CIRCULAR PAPER CHROMATOGRAPHY

I. A Technique for the Separation and Identification of Amino Acids

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(With 3 Figures)

Summary

1. A technique for the separation and identification of amino acids on partition chromatograms using circular filter-paper discs as the stationary phase is described. N-butanol—acetic acid—water has been used as the developing solvent.

2. The method of running mixed chromatograms for the identification of amino acids has been described.

3. Multiple development technique is found to be a very useful technique for achieving clear-cut separation of amino acids which do not readily separate into distinct bands by first development.

4. A scheme for the qualitative analysis of amino acids has been indicated.

5. The advantages of this technique over the other widely used paper chromatographic techniques have been discussed and the possibilities of the application of this technique in other fields of research are indicated.

In recent years with the advent of paper chromatography introduced by Consden, Gordon and Martin (1944), various adaptations of filter-paper to the separation and identification of amino acids have been reported. Rutter (1950) has described a method using circular filter-paper discs and indicated its application to the separation of dyes and inorganic substances. Although the method is very simple and convenient it could not be adapted, without considerable modification, to the separation and identification of amino acids by making use of mixed chromatograms with known amino acids run on the same paper, like the unidimensional chromatograms made by the "ascending" and "descending" techniques. Further, the separation of amino acids was not quite satisfactory when the paper was sandwiched between two glass plates as suggested by Rutter. Several modifications have to be introduced in order to make the method widely applicable to the separation of amino acids and other substances occurring in plant and animal tissues and biological fluids,

In previous publications (Giri, K. V., 1951; Giri, K. V. and N. A. N. Rao, 1952; Giri, K. V., Krishna Murthy, K. and Venkitasubramanian, T. A., 1952) new techniques were briefly described in which circular filter-papers served as the inert support for the separation and identification of amino acids. This technique has been termed as "Circular Paper Chromatography". It shows great promise in the field of separation and identification of amino acids, sugars, organic acids, vitamins, enzymes, proteins, antibiotics and other biological materials. The simplicity of apparatus, the speed with which the chromatograms can be run, reproducibility, sharpness of separation, compactness of apparatus and suitability for microanalysis are the special features of this technique, which is now in continuous use in this laboratory in the study of the amino acid metabolism in plants and animal tissues. The following account provides a more detailed description of the new techniques developed in this laboratory and the various modifications introduced from time to time in order to increase its applicability, reproducibility, efficiency and accuracy in the separation and identification of amino acids.

EXPERIMENTAL

Apparatus.—Various types of apparatus can be used from the equipment easily available in ordinary laboratories. The following description of the equipment required will serve as guide for constructing the apparatus.

The apparatus was simply assembled from two petri dishes. At the centre of one of the petri dishes was kept a small beaker containing the water saturated solvent. Another petri dish having the same diameter served as a cover. These are the essentials of the apparatus in its simplest form. The apparatus is shown in Fig. 1 *a*. It can accommodate circular filter-paper with a diameter of about 18 cm.

Alternatively, the petri dishes may be replaced by a bell jar or a desiccator with a small beaker containing the solvent kept inside, over which the paper rests.

For carrying out large size chromatograms (37·5 cm. diameter) it was convenient to construct the apparatus made of "Perspex"*. Fig. 1 *b* illustrates the essentials of the apparatus. It consists of a large basin with a flat bottom, covered with a glass plate. A beaker was kept at the centre of the basin and served as the container for the developing solvent. A circular ring made of "perspex" served as support for the paper. In order to saturate the atmosphere in the basin with respect to the solvent, a layer of the solvent was kept at the bottom of the basin in a petri dish or a beaker. A provision was also made where necessary, for the introduction of

* Made by Mysore Plexi Glass Co., Bangalore.

the solvent into the beaker kept inside for irrigation after the atmosphere was completely saturated with the vapour of the solvent by attaching a glass tube with a stopcock and funnel at the side of the basin as shown in Fig. 1 *b*.

