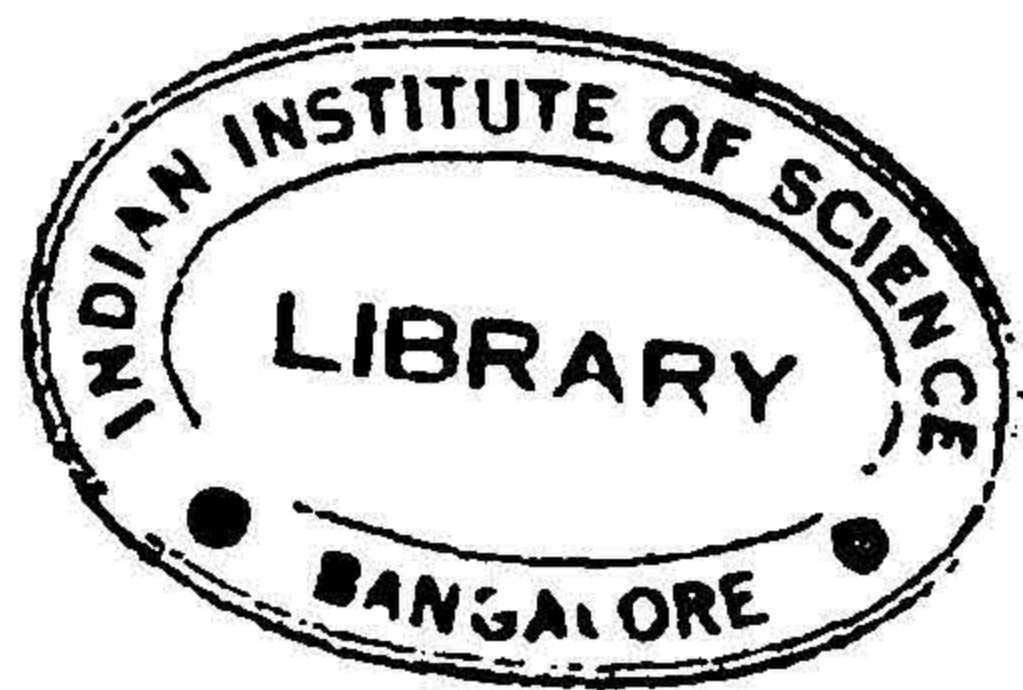


J 23395



JOURNAL OF
THE
INDIAN INSTITUTE OF SCIENCE
SECTION A

VOLUME 37

JANUARY 1955

NUMBER 1

CIRCULAR PAPER CHROMATOGRAPHY

Part IX. Preparative Circular Paper Chromatography for Large-Scale Separation and Isolation of Substances

BY K. V. GIRI

(Department of Biochemistry, Indian Institute of Science, Bangalore-3)

Received October 30, 1954

SUMMARY

A preparative paper chromatographic method for the separation and isolation of large quantities of substances is described.

The technique is developed to overcome one of the disadvantages of paper chromatography, namely the difficulty of separating components from a mixture in quantities large enough to perform additional tests and to prepare characteristic derivatives for confirming the identity of components.

The technique is simple, convenient and inexpensive.

It readily lends itself to the separation and isolation of amino acids and sugars and it should prove useful for other substances which can be separated by paper chromatography.

Chromatograms and details of a few specimen separations of amino acids and sugars from mixtures containing large quantities of these substances together with the yields obtained are given.

In the course of almost every investigation involving the separation and isolation of substances from biological sources or from chemical reactions, it is necessary to establish rigorously the purity and identity of the isolated material by the classical chemical and physical tests such as the determination of melting

point, optical rotation, X-ray diffraction, ultraviolet or infra-red absorption or by other procedures. In order to apply any or some of these criteria, it is necessary to isolate the individual substances in sufficient quantity.

Although paper chromatographic technique is now a well-established and useful method for the separation and identification of microquantities of many substances, it is not easily adaptable for large-scale chromatographic separation. The labour involved in the separation of even milligram quantities by the conventional paper chromatographic techniques is enormous. For example, Udenfriend and Gibbs (1949) have used 75 chromatograms to separate 10 mg. of C^{14} labelled fructose prepared by photosynthesis from a mixture containing fructose and glucose. A simple preparative method for isolation of large quantities of substances using filter paper will be of great use and of wide application to a large variety of substances of biochemical interest. It will have more advantages over the column chromatographic methods, which are restricted to certain types of compounds.

When a substance is separated and demonstrated to be homogeneous by paper chromatographic methods, *e.g.*, determination of the R_f value of the substance in different solvent systems, comparison of its behaviour on paper with known reference substances and colour reactions with specific reagents, conclusive evidence regarding the identity of the substance separated on the chromatogram should be obtained by isolating the substance in quantities sufficient for the determination of chemical and physical properties and preparation of characteristic derivatives. For identification where known substances are not available its identity can only be established by isolation and study of its chemical and physical properties.

Several methods have been proposed in which a pack or pile of paper discs or strips and paper columns are used for chromatographic separation of large quantities of substances. The first attempt in this direction was made by Mitchell and Haskins (1949), who have separated 50 mg. quantities of adenine, tryptophane, phenylalanine, *p*-aminocinnamic acid and anthranilic acid on a pile of paper discs (chromatopile) containing 865 discs of 9 cm. Whatman No. 1, clamped tightly by bolts at 4 corners of steel plates. The test solution is transferred to about 25 discs, by immersing the papers in the solution and drying at room temperature. The papers containing the substances are placed over a pile of 800 filter-paper discs, followed by 40 discs on the top. After careful alignment of the paper discs, the pile is clamped tightly between 2 stainless steel plates by means of wing nuts the top plate having perforations. The column is then placed in a battery jar with the solvent distributor at the top, which is filled with a solvent and developed. After development, the pile is taken out in sections and the location of the compounds determined by qualitative tests and finally extracted from the paper. Recoveries of the order of 82-92 per cent. have been reported for the substances investigated. One of the interesting features of the technique is that the paper discs containing a sample can be removed and incorporated into a new pile fo

further separation by using a different solvent mixture. The major drawbacks of this technique are:—

1. Large number of papers (more than 800) are required to make a pile.
2. The separation of the substances is difficult in some cases because of the formation of zones shaped like 'inverted cones' as observed by Porter (1951).
3. The method of locating the zones by removing many discs from the pile is very tedious.
4. In spite of the very large number of paper discs used, the quantities of the substances which can be handled are of the order of 50 mg. of each substance.

Porter (1951) has described a new apparatus termed "Chromatopak" consisting of a pack of Whatman No. 1 filter-paper strips (18×2 inches or wider) compressed between 2 stainless steel plates, for preparative work on semi-micro scale. In this method 200 strips are used and the sample (0.05 ml.) is applied on a line 2.0 cm. from one end of each strip. On each side of the pack of sample strips, 10 additional strips are placed and the whole pack is compressed between 2 stainless steel plates leaving about 5 mm. of the end of the pack containing the sample outside the plates, for immersing in the solvent kept in a stainless steel tray at the bottom of a glass cylinder (12×24 inches). The chromatogram is developed and the bands are located by spraying a sheet from each side and from the centre of the pack. After locating the bands, the pack is cut and the zones are isolated and eluted in the usual manner. The technique has been applied to the isolation of dyes, non-volatile organic acids and tobacco alkaloids from a mixture containing 3 components of each of the above class of substances. It has been possible to isolate upto 10 mg. of each constituent using 200 strips. As pointed out by the author of this technique, the procedure of placing the sample on separate sheets numbering more than 100 is tedious. It appears from the tabulated results, there is a tendency for considerable tailing of the bands. This is particularly noticed in the case of alkaloids, the band length extending to more than 10 cm. in length. This is not favourable for the separation of substances having R_f values closer than those selected for the tests. The large number of sheets used in this as well as in the "chromatopile" technique, is not an advantage from the point of view of economy and easy manipulation. The separations achieved are not quite satisfactory as broad and diffuse zones are obtained.

