. Methionine in presence of valine can be identified by brushing the area of the band with the platinic-iodide reagent described by Toennies and Kolb (1951). The presence of methionine is indicated by the appearance of bleached area (pale yellow) with a pink background.

Methionine can be oxidised quantitatively to methionine-sulphoxide by means of hydrogen peroxide. Thus when the hydrolysate was treated with hydrogen peroxide and developed in the usual way, the intensity of the combined band of methionine and valine, decreases, if methionine is present in the hydrolysate, and the serine-glycine band increases in intensity because the methionine sulphoxide band overlaps that combined band.

The colour intensity of the band in the chromatograms of silk fibroin, gelatin and zein is less than that of the corresponding band in the chromatograms of casein and egg albumin, thereby indicating the occurrence of these two amino acids in lower concentration in silk fibroin, gelatin and zein.

4. *Tryptophan.*—As this amino acid is destroyed by acid hydrolysis of proteins, the tryptophan band which takes the position below methionine-valine band, if present, does not appear in the chromatograms.

5. *Tyrosine.*—The band next to methionine-valine band in the chromatograms is that of tyrosine which can easily be identified by its bluish-violet colour resembling that of phenylalanine. Further tyrosine band is nearer to proline than to valine-methionine band and the space between the tyrosine and methionine-valine bands will be occupied by the tryptophan band, if present. The identification of tyrosine by specific colour test is discussed under histidine. The absence of visible tyrosine band in the gelatin chromatogram shows that the concentration of this amino acid in this protein is very low compared to other proteins.

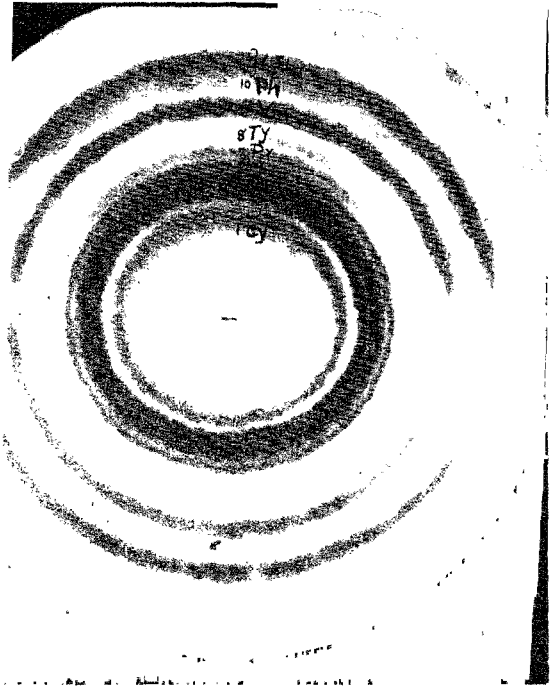


FIG. 1. Circular paper chromatogram of casein hydrolysate. (Solution containing 120 μ gr. of nitrogen spotted at the centre.)

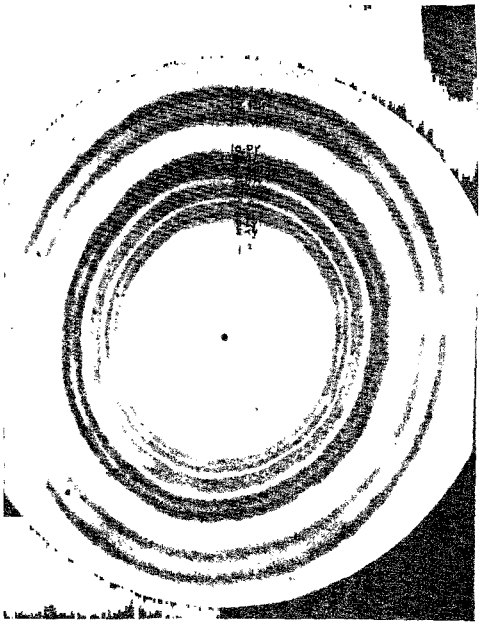


FIG. 2. Circular paper chromatogram of gelatin hydrolysate. (Solution containing 120 μ gr. of nitrogen spotted at the centre.)