Paper.—Whatman No. I filter-paper was used throughout this investigation. The circular filter-papers (having diameter of 15 cm. or more) were used in most of these investigations. For carrying out chromatograms on larger sheets of paper with diameter greater than 18 cm., the required size was cut out from large filter-paper sheets. All the papers were used as such without any treatment.

Solvent.—The success of chromatographic separation of amino acids depends largely on the proper choice of a suitable solvent. Although number of solvents like phenol, collidine, lutidine, normal butyl alcohol, tertiary butyl alcohol, mesityl-oxide, amylalcohol, *m*-cresol, etc., have been recommended, it was found that *n*-butanol-acetic acid-water mixture (40 : 10 : 50) by volume recommended by Partridge (1947) for sugars was found to be especially suitable for the separation of amino acids and the use of this solvent gave clear-cut separations on the paper. This solvent was, therefore, used throughout the present investigation.

The solvent was prepared by shaking four parts of *n*-butanol with one part of glacial acetic acid and five parts of water and rejecting the lower layer on separation, the upper layer being used as the solvent.

Procedure

Circular filter-papers (What. No. I) 15 cm. or more in diameter were used. The solution to be analysed was spotted at the centre of the filter-paper. Usually 5–10 μl. of the solution containing 5–10 μg. of each amino acid was applied at the centre of the paper from the tip of a capillary tube and dried at room temperature. A slit (about 0·5 cm. in length) was made at the centre of the filter-paper by making a short cut with a razor blade. A strip of filter-paper (0·5 cm. in width and about 4–5 cm. in length) was folded at the centre. The folded end was inserted into the slit keeping it in level with the adjacent surface of the paper. This served as a ' wick ' for irrigating the chromatogram with the solvent by capillary action. Alternately a strip of filter-paper rolled into the form of a ' wick ' (2–3 mm. thickness) and cut at the end into the form of a brush and inserting it into a hole (2–3 mm. diameter) made at the centre of the paper (Fig. 1 *a*) may be used for irrigating the paper with the solvent (Giri, *et al.*, 1952). The strip of filter-paper was kept perpendicular to the plane of the paper. The filter-paper with its ' wick ' was then placed on a suitable support in such a way that the ' wick ' was kept immersed in the developing solvent (*n*-butanol-

acetic acid-water; 40:10:10) contained in a small beaker or basin without the surface of the paper coming in contact with any other material. It was also found convenient to keep the paper taut by inserting the edge of the paper into a needle-work frame. The distance between the surface of the solvent and the paper was adjusted to about 1·0 cm. The whole set-up was kept in a large size petri dish covered with another of the same size, or under a bell jar or a desiccator, or in a specially designed chamber made of perspex (Fig. 1 *b*) and covered with a glass plate to make the atmosphere inside the chamber saturated with the vapour of the solvent.

The solvent was allowed to run a distance of about 8–10 cm. from the centre in 2–4 hours. The time taken for running larger size chromatograms (38 cm. in diameter) was about 12–14 hours. When the solvent had run the required distance, the paper was removed, the solvent boundary was marked with a pencil and dried at room temperature under a fan. After drying, the paper was sprayed with a solution of ninhydrin (0·1% in acetone or dipped into the reagent and dried at 65° C. for 10–15 minutes. The location of the amino acids was shown by the formation of coloured concentric circular zones, the radius of each varying with the type of amino acid.

Fig. 2 is the photographic reproduction of a typical circular paper chromatogram showing the separation of amino acids from a mixture into twelve distinct circular bands each representing a single amino acid, excepting the bands relating to glutamic acid and threonine, which always overlap.

The following abbreviations for the amino acids are used to indicate the position of the bands in the chromatograms.