From Tiselius laboratory (Danielsson, 1952) a new type of preparative chromatographic paper column has been described. The column consists of a cylinder (30 cm. high; 10 cm. in outside diameter) of paper rolled tightly on a cylindrical rod (1 cm. in diameter). It is prepared in a special machine in a paper factory in Sweden. The column is saturated with the solvent. The test solution is transferred to 7 concentric rings (inner radius 1.5 cm., and outer radius 4 cm.) of paper (Munktele filter papers, No. 3), by immersing them in the test solution and drying at 20° C. The rings are then placed on the top of the column. Another cylinder (10 cm. high) of similar type with its central hole (10 mm.) filled

with paraffin is placed on top of the filter-paper rings. After saturating the top cylinder with the solvent, the elution is carried out by adding the solvent into the cylindrical hole at the top of the cylinder. An inverted volumetric flask filled with the solvent serves to maintain the level of the solvent constant. By this method 25 mg. of each of the amino acids—glycine, alanine and valine have been separated from a mixture of these amino acids. The column can be coupled to a fraction collector. Although the method possesses the advantages of column and paper chromatography, the preparation of the column requires special machinery and careful alignment, which may not be easily available in many laboratories. The technique appears to be still in an experimental stage and requires further improvement and standardisation for routine use as a preparative method.

Cuendet *et al.* (1953), finding that the ordinary filter papers including Whatman No. 3 have a limited loading capacity, have used thick cellulose sheets* (one-eighth of an inch thick) as chromatographic supports for the separation of relatively large amounts of mixtures of sugars. Owing to their poor wet strength they can be used only in horizontal development chromatography. The apparatus consists of a horizontal glass tube (100 mm. diameter) closed at each end with rubber stoppers covered with aluminium foil. The cellulose strip is placed inside the tube supported by glass T pieces and the development is carried out by means of an adsorbent cotton bridge which is immersed in the solvent kept in a cylindrical dish at one end of the glass tube. Successful separation of the following pairs of sugars has been made:—

- (a) D-glucose (14 mg.) and L-Rhamnose (14 mg.), using phenol-water.
- (b) D-glucose and D-fructose using butanol-ethanol-water; and
- (c) 2, 3-dimethyl-D-glucose (14.2 mg.) and 2-3-4-6-tetramethyl-D-glucose (8.6 mg.) using methylethyl-ketone-azeotropes.

The R_f values of sugars in butanol-ethanol-water are higher than those obtained with Whatman No. 1 paper. The width of the sugar bands range between 3.2–5.5 cm. and the distance moved by the solvent front is 24.8–34.5 cm.

Frierson *et al.* (1954) have developed a technique using thick filter paper (Eaton-Dikeman No. 320 paper, 0.1 inch thick, 2 × 8 inches), for the separation of milligram quantities of uranium from synthetic test solutions containing uranium,^{VI} Copper^{II} and Aluminium^{III} by elution chromatography. The strip is cut into V-shaped edge at one end to hold the solvent. The other end of the paper is tapered to a point. The strip is supported in a vertical position, clamped between 2 glass plates, the largest edges of the strips being enclosed in platinum foil to prevent the solvent from creeping onto the glass. The tip of the lower end and the top cut portion (0.25 inch) of the paper are not covered by the glass plates. The test solution is deposited at the centre of the strip and about 1 inch from the top. The elution is carried out by allowing the solvent to drop from a funnel on

* The cellulose sheets from which ashless filter tablets are cut.

to the middle of the strip extending above the glass. The eluate is collected from the bottom tip of the paper. By this technique 0.72–1.43 mg. of uranium has been separated from a mixture containing Uranium, "aluminium" and copper." The recovery is more than 90 per cent.

Brockmann *et al.* (Brockmann and Gröne, 1953; Brockmann and Patt, 1953) who applied for the first time the multisector technique of Giri and Rao (1952) to the separation and identification of antibiotics—Actinomycins and Rhodomycins, have reported a preparative method in which a stack of filter paper (Schleicher and Schull No. 2043 *b*) containing 60 sheets (29.7 × 29.7 cm.) is used. By means of a special device of an automatic pipette, the paper pack is developed by allowing the solvent to flow from the pipette on to the centre of the pack. By this technique 600 mg. of the crude antibiotic rhodomycine has been separated into two components, rhodomycine A and rhodomycine B, by using 400 c.c. of butanol as solvent.

The techniques described above, particularly those employing paper pack, pile of filter-paper discs and paper columns are very involved and tedious. They require large number of papers, special devices and materials. In spite of the elaborate set-up, the amounts of substances which can be handled are of the order of a few milligrams. Brockmann and Patt (1953) have however separated 600 mg. of Rhodomycine on paper pack by circular technique. The number of components separated from a mixture are limited to 2 or 3, except in the case of "chromatopile" technique in which 5 substances have been separated from a mixture. It is also observed that the separations achieved are not ideal, diffuse bands being obtained in some cases. Considering the above limitations of the techniques so far developed, the one which is described in the present paper and termed "Preparative Circular Paper Chromatography" possesses the advantages of simplicity, easy manipulation and adaptability to the separation and isolation of large quantities of substances amounting to gram quantities from a mixture. In addition, the technique requires ordinary materials which can be easily obtained and assembled in any laboratory.

A brief description of this technique has been published in *Nature* (Giri, 1954). In the present paper full details of the procedure and the apparatus used are given and the merits and limitations of the technique are discussed.

EXPERIMENTAL

Sheets of Whatman No. 3 paper have been used throughout. The sheets are cut into circular shape (35–38 cm. diameter).

Solvents.—*n*-Butanol-pyridine-water (60:40:30) is used as the solvent mixture for the separation of sugars and *n*-butanol-acetic acid-water (40:10:50) is used for the separation of amino acids.

Reagents.—Aniline-diphenylamine-phosphoric acid reagent (Giri and Nigam, 1954) and ninhydrin in acetone (Giri and Rao, 1952) are used for sugars and amino acids respectively.

Technique.—The general procedure adopted in running the chromatograms resembles that employed before for the separation of microquantities of sugars and amino acids on a single paper. In view of certain modifications made in the original method for adaptation to preparative chromatography for large-scale separation using paper pack, a description of the technique which has been found most satisfactory for the separation and isolation of sugars and amino acid is given below.