FIG. 3. Circular paper chromatogram of egg albumin hydrolysate. (Solution containing 120 μ gr. of nitrogen spotted at the centre.)

FIG. 4. Circular paper chromatogram of whole egg protein hydrolysate. (Solution containing 120 μ gr. of nitrogen spotted at the centre.)

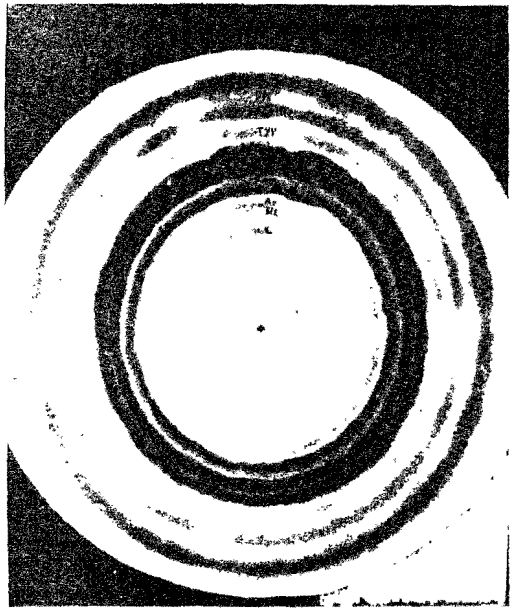


FIG. 5. Circular paper chromatogram of Zein hydrolysate. (Solution containing 120 μ gr. of nitrogen spotted at the centre.)

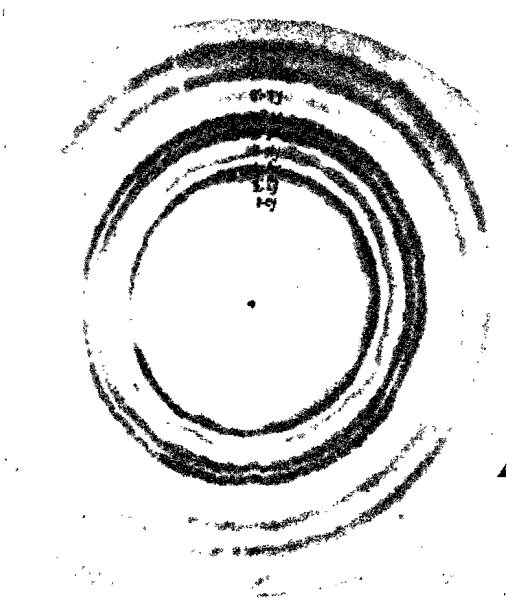
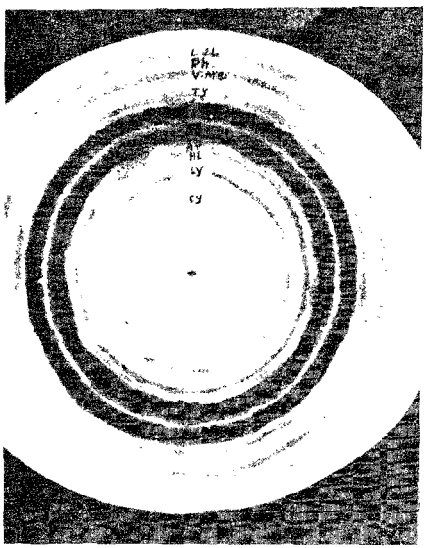


FIG. 6. Circular paper chromatogram of rice glutenin hydrolysate. (Solution containing 120 μ gr. of nitrogen spotted at the centre.)