Alanine	.. *Al.*	Lysine	.. *Ly.*
Arginine	.. *Ar.*	Methionine	.. *Me.*
Asparagine	.. *Asp.*	Ornithine	.. *O.*
Aspartic acid	.. *A.A.*	Phenylalanine	.. *Ph.*
Cystine	.. *Cy.*	Proline	.. *Pr.*
Glutamic acid	.. *Glu.*	Serine	.. *Se.*
Glycine	.. *Gly.*	Threonine	.. *Th.*
Histidine	.. *Hi.*	Tryptophan	.. *Try.*
Hydroxy proline	.. *H.Pr.*	Tyrosine	.. *Tyr.*
Iso-leucine	.. *I.L.*	Valine	.. V.
Leucine	.. *L.*		

MULTIPLE DEVELOPMENT TECHNIQUE

Amino acids having very nearly the same Rf. values may not separate into distinct bands. They often overlap, and the bands tend to diffuse. It

was also found that when the amino acids were present in somewhat higher concentration the bands appeared rather diffused. and merged with the other bands. Where this happens, succession of developments (multiple development) separate the amino acids into distinct and well-defined bands. After the first development, the chromatogram was dried at room temperature and again developed with the same solvent (*n*-butanol-acetic acid-water), until the solvent boundary occupied the same position as before and dried. The same process could be repeated, if necessary, several times. Distinct improvement in the separation of the amino acid was seen after each development. Fig. 3 illustrates the application of this technique to the separation of the slow running amino acids.

This method of operation is extremely important inasmuch as it brings together into a narrow band, the amino acid which has tended to diffuse on either side of the band in the course of the first development. On repeating the development again, the bands become more clear and well defined. This repeated operation of multiple development results in the distribution of amino acids as fine lines in the form of circles or arcs as the case may be, resulting in efficient separation of amino acids.

IDENTIFICATION OF AMINO ACIDS

Mixed Chromatograms

The technique described above serves as a rough qualitative test for the identification of amino acids separated by circular paper chromatography. In many chromatograms obtained by the above technique, the separation of the amino acids into circular bands was so distinct and clear-cut that in many cases they may be identified simply from their relative positions and characteristic shades of colour. It has been observed by several workers on paper chromatography that the R_f values vary with the type of paper, the nature and purity of the solvent, the pH of the paper and solvent, temperature, degree of saturation with water, distance between the starting point and solvent boundary, and other unknown factors. The amino acids cannot, always, be identified with assurance by referring only to their R_f values. It is, therefore, desirable to run mixed chromatograms on the same paper with known amino acids since R_f values are not easily reproducible. The unknown amino acids can be identified by reference to the chromatogram of the known amino acids or determination of the position relative to a known amino acid run on the same paper.

The technique described above cannot be adapted to the identification of amino acids by running mixed chromatograms as in the case of the widely used unidimensional chromatograms made by the "ascending" and

"descending" techniques. A new technique eliminating the limitation of this method was developed and briefly described before by Giri and Rao (1952). Further details of this technique are described below.

A small circle of about 4 cm. diameter was drawn with a pencil from the centre of a circular filter-paper. The solution (about 3–8 μl.) containing mixture of amino acids to be analysed, was applied along the circumference of the circle from the tip of a capillary tube. Usually maximum number of eight spots could be placed, leaving some space between the adjoining spots. Some of the spots, usually the alternate ones, were of known amino acids and the others were of the unknown mixture of amino acids. After application of the amino acid mixture, the spots were dried at room temperature and the paper together with the "wick" introduced at the centre of the paper for irrigation with the solvent was then transferred to the chamber. As usual the chromatogram was developed and the position of the amino acids was located by spraying or dipping the paper in a solution of ninhydrin and drying at 37° C. for 15–20 minutes. The location of amino acids was shown by the formation of coloured concentric arcs, the radius of each varying with the type of amino acid present in the mixture. The amino acids present in the unknown sample could be easily identified by reference to the arc of a known amino acid formed on the circumference of the same circle. By placing the spots very near each other, it is possible to obtain a chromatogram in which the arcs of the known amino acids and those of the unknown join together to form a circle.