The assembly for the preparative chromatography on paper pack (Fig. 1) is quite simple and consists of two thick glass plates (18×18 inches, $\frac{1}{4}$ inch thickness) having a hole ($\frac{1}{4}$ inch diameter) at the centre. A small beaker kept at the centre of a basin, serves as the container for the solvent employed for developing the chromatogram. The basin serves as support for the glass plates. Four

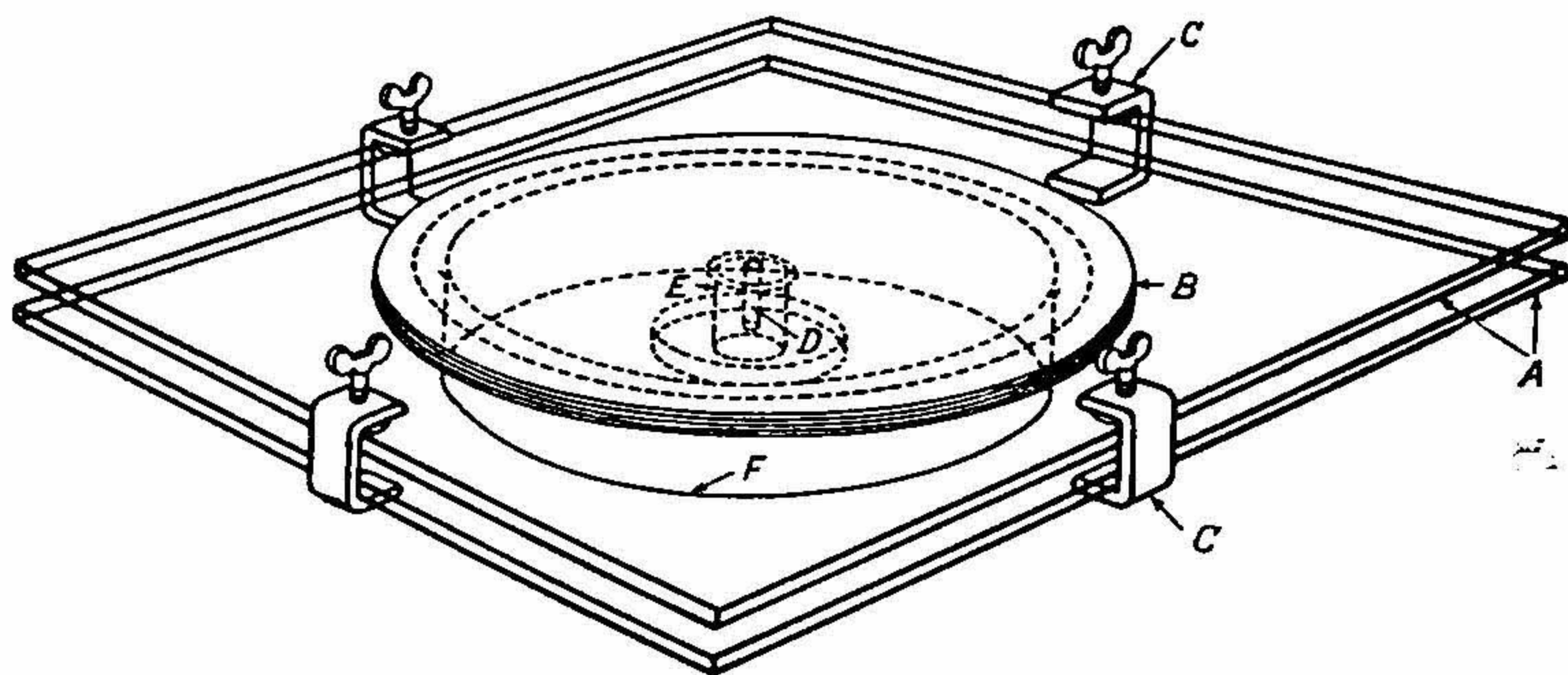


FIG. 1. Schematic diagram of the experimental arrangement for preparative circular paper chromatography on paper pack.

- A Thick glass plates (14×14 inches, $\frac{1}{4}$ inch diameter).
- B Paper pack (containing 5 or 6 Whatman No. 3 papers of 35–38 cm. diam.)
- C Clamps.
- D Cylindrical paper wick.
- E Beaker containing the solvent.
- F Basin for supporting the glass plates.

screw clamps are fixed at the four sides of the two glass plates and these serve to keep the filter paper pack containing five papers tightly sandwiched between the two glass plates.

Procedure for separation and isolation of substances on single paper.—The method of running the chromatogram on single paper is substantially the same as in the earlier description (Giri and Rao, 1952), except that for large-scale separation the volume and concentration of test solution used for spotting are much higher than those used in the micro-chromatographic method. For preparative work, the method consists in spotting 0.2 ml. at a time of the solution containing a mixture of sugars (about 20 per cent. each of 3–5 sugars) or amino acids

(about 5 per cent. solution) at the centre of the paper and drying before another aliquot is superimposed on it until 0.5–1.0 ml. of the solution has been applied. The spotting should be done evenly without much concentration at the centre of the spot. Cold air from a fan or hair dryer can be blown over the surface of the filter paper to facilitate the drying. The large quantity of the solution used for spotting forms a wet patch about 1–1½ inch in diameter. In spite of the fact that the solution spreads to a larger area, little trouble has been experienced in the separation. If the concentration of the substances in the solution is large, the area of the spot should not be smaller than 1 inch in diameter. By reducing the area of the spot, the solute will be concentrated at the centre resulting in the obstruction of the movement of the solvent. If this happens at any time, a drop of water added at the centre will distribute the solute to a larger area reducing its concentration at the centre. It is then irrigated with the solvent by means of a wick inserted at the centre.

Multiple development.—One of the essential features of the technique consists in the application of the multiple development technique, which is found to be very effective in improving the resolution of the substances. After the first development and drying, it is again developed for the second time until the solvent boundary comes near to the edge of the paper. The development is repeated until the substances separate into distinct bands without any overlapping. The number of developments to be used depends on the number and concentration of the substances to be separated. This must be tested in each case by cutting a small sector after each development and observing the bands after treatment with the reagent. The bands can also be seen under filtered ultraviolet lamp (Philips H.P., 125 W. analysing lamp), if the substances are present in high concentrations. In such cases, it is very easy to visualise the bands and control the number of developments to be used for satisfactory separation.

A typical chromatogram showing the improvement in the resolution of the substances achieved by the application of multiple development technique is illustrated in Fig. 2.

Location of the bands.—After final development and drying at room temperature, narrow segments are cut at different regions of the paper and treated with the chromogenic reagent. Using these segments as guide strips, the bands relating to the individual sugars or amino acids can be marked with a pencil.

Elution of the substances from the paper.—The marked areas of the paper containing the desired substances are cut into small pieces and extracted with water or 80 per cent. alcohol. The substances can be extracted effectively from the paper by disintegrating the paper pieces in a suitable solvent either by vigorous shaking or in a Waring blender followed by filtration of the pulp and further washing the paper fibres on the filter. If the substances are stable, they can be eluted from the paper by boiling the cut pieces with 80 per cent. alcohol followed by Soxhlet extraction. The alcohol is removed by vacuum distillation or on water-bath if

the substances will stand high temperature. When solvents like phenol are used for chromatography, it is desirable to extract the concentrated solution with ether to remove traces of phenol. Ion-exchange resins can also be used with advantage in the case of sugars to remove other impurities present in the solution. The solution can be finally concentrated and crystallised in the usual manner.