G. 7. Circular paper chromatogram of silk fibroin hydro. (Solution containing 120 μ gr. of nitrogen spotted at the)

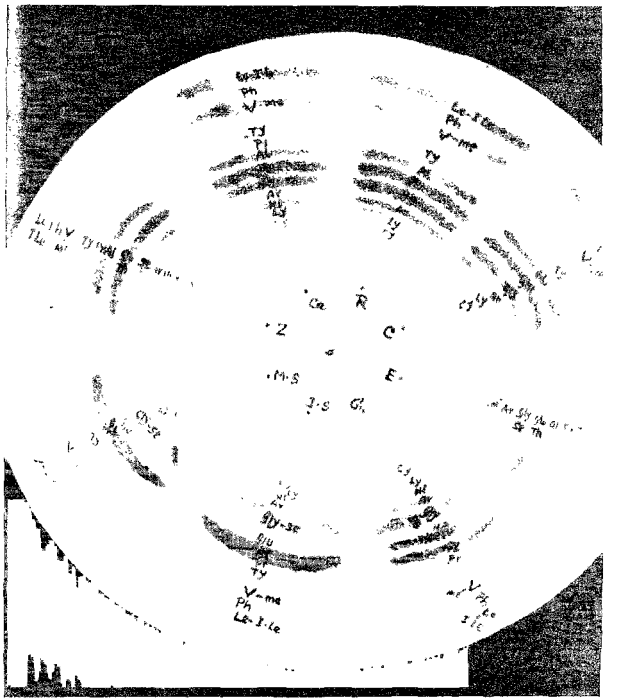


FIG. 8. Circular mixed chromatogram of the hydrolysates of different proteins. *Ca*, Casein, *R*, Rice glutenin, *C*, Cambu glutenin, *E*, Whole egg protein, *G*, Gelatin, *J. S.*, Japanese silk, *M. S.*, Muscle silk, and

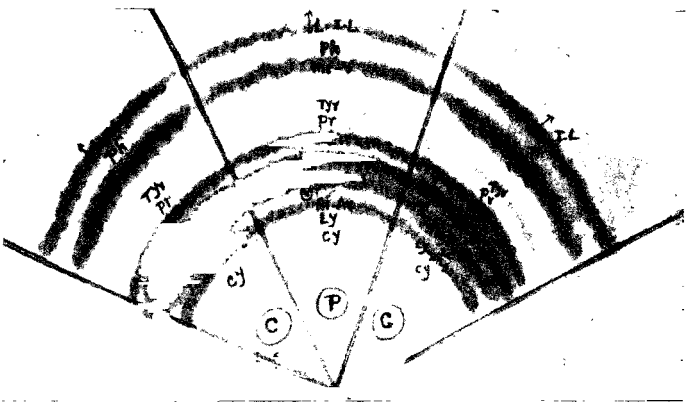


FIG. 9. Circular paper chromatogram of casein hydrolysate showing the disappearance of Threonine and Serine on per-iodate treatment.

C., Casein hydrolysate without any treatment. *P.*, Casein hydrolysate after treatment with per-iodate.

6. *Proline.*—The presence of this amino acid in proteins can be easily identified by its characteristic yellow colour. Proline occupies the position immediately below the tyrosine band. This band being yellow in colour is not clearly visible in the photograph. However, the presence of this amino acid will be clearly seen by its characteristic yellow colour on the chromatograms.

7. *Alanine.*—Alanine takes the position immediately below the proline band. The greater intensity of colour and the greater width of the alanine band of silk fibroin hydrolysate compared to those of other protein hydrolysates indicate the occurrence of this amino acid in very high concentration in silk fibroin, confirming the results obtained by other well-known methods.

8. *Threonine-glutamic acid.*—These two amino acids always overlap each other in all the chromatograms of the proteins. This band occurs below the alanine band. Threonine is oxidised quantitatively by periodic acid. This reaction is used for the identification of this amino acid. The presence of threonine can be identified by subjecting the protein hydrolysate to periodate oxidation and running the chromatogram simultaneously with the untreated sample. As threonine is oxidised by periodic acid to glyoxalic acid, acetaldehyde and ammonia, the intensity of the colour of the band not only decreases but also a clear space above the band will be visible owing to the absence of this amino acid in the treated sample. This is clearly shown in Fig. 9.