The amino acids can also be identified by running the unknown mixture with the addition of known amino acids which are believed to be identical with the unknown and comparing the bands in the mixed chromatogram run in the same manner as described above. The increase in intensity of the bands of the mixture containing the added known amino acids when compared with those of the unknown mixture located on the same circumference of the circle indicates the presence of those particular amino acids in the experimental solution.

In addition, known amino acids which occupy positions very near to the amino acid which is to be identified, may be added to the unknown mixture and run on the same paper, and the relative positions of the amino acids with respect to the known amino acids may be determined. By these methods it is possible to identify the amino acids with assurance and accuracy.

IDENTIFICATION OF AMINO ACIDS BY SPECIFIC TESTS

The identification of the amino acid bands, made by the above described techniques can be further confirmed by the application of specific tests,

Many colour reactions have been developed for some of the amino acids, which are described in detail by Block (1950). These specific colour tests give very satisfactory results when the reagents are applied with a brush on to the paper containing the amino acid band.

A sector was cut out from the chromatogram after running with the solvent and after drying the bands of the amino acids were located by spraying the sector with ninhydrin reagent. The sector was again replaced in its original position and the bands relating to the amino acids, were marked with a pencil. The reagents were then applied on to the position of the band with a brush. When several reagents were used for successive applications, the paper was dried before each application of the specific reagent. The presence of the amino acid was indicated by the development of the colour characteristic of the amino acid.

Identification of Overlapping Amino Acids

Some of the amino acids are difficult to separate even by multiple development technique. For example, valine-methionine, glutamic acid-threonine are very difficult to separate from each other. In such cases other methods of identification must be adopted. Methionine, if present, can be identified in a separate run by converting it to methionine sulphone, using the oxidation technique involving hydrogen peroxide and ammonium molybdate (Dent, C. E., 1948). The methionine sulphone band appears in a different position lower than the valine band. The presence of threonine can be identified by treatment with periodate and observing the loss in intensity of the glutamic acid-threonine band when compared with the band occupying the same position in the chromatogram run with the sample without treatment.

It is often necessary to run several chromatograms with varying concentrations of the experimental solution, in order to identify all the amino acids, which may be present in different concentrations. This is necessary, particularly, when some of the amino acids, which are present in very low concentrations, compared to others, may not be visible in the chromatogram. On the other hand, if the amino acids are present in too high concentrations, the bands appear too wide and congested. Under such circumstances, it is necessary to strike a balance between obtaining broad and congested bands and diluting the solution to such a degree as to exclude many of the amino acids present in lower concentration.

Discussion

From the above results of investigations certain generalisations on the novel features of this technique and its application to the separation and identification of amino acids may be made,

1. Clear and distinct bands are obtained indicating more perfect separation of amino acids than that obtained in the widely used unidimensional chromatography by the "ascending" and "descending" techniques.

2. The technique is simple and reasonably rapid, and permits the conservation of solvents, reagents, filter-paper. Costly special apparatus and chemicals are not required. The equipment can be easily assembled from the apparatus readily available in ordinary laboratories.

3. The unit occupies little space and can be accommodated in any refrigerator or an incubator and thus facilitates the running of the chromatograms at low or constant temperatures.

4. The unit can be made easily airtight in order to carry out the separation using highly volatile solvents. It can be easily adapted to the separation of substances which are labile or easily susceptible to oxidation by carrying out the development in an atmosphere of a gas or vapour.

5. The technique is especially convenient and suitable in all laboratories where routine work on amino acid analysis is being carried out, as it provides at a glance. information, which can only be obtained by carrying out numerous experiments involving considerable amount of time, expenditure on chemicals and experimental skill necessary for obtaining accurate results.

6. The technique is capable of giving good results in the hands of relatively inexperienced workers. In routine work such as is used in the study of the nitrogen metabolism in bacteria, the method does not require continuous attention and precautions to be observed with respect to the saturation of the atmosphere with the solvent, which is very essential for obtaining good resolution of the amino acids by the widely used one and two-dimensional techniques.