Procedure for separation and isolation of substances on paper pack.—For separation of larger quantities of substances than those possible on single paper, a paper pack containing 5 papers can be used for chromatography. After spotting the solution containing the substances on each paper, they are placed one over the other after drying the spots at room temperature. The paper pack is pressed well between two glass plates by means of clamps at the four sides of the plates (Fig. 1). A hole is made through the papers with a pointed glass rod and a cylindrical wick of paper is inserted through the holes to reach the solvent kept in a beaker underneath the glasses, which rest on a basin. Multiple development is carried out as described before. The separation of bands at the end of each development can be observed under a filtered ultra-violet lamp (Philips H.P., 125 W. analysing lamp) and further development is discontinued, after ascertaining that the desired separation has been achieved. The paper pack is then removed intact and the position of each paper with respect to the other is marked with a pencil on the edges of each paper or by making a short cut with a pair of scissors on the edge of the pack. The papers are then dried at room temperature and replaced one over the other keeping the position of the papers exactly the same as before in the pack. The top paper is taken out and treated with the chromogenic reagent to reveal the position of the bands on the paper. The paper with the visible bands is placed over the paper pack and the position of the bands in the other papers are marked with a pencil. In order to prevent the contamination of the papers with the chromogenic reagent used for treating the top paper the position of the bands can be traced on another fresh paper, which can be used for tracing the bands on the paper pack. The bands in most cases, can be seen under the filtered ultraviolet lamp. In such cases the treatment of the paper with the reagent can be avoided and the position of the bands can be located and traced under the ultra-violet lamp. As the bands in all the papers occupy the same position, the bands relating to each substance are superimposed, thereby facilitating the location of the bands on each paper by using the top paper as guide for tracing the position of the bands. The paper pack, in fact, behaves like a single thick pad of paper in the distribution of the bands uniformly in all individual papers. The first development takes longer time (about 20–24 hours) than the subsequent developments (8–15 hours). The elution of the substances can be carried out by any of the methods described before. The eluate is concentrated and crystallized in the usual way. The purity of the components can be tested by circular paper chromatographic technique.

Typical chromatograms showing the separation of sugars and amino acids on single paper and on paper pack are illustrated in Figs. 3–7.

SUMMARY OF EXPERIMENTAL RESULTS OBTAINED USING SINGLE PAPER AND PAPER PACK

TABLE I (a)
Single Paper

Expt. No.	Type of mixture	Components of mixture (mg. spotted)	Temperature of development	No. of developments	Yield of the substances after elution	Purity as determined by circular paper chromatography	Remarks
1	Sugars	(1) Raffinose (2) Molibiose, (3) Lactose (4) Maltose (5) Sucrose (6) Glucose (7) Arabinose (8) Xylose (9) Ribose (10) Rhamnose 10 mg. of each sugar present in the mixture used for spotting	Room temperature 22-24° C.	Three	Separate into ten distinct bands
2	do	(1) Kaffinose 100 mg. (2) Maltose 100 " (3) Glucose 100 " (4) Xylose 100 " (5) Rhamnose 100 "	do	Four	Separate into five distinct bands
3	do	(1) Raffinose 240 " (2) Maltose 240 " (3) Glucose 240 "	50-55° C.	Four	Clear separation into three distinct bands
4	do	(1) Raffinose 200 " (2) Maltose 200 " (3) Glucose 200 "	Room temperature	Four	(1) 120 mg. (2) 180 " (3) 160 "	(1) Pure (2) do (3) Traces of maltose	Satisfactory separation is achieved with the bands of maltose and glucose close to each other
5	do	(1) Raffinose 200 " (2) Maltose 200 " (3) Glucose 200 "	do	Five	(1) 170 mg. (2) 179 " (3) 140 "	(1) Pure (2) do (3) do	Clear separation into three bands
6	Amino acids	(1) Glycine 25 " (2) Alanine 25 " (3) Valine 25 "	Room temperature	three	Clear separation into three distinct bands
7	do	(1) Histidine 30 " (2) Glutamic acid 30 " (3) Valine 30 "	do	three	Clear separation into three distinct bands

TABLE I (b)
Paper Pack (Containing 5 Papers)

Expt. No.	Type of mixture	Components of mixture (quantity spotted)	Temperature of development	Number of developments	Yield of the substances after elution	Purity as determined by circular paper chromatography	Remarks
1	Sugars	(1) Lactose .. 1 gm. (2) Xylose .. 1 "	Room temperature	Four	(1) 0.680 gm. (2) 0.720 "	(1) Pure (2) Pure	..
2	do	(1) Raffinose .. 1 " (2) Sucrose .. 1 " (3) Rhamnose .. 1 "	do	Four	(1) 0.650 " (2) 0.610 " (3) 0.720 "	(1) Pure (2) Pure (3) Pure	..
3	do	(1) Raffinose .. 1 " (2) Glucose .. 1 " (3) Rhamnose .. 1 "	45-50° C.	Three	Separate into three distinct bands
4	do	(1) Raffinose .. 600 mg. (2) Glucose .. 600 " (3) Maltose .. 600 "	do	Four	Very clear separation into three distinct bands. The movement of the solvent is fast at higher temperatures
5	Amino acids	(1) Lysine .. 150 mg. (2) Alanine .. 150 " (3) Valine .. 90 "	Room temperature	Five	Excellent separation into three distinct bands
6	do	(1) Asparagine .. 250 " (2) Glutamic acid .. 250 " (3) Phenylalanine .. 100 "	do	Four	(1) 190 mg. (2) 180 " (3) 75 "	(1) Contains traces of aspartic acid (2) Pure (3) Pure	The presence of aspartic acid is due to the conversion of asparagine into the corresponding dibasic acid during evaporation

When more than 100 mg. quantities are used, the width of the bands range between 0.5-2.5 cm. Lower concentrations (10 mg.) give bands of smaller width.

The summary of the experimental results obtained using single paper and paper pack is given in Table I (a), (b). Only a representative few of the large number of separations carried out successfully have been given in the table.

DISCUSSION

It may be seen from the experimental results that the separation and isolation of large quantities of substances by the circular technique is much simpler than the methods suggested by other workers using various types of paper support with downward flow development. As the bands in circular technique spread over a large area without considerable broadening of the bands after multiple development larger quantities of substances than those that are possible in linear flow chromatography can be separated. The separations are sharp and well defined compared to the linear flow chromatographic techniques, in which the bands spread in the direction of the flow of the solvent.

The following are some of the special features of the technique:—

1. Two hundred milligram quantities can be separated on a single paper. This is not possible with the linear flow paper chromatographic methods.
2. By superimposing number of papers the quantity of material used for separation can be correspondingly increased. By using a paper pack containing five papers gram quantities of substances can be separated and isolated. The use of still larger size of filter paper may be found to be of advantage for the separation of larger quantities of substances. The effect of increasing the number of papers above five, which is the maximum number used in the present investigation, has not been attempted. Possibly larger quantities of substances can be handled by increasing the number of papers.
3. The bands in all the papers in the paper pack occupy the same position, thereby facilitating the location of the bands by treating the top paper with the chromogenic reagent and using it as guide paper.
4. The multiple development technique is very useful in improving the resolution of the substances.
5. The apparatus can easily be maintained at any desired working temperature. Sugars will separate well at high temperatures (50–55° C).
6. By spotting large concentrations of substances the presence of minute amounts of impurities and other substances present in the sample can be easily detected.
7. The central feed method by means of a wick adopted in the technique affords a simple device for controlling the rate of flow of solvent. The size of the wick can be adjusted to reduce the rate of flow for better separation. In linear flow chromatography, where the use of special high grade of filter paper is suggested for preparative work, it is not possible to reduce the speed of flow unless a device is adopted by attaching a strip of Whatman No. 1 paper to one edge of the thick paper by fastening with stitches by means of sewing machine (Müller, 1950).

