Gravimetric Evaluation of Paper Chromatograms I

Theory, Instrumentation, and Procedure

By ALBERT E. H. HOUK

Theory, instrumentation, and procedure are given for the gravimetric determination of the distribution of compounds over an entire paper chromatogram. After the chromatogram is developed, the paper is cut into a series of $^3/_{16}$-in. strips at right angles to direction of development. The portion of the sample on each strip is eluted into a tared cup. The results are graphed, using the weights of the dried residues in the cups as the ordinates and the number of the strips from the bottom of the chromatogram as the abscissas. This procedure permits the quantitation and isolation of components in low concentrations which may be missed by conventional methods of detection and eliminates the need for a reference standard for quantitative evaluation. The isolated fractions may be used for additional studies.

PAPER CHROMATOGRAPHY has been used successfully for the separation and determination of chemicals in almost every field of science. Most of the standard procedures are inadequate since unknown impurities present may escape detection if they do not respond to the test used to detect the major components of the mixture. In many cases, the quantitative evaluation of the results is based on a visual comparison with reference standards (1–4).

In investigations in this laboratory of the purity of some U.S.P. reference standards and commercial drugs, these limitations of paper chromatography have been overcome by rapidly eluting fractions covering the entire paper chromatogram and by weighing the dried fractions obtained. The theory, instrumentation, and procedures developed and used during the past 3 years for obtaining weight-distribution curves from paper chromatograms are given. The application of the procedure to ouabain, other glycosides, and prednisone is presented in another paper (5).

THEORY

A solution of Sample A (Fig. 1) was streaked on the starting line, and the paper chromatogram developed by ascending technique in a sealed tank or by the continuous process (6). Where possible, volatile solvents were used as the mobile and immobile phases. Sample A was separated into four apparent components: *a*, *b*, *c*, and *d*. The chromatogram was cut into a series of narrow strips parallel to the starting line but were held together at the left edge by an uncut area. Components on the strips were eluted simultaneously into tared Teflon[1] cups. The solvent was evaporated, and the weight of the residue in each cup was determined. The results were graphed, using the weights of the residues as the ordinates and the number of the strip (counting from bottom of paper) as the abscissas. Usually, inflections obtained over those given by blank chromatograms indicated the presence of a component of the mixture, and its quantity was the sum of the weights from the strips producing the inflection less the weight given by the blanks or overlapping components.

The weight-distribution curve of Sample A shows that components *a*, *c*, and *d* were successfully isolated in a relatively pure form. The curve obtained for *b*, however, indicated the presence of more than one compound. The residues from the fractions in this area were combined, and a better separation of the components was achieved on another chromatogram with a modified solvent system.

INSTRUMENTATION

Sample Applicator Box (A, Fig. 2).—To prevent excessive evaporation of the more volatile immobile solvents from the chromatographic paper during application of sample to the starting line, a box of the following design is used: the box is 11 × 18 in. inside dimensions, and 5 in. in height at the sides and back; the front of the box is $4^1/_4$ in. in height, extending within $^3/_4$ in. to the top of the sides.

Place a crosspiece 4 in. from the inside front of the box and extending within $1^1/_2$ in. to the top. Inside and near the bottom of the front compartment thus formed, install two light sockets to take 25-watt light bulbs for back-lighting the starting line. Immediately above the light bulbs, insert a glass plate for heat insulation. Cut a groove around the inside periphery of the box $1^1/_2$ in. below the top and extending through the front panel to allow for the introduction of a glass plate to support the chromatographic paper. To prevent paper from adhering to the glass plate, it has been found advisable to cover the plate with a sheet of 100-gauge Teflon film[2] which can be replaced as needed. Place two $^5/_{16}$-in. glass rods (covered with closely fitting

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[1] Marketed by E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

[2] Type A, FEP-fluorocarbon film, Film Department, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