The relative rates of movement of the amino acids are always found to be constant, provided the same solvent is used. The bands can be readily identified by their position relative to known bands. A system of qualitative amino acid analysis of proteins and biological fluids can be developed on the basis of the results obtained in the present investigation. It was found that the amino acids tend to fall into the following three groups when *n*-butanol-acetic acid-water was used as the developing solvent.

Group I.—Amino acids occupying positions between leucine (or iso-leucine), and proline bands in the following order;

Leucine and Iso-leucine (Purple)
Phenylalanine (Blue purple)
Valine and Methionine (Purple)
Tryptophan (Light blue purple)
Tyrosine (Blue purple)
Proline (Yellow).

Group II.—Amino acids occupying positions between proline and hydroxy-proline in the following order:

Proline (Yellow)
Alanine (Purple)
Glutamic acid—threonine (Intense purple)
Hydroxy-proline (Yellow).

Group III.—Amino acids occupying positions between hydroxy-proline and cystine in the following order:

Hydroxy-proline (Yellow)
Glycine and Aspartic acid (Purple)
Serine (Purple)
Arginine (Purple)
— Asperagine (Tan colour)
Histidine (Grey purple)
Lysine and Ornithine (Purple)
Cystine (Purple).

Based on the above classification of amino acids, the following scheme of procedure has been evolved for the identification of amino acids in protein hydrolysates or in a mixtures of unknown amino acid composition.

1. The first step consists in preparing a complete circular chromatogram with the addition of proline and hydroxy-proline to the experimental solution, if it does not contain these amino acids and identifying the amino acids by their characteristic colour and their relative position with respect to the amino acids falling within the respective groups. Many of the amino acids, particularly those of Groups I and II, with few exceptions of pairs of amino acids which do not separate into individual bands like valine-methionine; glutamic acid-threonine, can be readily identified.

2. The second step consists in running mixed chromatogram with a mixture of known amino acids and identifying the unknown by reference to the arcs of the known amino acids formed on the circumference of the same circle, or by adding known amino acids to the unknown mixture and observing the intensity of the bands.

3. The identification of the amino acids may be further confirmed by carrying out the specific colour reactions for some of the amino acids. The reagents are best applied with a brush at the point of the band, which can be easily located by the technique of cutting the sector as described before.

Some of the amino acids (glutamic acid-threonine, glycine-serine-aspartic acid, arginine, asparagine, histidine-lysine) overlap each other and identification of these amino acids is rendered difficult on first development. By repeating the development (multi-development technique), however, distinct improvement in the separation of some of the amino acids can be seen after each development and clear-cut bands can be obtained for each amino acid.

Circular paper chromatography has certain advantages over the widely used unidimensional chromatography using filter-paper strips by the "ascending" and "descending" techniques. It may be of interest to make a few comments on some of the good features of this technique as compared with the one-dimensional chromatography technique. In the latter technique the amino acids spread in the form of spots and very often somewhat elongated spots are obtained resulting in the frequent occurrence of overlapping of the spots. On the other hand, in the case of circular paper chromatography the amino acids spread on the paper along the direction orthogonal to the line or direction of flow of the solvent, resulting in the formation of well-defined narrow bands. This distribution of amino acid on paper in the direction orthogonal to the line of flow of solvent is undoubtedly more favourable to the separation of the amino acids into clear-cut narrow bands in contrast to the spreading of the amino acids into large size spots, sometimes elongated in the direction of flow of the solvent resulting in overlapping. It was found that most of the amino acids which are present in plants, animal tissues and biological fluids can be readily identified with less effort and in comparatively short time compared to the widely used unidimensional and two-dimensional chromatographic techniques.

The technique of circular paper chromatography is now in continuous use in these laboratories and has proved applicable to the amino acid analysis of proteins, cereals, biological fluids and to the study of the nitrogen metabolism in plants, bacteria and molds. However, much more work is necessary to make the technique a good and perfect analytical method. This technique can also be applied with equal success to carbohydrates, organic acids, vitamins, enzymes, proteins, organic compounds and inorganic analysis.