Threonine can also be identified by the method suggested by Synge (*cf.* Block, 1950). The method consists in preparing Nessler's reagent and adding it to solid periodic acid until the precipitate first formed is just dissolved. The resulting solution can be used to identify both serine and threonine, which liberates ammonia when treated with periodate and the ammonia thus liberated produces colour with the Nessler's reagent.

The glutamic acid-threonine band is very prominent in all the chromatograms of the proteins investigated, with the exception of silk fibroin chromatogram, in which the band is very feeble, thereby indicating that the glutamic acid content of silk fibroin is very low compared to other proteins, as determined by the chromatographic method. This is in conformity with the analytical data obtained by other methods of analysis.

9. *Hydroxyproline.*—This amino acid occurs in gelatin hydrolysate. It takes the position immediately below threonine-glutamic acid band and just above the glycine-serine band. It can be readily identified by its yellow colour. Gelatin is the only protein among those examined in the present investigation which contains this amino acid (Fig. 2).

10. *Glycine-serine-aspartic acid.*—These two amino acids which like glutamic acid-threonine, overlap each other, take the position below the threonine-glutamic acid band. It was, however, found that by multiple development and using larger size circular papers (35 cm. diameter), glycine and serine can be separated from one another, serine taking the position just below glycine. Serine in presence of glycine can be identified by periodate oxidation method and by treatment of the band with the Nessler's reagent in presence of periodic acid as described before for identification of threonine (Fig. 9).

It can be seen from Fig. 7 the glycine-serine band of silk fibroin is wider than the bands relating to these amino acids present in other protein hydrolysates investigated, thereby indicating the presence of these amino acids in highest concentration in this protein compared to other protein hydrolysates. Next to silk fibroin, gelatin hydrolysate also shows higher concentration of glycine compared to other proteins. The intensity of these bands in the chromatogram of these protein hydrolysates is also more than those of the other protein hydrolysates. In the figure, however, this marked difference in intensity of the bands is not clearly visible, as the intensity of light used when taking the photograph was not even throughout. It may, however, be mentioned that both the width of the bands and intensity are rough indications of the concentration of the amino acids present in the protein hydrolysates.

11. *Arginine.*—The arginine band takes the position immediately below that of glycine-serine. The identification of arginine band can be confirmed by the Sakaguchi test (The α-naphthol-hypochlorite reaction). The test can be carried out by the brushing technique described by Giri and Rao (1952). α-Naphthol reagent (0·1%) in normal sodium hydroxide solution should be applied to the band with a brush and after drying in air, the band is touched lightly with a brush dipped in sodium hypochlorite reagent. The appearance of red colour indicates the presence of arginine.

The width and intensity of colour of this band in the chromatograms of gelatin and rice protein hydrolysates are more than the corresponding bands in the chromatograms of other protein hydrolysates investigated, indicating thereby the occurrence of this amino acid in gelatin and rice protein hydrolysates in somewhat higher concentration compared to other protein hydrolysates.

12. *Histidine.*—This amino acid takes the position below the arginine band. It can be identified by the Pauli reaction. Freshly diazotised sulfanilamide in *n*-butanol is applied with a brush on the paper at the position

ccupied by the amino acid and after drying in air, saturated solution of odium carbonate is applied. By means of this reaction red coloured band haracteristic of histidine, will be formed. Tyrosine also gives this reaction Block, 1950). Recently Sanger and Tuppy (1951) have suggested a modifica- ion of the Pauli reaction which avoids the use of aqueous solutions and it hus affords a convenient test for identification of the amino acids, as the se of aqueous solutions makes the amino acid spread on the paper resulting n the diffused appearance of the colour. The reagent consists of a mixture f equal volumes of 1% (W/V) *p*-anisidine in alcoholic 0·11 N HCl and 0% (V/V) amylnitrite in alcohol. A few minutes after preparation, the eagent can be applied to the paper by means of a brush. After drying n air, the paper is exposed to ammonia vapour or alcoholic potash solu- on may be applied to the paper with a brush. The development of dark ed band indicates the presence of histidine. Tyrosine gives an orange oloured band.