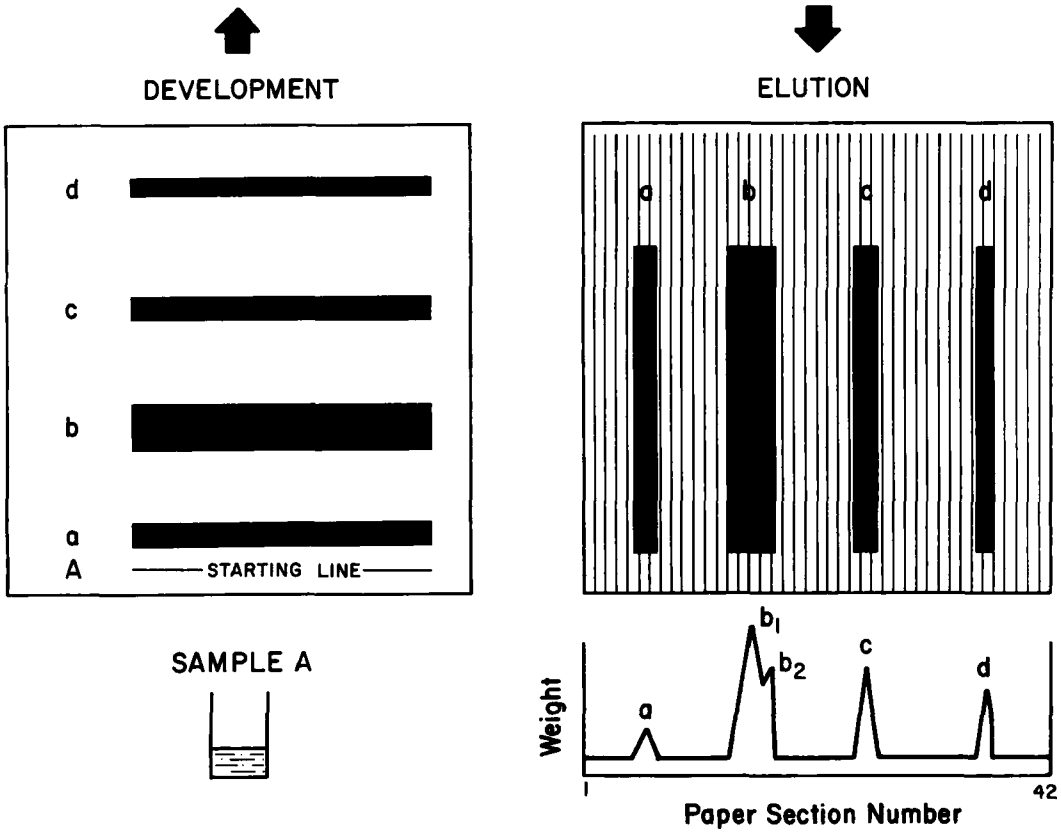

Fig. 1.—Determination of a weight-distribution curve of a paper chromatogram.

spaghetti tubing,[3]) 1/8 in. above the upper glass plate and 1 and 3 in., respectively, from the inside front of the box. Suspend these rods in the U-slots of two brackets made of 1/8-in. metal and fastened to the sides of the box. Cut grooves in the sides and back of the box 1/2 in. below the top to permit the introduction of a cover made from 1/16- in. aluminum sheet. Near the front of the cover, a slot 1/4 in. wide and 10 in. long is made so that (with the cover in position) the slot is midway between the glass rods. Fasten cross ribs to support blotting paper wetted with a suitable solvent to the underside of the cover. Mount the box on 3/4-in. thick blocks at each corner for ventilation.

Cutting Equipment (B, Fig. 2).—As a guide to be used in cutting the chromatogram, make a template 6 1/8 × 12 in. from 3/16-in. hardened aluminum plate. Using a milling machine equipped with a 1/32-in. circular saw, cut thirty 10-in. slots through the plate at 3/16-in. intervals, leaving a 1/2-in. border at the left and a 1-in. border at the top and bottom. With a scriber, make two guide lines at the top of the plate, perpendicular to and 3/8 and 1/2 in., respectively, below the top of the slits. Through the upper line, drill six 7/64-in. holes, approximately evenly spaced across the plate so that the top of the chromatogram can be brought even with the top line. Sometimes difficulties may be enountered in sawing the slots in the plate. In this case, several smaller sections (satisfactorily made) can be fastened together with metal strips at the top and bottom.

Place the paper and the template on a hardwood cutting board. Place two metal bars across the top and bottom of the template and bolt the extremities of the bars to the board to prevent the movement of the template during use. The bars should be long enough to permit the template to be moved across the entire width of the paper.