FIG. 1 *a*

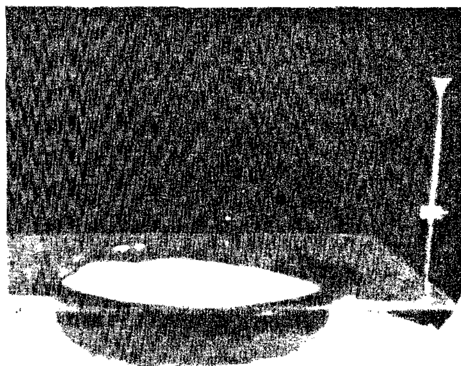

FIG. 1 *b*

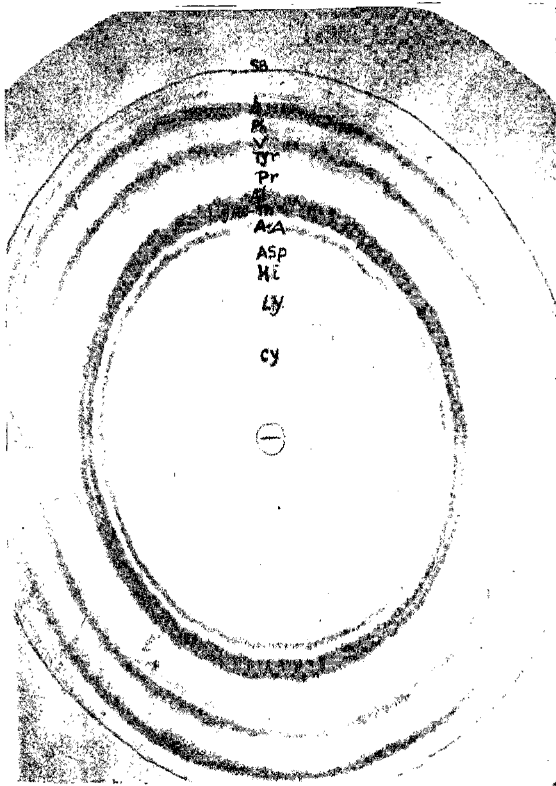

FIG. 2

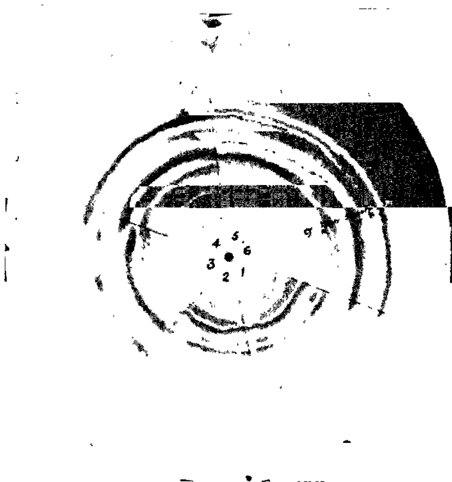

Fig. 3

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Explanation of Photographs

FIG. 1 *a* and *b*. Circular paper chromatography apparatus (for explanation see text).

FIG. 2. Circular paper chromatogram showing the separation of amino acids from a mixture containing Cystine (*Cy.*), Lysine (*Ly.*), Histidine (*Hi.*), Asparagine (*Asp.*), Aspartic Acid (*A.A.*), Glutamic Acid (*Glu.*), Threonine (*Th.*), Alanine (*Al.*), Proline (*Pr.*), Tyrosine (*Tyr.*), Valine (*V.*), Phenylalanine (*Ph.*), Leucine (*L.*).

FIG. 3. *Multiple development technique.*—The mixture of amino acids was spotted at position (6) and the chromatogram was developed and dried. Before second development the mixture was spotted at position (5) and again developed. This was repeated four times. The mixture spotted at position (6) was thus run six times, while the one spotted at position (1) was run once only.