8. Thick papers having poor wet strength can be used in this technique, as they can be conveniently supported on glass and held horizontally.

9. The technique possesses certain advantages over the column chromatographic techniques using powdered cellulose. Although hundred milligram quantities of sugars can be separated on such columns (Hough *et al.*, 1949) large volumes of solvent are required to elute the individual sugars. When substances having very low R_f values, *e.g.*, Raffinose and other tri- and higher saccharides are present in the mixture the volume of solvent required to elute the substances is very considerable and highly dilute solutions are obtained. To concentrate such dilute solutions by evaporation is tedious and time consuming. The identification of the components by paper chromatographic method is rendered difficult on account of the high dilution of the components in the fractions. Furthermore, as the time of elution is inversely proportional to the R_f value of the substance, it takes long time to elute such compounds from the column. These difficulties are not encountered in the present technique in which the amount of the solvent used for elution of the substances from the paper is reasonably small and the technique lends itself to easy identification of the substances in the eluate by paper chromatographic method and concentration by evaporation.

As the paper contains some impurities such as metallic ions and organic matter, it is necessary to pre-wash the paper with dilute acid and water.

The recovery of the substances, as determined both by weighing the residue and by quantitative paper chromatography ranges between 60–90 per cent. Known sources of loss include losses in extraction, losses by discarding the small boundary area of the band at which the substances overlap, and losses due to the removal of a portion of the paper used for treatment with the chromogenic reagents to serve as guide strips for locating the bands on the paper. It is possible to reduce the losses caused by more efficient and repeated extraction and using strips of small width for treatment with the reagents. In preparative procedure, however, the object is to recover the maximum possible yield of the substance with highest purity although there may be losses due to incomplete separation or extraction.

The quantities of the substances which can be separated without overlapping depends on the number of components present in the mixture and the difference in their R_f values. A mixture containing 10 mg. of each of 10 sugars has been separated into 10 distinct bands (Fig. 3). At higher concentrations of these sugars the bands overlap each other. Mixtures containing 2 or 3 sugars with wide difference in R_f values can be separated in large quantities (200 mg.). The larger the concentration of the substances present in the mixture spotted on the paper, the wider the bands become and unless the difference in the R_f values of the substances is sufficiently large, there will be overlapping of the bands. It is, therefore, necessary to carry out preliminary tests on a single paper to determine the suitable concentration of the solute mixture to be used for spotting before a large-scale separation is attempted on paper pack.