Weighing Cups (C, Fig. 2).—The weighing cups are made from disks of 50-gauge Teflon film 1 1/4 in. in diameter. Using a film 2 in. in width, attach the end of the film with adhesive tape to a cardboard strip 1 1/2 in. in width and make 50 superimposed layers of film around the strip. Fasten the end of the film with adhesive tape to the top layer on the cardboard. Place the cardboard strip with film between two sheets of bond paper and lay on a 1/4-in. sheet of polyethylene. Cut a block of disks using a 1 1/4-in. steel punch.

The molds for forming the cups are made from 13 1/2 × 2 7/8-in. strips of 1/2-in. hardened aluminum plate. Starting with the centers 2 1/4 in. from one end and 3/4 in. from either side of the strip, inscribe two parallel rows of nine 1 1/4-in. circles. At the center of each circle, drill a 15/32-in. hole through the plate. Remove sharp edges of the holes by reaming. For molding cups, make cylindrical plugs from 1/2-in. aluminum rod reduced in diameter on a lathe until it and a Teflon disk can be fitted easily but firmly into the holes of the mold. Cut plugs

[3] Teflon tubing, Pennsylvania Fluorocarbon Co., Inc. Philadelphia 4, Pa.

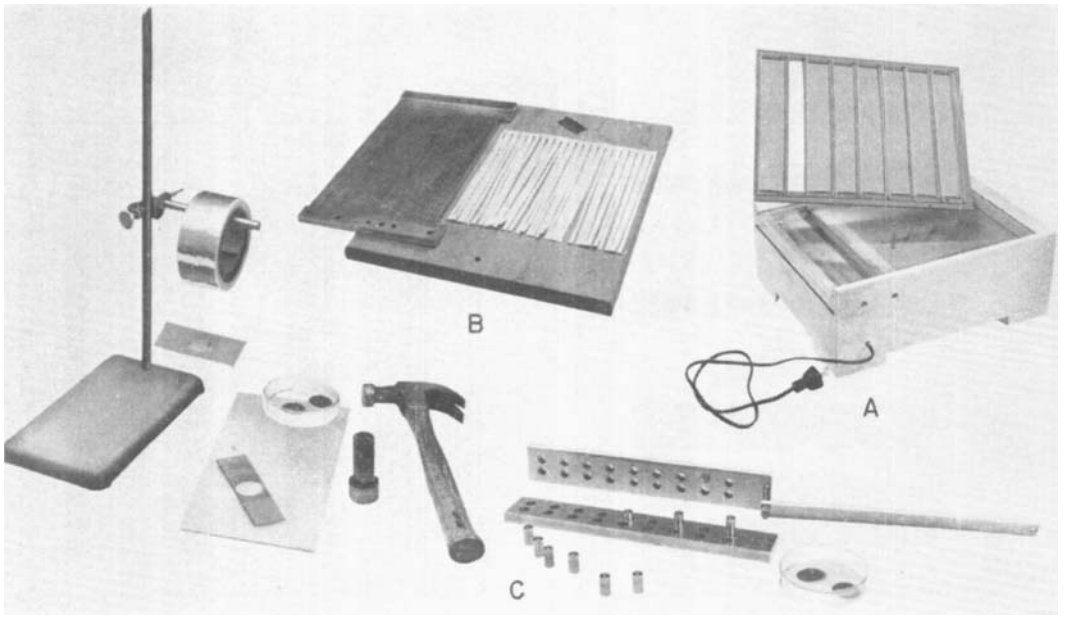

Fig. 2.—*A*, Sample applicator box; *B*, equipment for cutting paper chromatogram; and *C*, equipment for making Teflon weighing cups.

7/8 in. in length from the rod and round the edges with a cutting tool and a fine file. Polish both the holes of the mold and the plugs consecutively with 200-mesh aluminum oxide and talc wetted with alcohol.

To handle the hot molds, drill two 5/16-in. holes, 5/8 in. apart, 1/4 in. from the clear end of the mold. Make a detachable handle from a 14 × 1-in. strip of 1/4-in. aluminum. Attach to it two 1/4-in. brass rods so placed as to fit into the holes at the end of the mold.