13. *Lysine.*—Immediately below histidine is the lysine band which is omewhat diffused in appearance. The absence of lysine band in the hromatogram of zein hydrolysate and the presence of a very faint one in iat of the hydrolysate of silk fibroin and strong ones in that of the hydro- sates of casein and egg albumin are clearly shown in the mixed chromatogram f the protein hydrolysates (Fig. 8). These observations confirm the results btained by other analytical methods, on the occurrence of the amino acid n proteins in varying proportions.

14. *Cystine.*—Cystine occupies the lowest position in the chromato- ram, with a bluish-violet colour. It can be very readily identified by its osition on the chromatogram. It can also be identified by its bleaching ction when the paper is brushed with platinic iodide reagent, as described efore for the identification of methionine.

Discussion

The chromatograms of the proteins investigated show clear separation f the amino acids present in the hydrolysates into distinct bands each one epresenting one or two amino acids. Except for some pairs of amino acids aline-methionine, glutamic acid-threonine, and glycine-serine, which always verlap each other, all other amino acids can be easily identified by their espective positions in relation to other acids. Some of the bands of the low running amino acids (lysine, histidine and arginine) are often crowded ogether rendering the identification somewhat difficult. In such cases, multiple development technique will considerably improve the separation f these amino acids into distinct zones. Some of the overlapping amino

acids (methionine, serine, threonine, arginine, histidine) can be identified by the application of specific reactions characteristic of the amino acids, as described before. Serine and glycine which always overlap each other can also be identified by multiple development technique. After repeated development with a mixture of serine and glycine spotted on the same paper along with the protein hydrolysate, it was found that the serine band occupied a position just below the glycine band. These two amino acids can, therefore, be readily identified by running mixed chromatograms and applying the multiple development technique.

Leucine and isoleucine which overlap forming the outermost band in all the chromatograms are always considered together under the name "leucines". The absence of tryptophan band, which, if present, will appear between the tyrosine and valine bands, is due to its decomposition during acid hydrolysis of the proteins.

Although the visual comparison of the chromatograms of the proteins investigated has little absolute quantitative significance, it is, however, useful as rough indication of the major differences in the amino acid composition of the proteins. The method is undoubtedly rapid, simple and convenient for routine analysis and characterisation of proteins compared to other paper chromatographic techniques widely used at present. It is capable of detecting major differences in the amino acid composition of proteins. It is possible to get some rough idea of the amount of amino acid deficient in a particular protein from the position and intensity of the bands relative to others. Thus in Fig. 8, which is a reproduction of the photograph of the mixed chromatogram of the hydrolysates of various proteins, it becomes at once apparent that gelatin contains less tyrosine than casein, silk protein, rice protein and zein, and that silk protein is very low in leucines and other fast running amino acids compared to other proteins. The predominance of glycine, serine and alanine in silk protein is clearly seen in the chromatogram.

It is thus clear from the mixed chromatogram (Fig. 8) of the protein hydrolysates, that proteins can be characterised nearly sharply enough to provide an adequate method for detecting chemical differences between proteins from different sources. It is, therefore, ideally suited to the study of the changes in the amino acid composition of proteins, and their variation brought about by processing of foods, such as heat, irradiation, storage, cooking and enzymic digestion. The application of this technique to the investigation of these proolems is in progress.

REFERENCES

Block, R. J. .. *Food Industries*, 1950, **22**, 824.

Giri, K. V. and Rao, N. A. N. .. *J. Ind. Inst. of Sci.*, 1952, **34**, 95; *Nature*, 1952, **169**, 923.

——, Krishnamurthy, K. and Venkitasubramanyan, T. A. *Curr. Sci.*, 1952, **21**, 11.

Sanger, F. and Tuppy, H. .. *Biochem. J.*, 1951, **49**, 463.

Tadokoro, T. .. *Proc. Imp. Acad. of Japan*, 1926, **2**, 498.

Toennies, G. and Kolb, J. J. .. *Anal. Chem.*, 1951, **23**, 823.