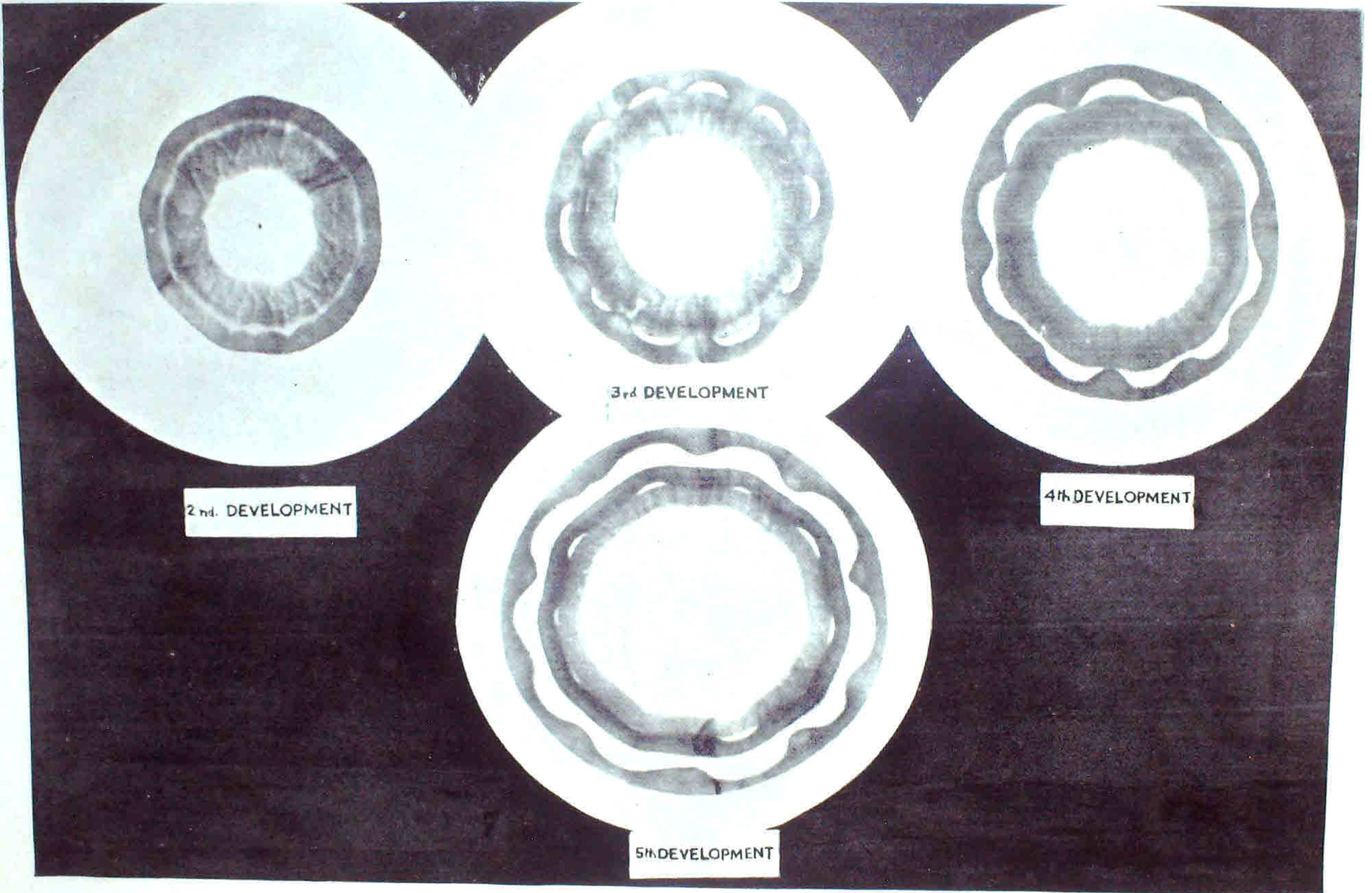


FIG. 2

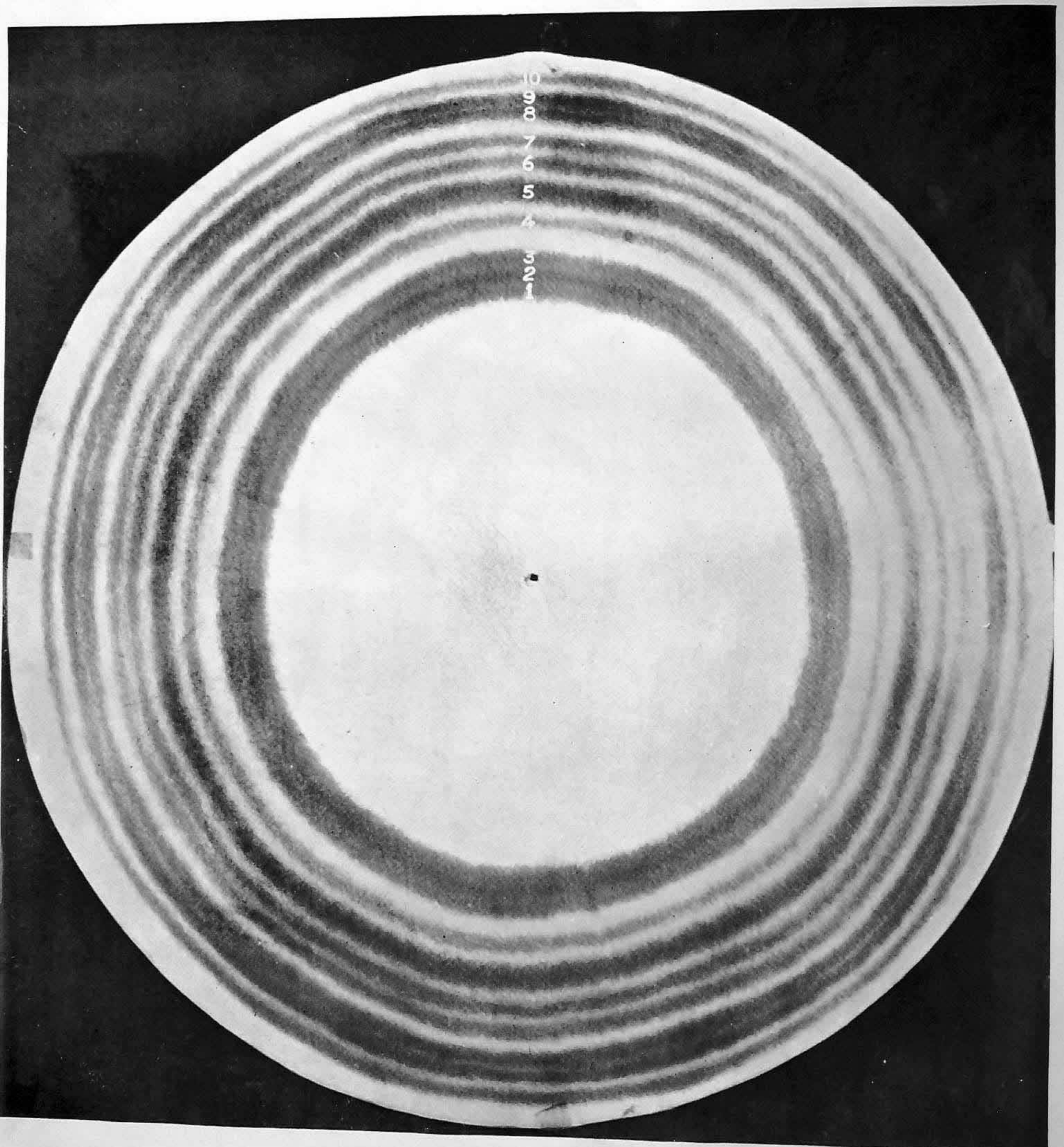


FIG. 3

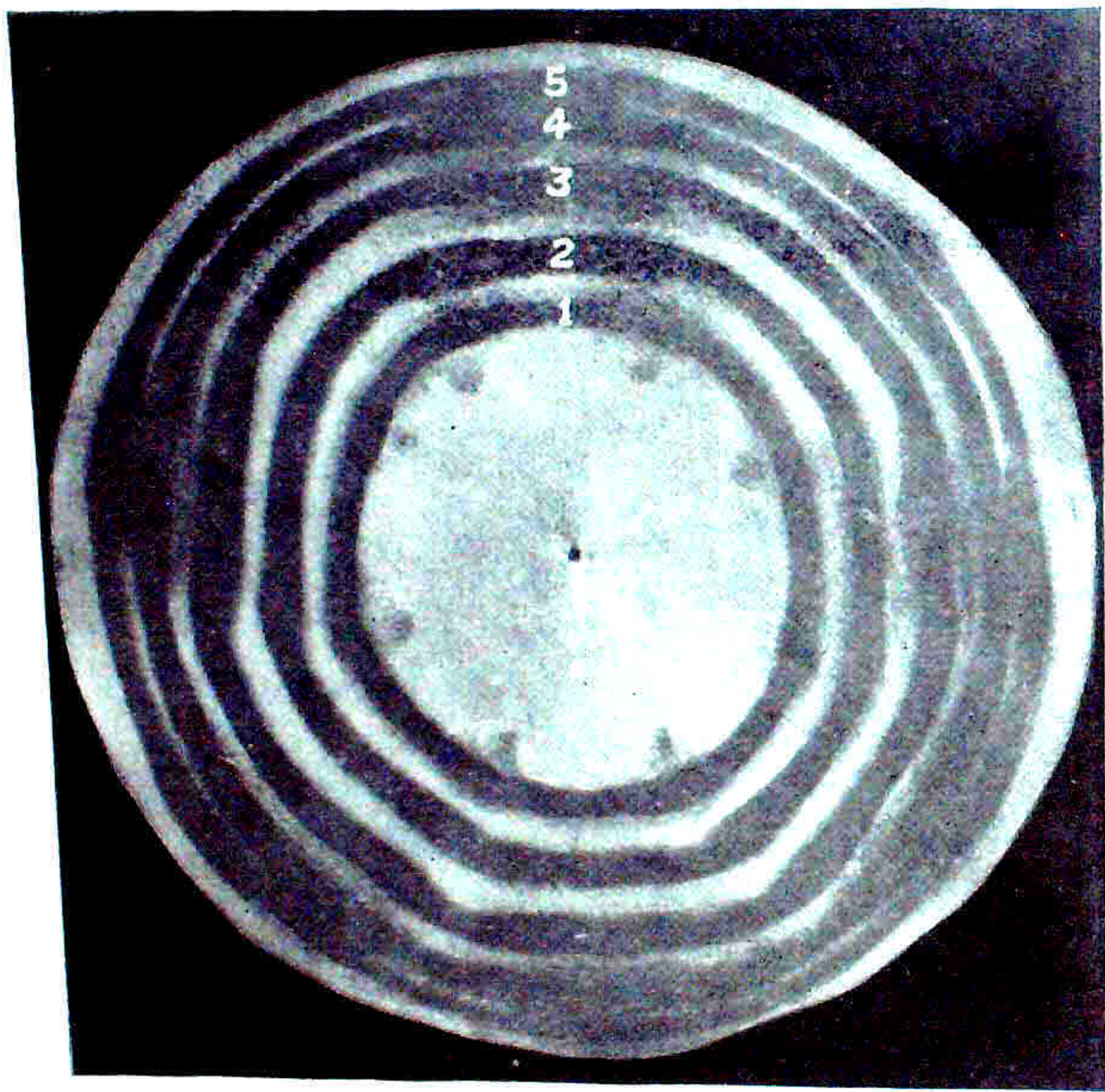


FIG. 4

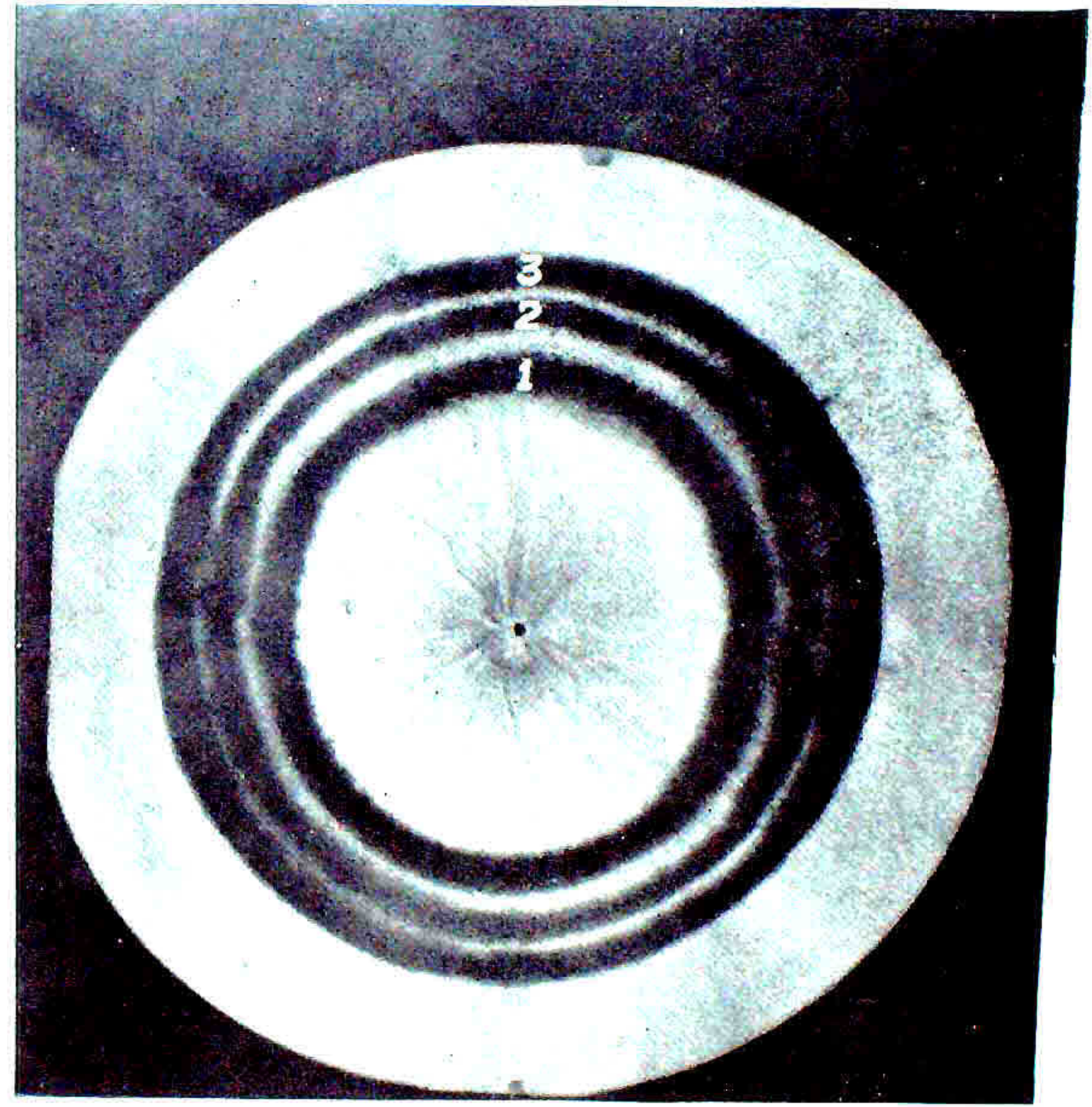


FIG. 5

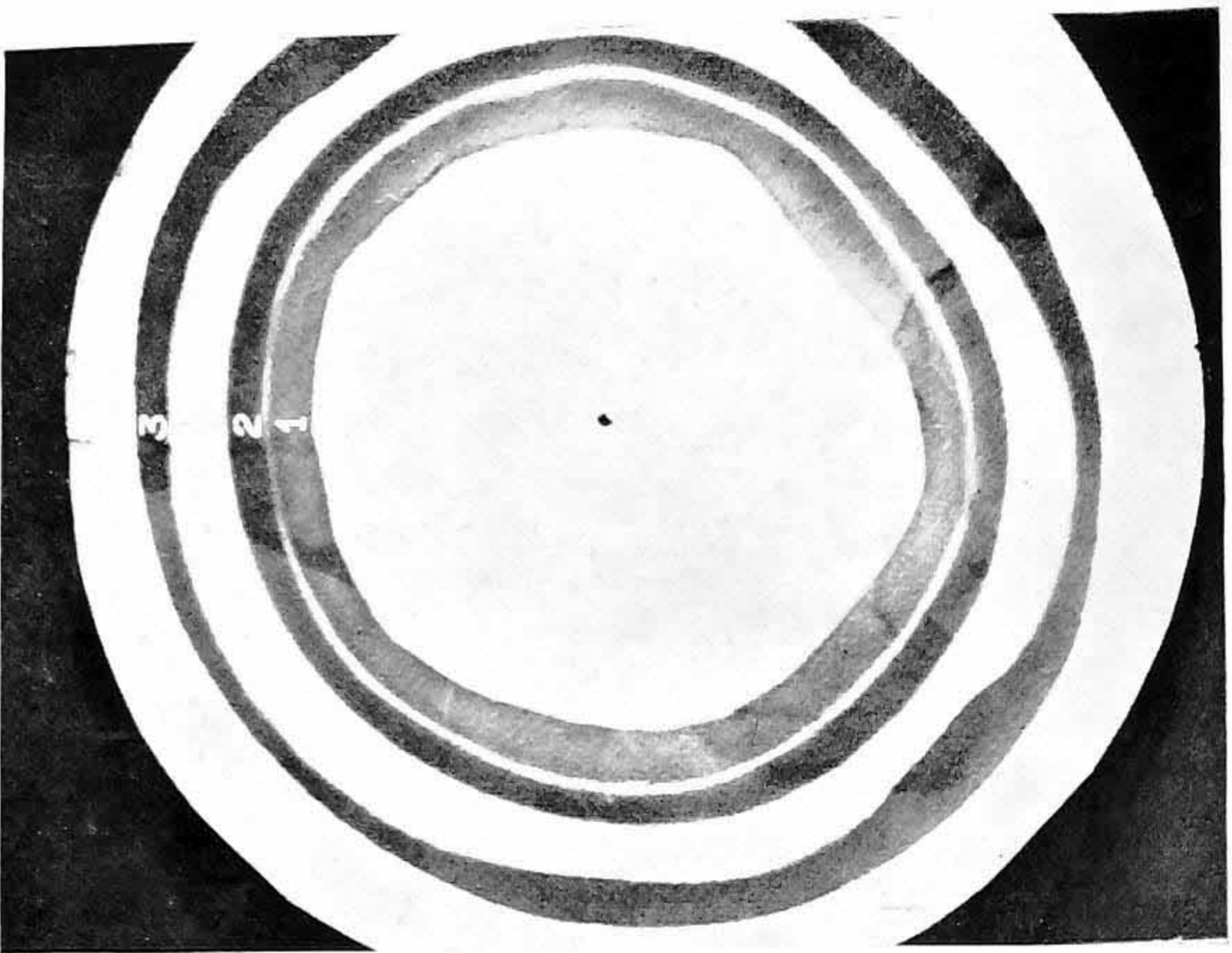


FIG. 7

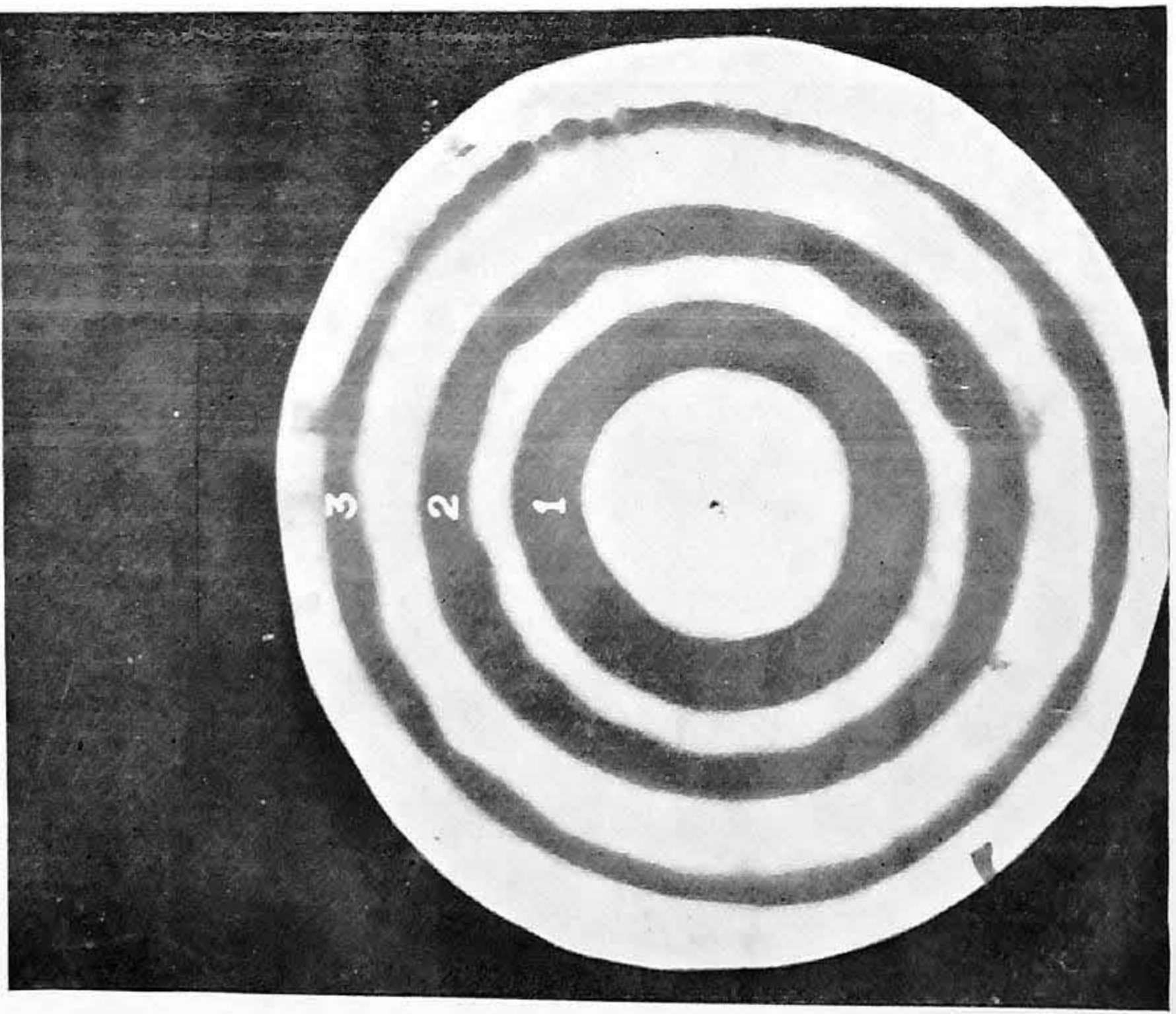


FIG. 6

The first step is to find out the loading capacity of the paper for the separation of the substances under test. This can be determined by running a few chromatograms with increasing concentrations of the test solution spotted at the centre and determining the maximum quantity of the sample which can be spotted without overlapping of the bands. After determining the maximum load for a single paper, the same quantity of material can be spotted on 5 papers and chromatographed after superimposing the papers into a paper pack.

REFERENCES

1. Brockmann, H. and Gröne, H. .. *Naturwissenschaften*, 1953, 40, 222.
2. Brockmann, H. and Patt, P. .. *Ibid.*, 1953, 40, 221.
3. Cuendet, L. S. Montgomery, R. and Smith, F. .. *J. Amer. Chem. Soc.*, 1953, 75, 2764.