Analytical Balance.—Balances with a suitable counterbalancing device and a sensitivity of 1 mcg. can be used for weighing the cups. The Cahn gram electrobalance[4] is satisfactory. However, an experimental automatic model 1700 AH electrobalance[4] (*A*, Fig. 3) developed for this study simplifies the weighing procedure greatly. This balance is connected to a precision four-digit voltmeter[5] which automatically indicated the weight. A 500-μc. radioactive ionizing unit is placed inside the weighing chamber to aid in the weighing of cups having a static charge. When weighing hygroscopic residues, two 5-ml. beakers containing P_2O_5 desiccant are placed in the weighing chamber.

Multifraction Elutor (B, C, Fig. 3).—The details of the construction of the component parts of the elutor canot be described adequately in a presentation of this type. Detailed drawings and instructions for making the complete elutor are available upon request to the author. The following is a functional description of this part of the equipment.

The assembled elutor is represented by *B*, Fig. 3. In *C*, Fig. 3, the elutor is pictured partly disassembled to show the component parts. The rigid frame work of the elutor consists of two sides and a bottom made of hardened aluminum plate. A crosspiece placed on either side lends rigidity to the sides Two bars are fastened to the bottom plate to prevent tipping.

A spacer bar (4, Fig. 3) made of extruded aluminum tee is used to hold the paper in position. The leg of the tee is sharpened to a wedge shape; the base of the tee is cut to have 21 slots on each side so positioned to accommodate the strips of paper. The spacer bar is placed on shelves fastened to the inside of the frame.

A cup holder (2, Fig. 3) containing four staggered rows of 12 holes each is placed on the bottom of the frame. The ends of the paper strips are held immediately above the proper cups by a needle rack (3, Fig. 3) fastened to the median line of the cup holder. To decrease the possibility of creeping of the eluting solvents, the cup holder and needle rack are coated with a TFE-fluorocarbon resin primer.[6] A flat heating element connected to the electric receptacle on the side of the frame is placed in the base of the frame under the cup holder to aid in the evaporation of the solvent collected in the cups.

A syringe rack (6, Fig. 3), carrying twenty-one 1-ml. syringes in two staggered rows, is mounted on each side of the frame. When in position, the tip of each syringe is in contact with a strip of paper. The plungers of the syringes are moved by crosspieces which are driven by a demountable clock motor and gears (8, Fig. 3).

To prevent the evaporation of volatile eluting solvents from the paper, two side panels (5, Fig. 3), equipped with metal ribs to hold sheets of blotting paper wetted with the solvent, are placed in grooves in the sides and base of the frame. A lid (7, Fig. 3) with a silicon rubber gasket is also provided so that with the side panels, the syringe racks, and the lid in place, the elutor is essentially airtight.

[4] Cahn Instrument Co., Paramount, Calif.
[5] Non-Linear Systems, Inc., Del Mar (San Diego), Calif.
[6] Finishes Division, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

PROCEDURE

Preparation of Cups.—Using disks prepared as described in the preceding section, center a disk in each of the circles on the mold. Form the cups by pushing the disks into the holes with the cylindrical plugs and leave the plugs in place. Using the mold handle, place the mold on its side in a muffle furnace at 365°. Avoid movement of plugs while the mold is hot. After 17 minutes, remove the mold, cool at room temperature for 5 minutes, then quench with cold water. Rinse the mold with 95% alcohol, remove plugs, and separate cups. Cups are usually made in lots of 1,000 and sorted by weight into groups covering a 1-mg. weight range.

The plug and plastic disk should fit snugly into the hole in the mold to prevent the plug from falling out and to assure bonding of the folds in the sides of the cup. If the plug is too large, it may stretch or tear the plastic disk. The heating time can be varied to achieve good bonding of the folds and to prevent the film from adhering to the mold. Detect improper bonding of the folds or the presence of small leaks by placing a few sample cups on a piece of absorbent paper and half filling each cup with methanol. Press the cups firmly against the absorbent paper with a smooth glass rod. Dry the rod and rotate it around the outside of the cups. If the rod adheres to the outside of the cup or a moist spot appears on the paper, a breakage of the film is indicated. Place a few crystals of a methanol-soluble fluorescent material in the cup and allow the solvent to evaporate overnight at room temperature. Appearance of capillary streaks up the sides of the cup or the presence of fluorescent material on the outside of the cup when it is examined under ultraviolet light indicates poor bonding or breakage of the film. Repetition of this test is unnecessary when satis-

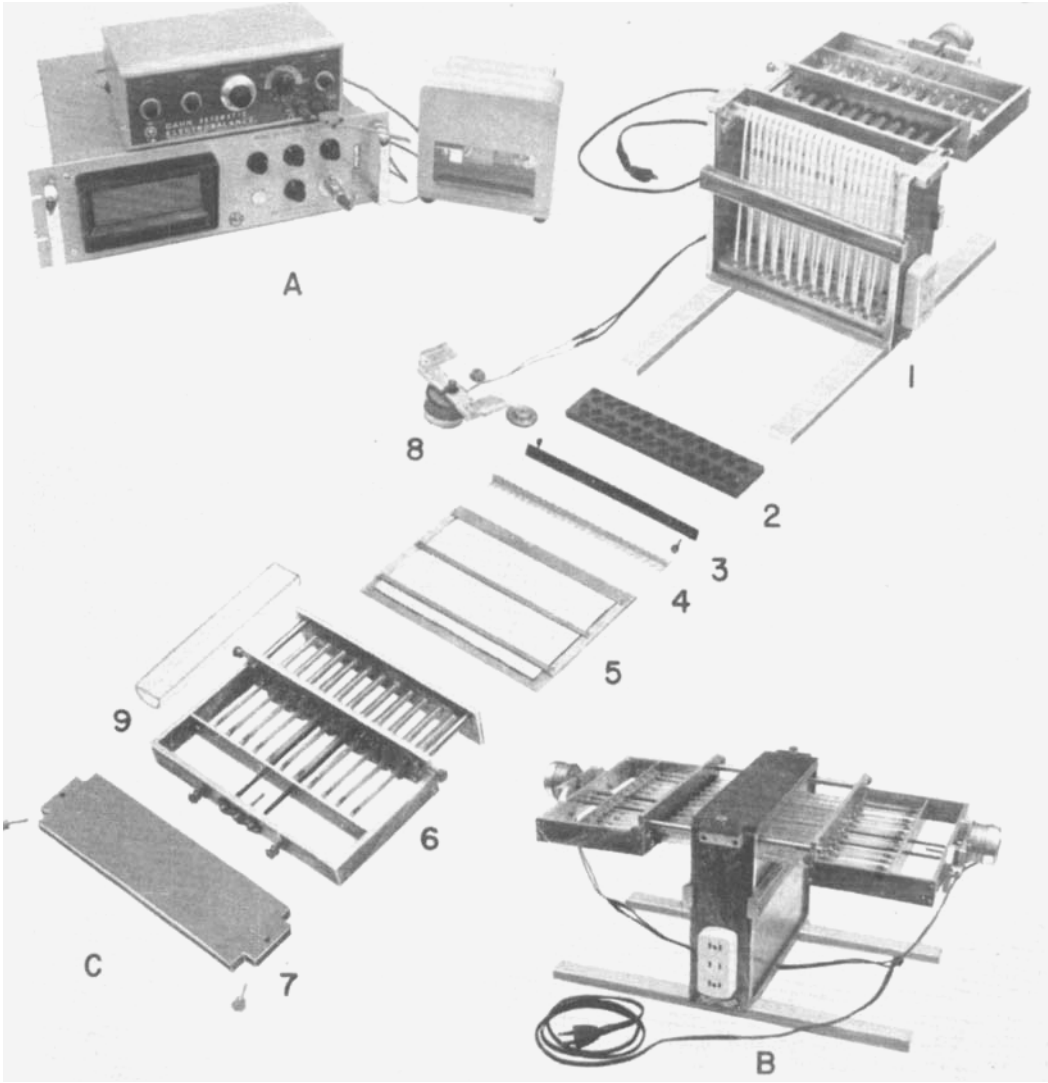

Fig. 3.—*A*, Automatic electrobalance with digital voltmeter; *B*, assembled; and *C*, breakdown view of multifraction elutor showing: 1, elutor with one side removed; 2, cup holder; 3, needle rack; 4, spacer bar; 5, side panel; 6, syringe rack; 7, lid; 8, motor with supporting frame; 9, glass trough.

factory conditions for producing suitable cups have been established.