4. Danielsson, C. E. .. *Arkiv. für Kemi*, 1952, 5 (14), 173.
5. Frierson, W. J., Thomason, P. F. and Raaen, H. P. .. *Anal. Chem.*, 1954, 26, 1210.
6. Giri, K. V. and Rao, N. A. N. .. *Nature*, 1952, 169, 923; *J. Ind. Inst. Sci.*, 1952, 34, 95.
7. ——— and Nigam, V. N. .. *Naturwissenschaften*, 1953, 40, 343; *J. Ind. Inst. Sci.*, 1954, 36, 49.
8. Giri, K. V. .. *Nature*, 1954, 173, 1194.
9. Hough, L., Jones, J. K. N. and Wadman, W. H. .. *J. Chem. Soc.*, 1949, 2511.
10. Mitchell, H. K. and Haskins, F. A. .. *Science*, 1949, 110, 278.
11. Müller, J. H. .. *Ibid.*, 1950, 112, 405.
12. Porter, W. L. .. *Anal. Chem.*, 1951, 23, 412.
13. Udenfriend, S. and Gibbs, M. .. *Science*, 1949, 110, 708.

EXPLANATION OF PLATES

- FIG. 2. Chromatograms showing the improvement in the resolution of large amounts of amino acids by multiple development technique. 1. Lysine (150 mg.). 2. Alanine (150 mg.). 3. Valine (90 mg.). A pack of five papers is developed after spotting the mixtures on each paper. After each development one sheet is taken out and treated with the reagent.