Push the bottom of the cup outward by gentle pressure with a forceps so that the cups will rest securely on the balance loop. Immerse the cups successively in water, acetone, and petroleum ether; dry for 2 hours at 120° and store in a desiccator.

Preparation of Chromatographic Paper.—Draw lightly (with a lead pencil) a starting line 1 in. from the bottom of the paper, beginning $2^1/_2$ in. from the left edge and ending $^3/_4$ in. from the right edge of the paper.

Chromatographic paper may contain substances which will be removed by the eluting fluid and influence the weight of material determined. In most instances the major portion of these substances can be removed by successively washing the paper by descending chromatography with a solution of nitric acid in water (1–500), water, 95% ethanol, acetone, and petroleum ether. Dry the paper at room temperature and store to prevent contamination with dust or exposure to ammonia or other fumes. The procedure for washing the paper may be varied to suit the requirements of the problem.

Sometimes the successful separation of components of a mixture will require the addition of a chemical to act as an immobile phase. Since these chemicals may be removed from the paper by the eluting solvent, they should (whenever possible) be volatile or insoluble so that they will not contribute to the weight of the material in the cup after drying. When a nonvolatile substance is desirable as an immobile phase and it is soluble in the eluting solvent, correction for its weight may be possible through the use of a blank or from analysis of the residues in the cups. To prevent the contamination of the paper through handling following the application of the immobile phase, attach a glass rod to the top edge of the paper with Teflon-coated clips. This rod should be of the length required to support the paper in the development tank. After the paper has been saturated with the immobile phase, it is allowed to drain without blotting.

Application of Sample to the Paper.—Place the prepared paper in the applicator box (*A*, Fig. 2) with the starting line supported by the rods and immediately beneath the slit in the top of the box. If the paper carries a volatile material as the immobile phase, prevent excessive evaporation by wetting the blotting paper in the top of the box with the same material.

Weigh the desired quantity of the sample into a tared Teflon cup and dissolve in about 0.3 ml. of solvent. Using a weighed capillary tube with a fire-polished tip, transfer the sample solution to the paper by repeatedly streaking it uniformly along the entire length of the starting line. Rinse the cup and tube by adding two successive portions of about 0.1 ml. of the solvent and streak the resulting solutions on the starting line. The cup and the capillary tube are dried with the cups from the elution apparatus and reweighed to determine the weight of sample placed on the paper. Remove the paper from the box and evaporate the solvents used to dissolve the sample and the immobile phase by air drying in a hood briefly.

Development of Chromatogram.—The chromatogram may be developed by ascending chromatography using either a sealed tank or the continuous slit technique (6). If the latter technique is used, coating of the slit in the cover of the chamber with TFE-fluorocarbon resin primer[6] prevents the developing solvent from depositing some of the sample on the cover. Reattach the glass rod to the top of the paper after development. Remove the paper from the tank and evaporate excess mobile solvent by air drying in a hood. If the paper is to be cut after drying, wrap the paper loosely in Teflon-resin-coated aluminum foil and complete the drying in a vacuum oven. If the paper is to be cut while wet, place it between two Teflon sheets prior to cutting.

Cutting of the Paper.—Using the cutting equipment (*B*, Fig. 2), the paper is cut into strips at right angles to direction of development. Place the chromatogram and the template on the hardwood cutting board so that the right-hand edges coincide and the top edge of the paper is centered in the holes of the upper guideline of the template. Place the metal bars across the template to hold it in position. With either razor or surgical blades (the latter being preferred), cut paper into strips starting at the lower guideline. Any fractional strip remaining at the bottom of the chromatogram may be discarded.

Elution.—The required number of cups with a weight range of not more than 1 mg. are weighed to the nearest mcg. by counterbalancing and placed in the holes of the cup holder. Extra holes in the cup holder not required by the number of strips to be eluted can be used for controls which are desired. Attach the needle rack to the cup holder and place the holder in the elution apparatus.