- FIG. 3. Chromatogram showing the separation of ten sugars (1. Raffinose, 2. Melibiose, 3. Lactose, 4. Maltose, 5. Sucrose, 6. Glucose, 7. Arabinose, 8. Xylose, 9. Ribose and 10. Rhamnose) from a mixture containing 10 mg. of each sugar. (Three developments; Single paper 35 cm. dia.)
- FIG. 4. Chromatogram showing the separation of: 1. Raffinose, 2. Maltose, 3. Glucose, 4. Xylose, 5. Rhamnose, from a mixture containing 100 mg. of each sugar. (Four developments; Single paper, 35 cm. dia.)
- FIG. 5. Chromatogram showing the separation of: 1. Raffinose, 2. Maltose and 3. Glucose from a mixture containing 100 mg. of each sugar. (Four developments; Single paper, 35 cm. dia.)
- FIG. 6. Chromatogram showing the separation of: 1. Raffinose, 2. Glucose and 3. Rhamnose from a mixture containing 1 gr. of each sugar. (Four developments; paper pack containing five papers, 35 cm. dia.)
- FIG. 7. Chromatogram showing the separation of: 1. Glycine, 2. Alanine and 3. Valine from a mixture containing 25 mg. of each amino acid. (Three developments; Single paper, 35 cm. dia.)