With the aid of forceps, insert a $^1/_8$-in. aluminum rod immediately below the uncut portion of the chromatogram so that adjacent strips are on alternate sides of the rod. If Teflon sheets were used in cutting the chromatogram, they are now removed. With the aluminum rod, transfer the chromatogram to the spacer bar. When in position, adjacent strips should be on alternate sides of the spacer bar and in the appropriate slits. The tip of the strips should be about $^1/_8$ in. above the top of the cups. Adjust this distance by placing aluminum strips between each shelf and the ends of the spacer bar. Draw each strip taut and center it in front of the corresponding needle with metal forceps. Using a thin-walled glass tube of about $^1/_8$ in. in diameter, push the paper on the needle until the strip is centered over the cup.

The strips have a tendency to sag and buckle when wet with some eluting solvents. To prevent this, raise the spacer bar further by placing an additional metal strip $^1/_8$-in. thick between each end of the bar and the shelves. Thus the weight of the suspended cup holder exerts a constant tension on the strips.

Adjust the position of the movable crosspiece in each syringe rack so that the plungers can be pulled out slightly past the 1-ml. mark. Loosen the knurled knobs on the sides of the syringe rack, move the front plate back so that the ends of the syringes protrude about 1 in. from its face, and retighten the screws. Fill the trough (9, Fig. 3) with the eluting solvent. Immerse the ends of the syringes in the solvent. Remove the air in the syringes by filling and discharging the solvent. Finally, fill each syringe by drawing out the plunger until it is in

contact with the movable bar. Readjust the front plate of the carrier so that the syringes protrude about 1/2 in.

Moisten the blotter paper in the side panels with eluting solvent and put them in place. Insert the syringe racks into the appropriate slots and adjust so that the tips of the syringes are in firm contact with the centers of all strips. Attach the lid and the motor assemblies.

The motors are started after about 10 minutes to permit the partial saturation of the air in the elutor with the solvent. The time of elution can be varied by using different gears on the motor assembly. A short elution period is desirable with highly volatile, nonpolar solvents. The elution process can be automatically stopped when the plungers are near the bottom of the syringe barrel by powering the motors through a timer.

After allowing the solvent to drain from the strips into the cups for 10 minutes, remove the lid and the motor assemblies of the elutor. Adjust the syringe rack so the ends of the syringes will clear the spacer bar; remove the racks and the side panels. Evaporate the more volatile solvents by connecting the variable heater in the base of the elutor. Detach the needle rack from the cup holder and remove the spacer bar with attached paper and needle rack. Retain this assembly for additional studies which are indicated. Complete the drying of the cups by loosely wrapping the cup holder in aluminum foil and placing it in a vacuum oven. After drying, cool the cup holder to room temperature in a desiccating cabinet and reweigh the cups. Treat the results as indicated in the section on *Theory*.

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Gravimetric Evaluation of Paper Chromatograms II

Studies on Ouabain, Other Glycosides, and Prednisone

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Results are reported on the analyses of milligram quantities of some pure and crude steroid preparations. Samples are chromatographed on paper, and the entire sheet is rapidly eluted by a previously described technique. Components, in concentrations of 0.2 per cent or less, can theoretically be separated and detected. This technique may be found of equal benefit in the study of other compounds which can be separated by paper chromatography.

ANALYSES of steroid preparations by paper chromatography have been impeded by the difficulty in locating, rapidly eluting, and quantitating all components separated. This led to the development in this laboratory of new instrumentation and procedures reported previously (1). Results of some applications of this technique to steroid analyses are now given.

EXPERIMENTAL

Solvents

Neutral, readily volatile solvents were used. All solvents were percolated rapidly through a column of anion-cation exchange resin.[1] The first effluent through the column was discarded; the remainder was collected and redistilled. Formamide was redistilled under vacuum and stored in a desiccator over sulfuric acid until free of ammonia. Constant boiling fractions were returned to their original glass containers. Teflon[2] liners were placed in each bottle cap.

Chromatographic Paper

Whatman No. 3-mm. chromatographic paper in 8 × 8-in. sheets was ordinarily used. When required, 8 × 11 1/4-in. sheets were cut from a 23-cm. roll. Guidelines for streaking and development of paper were marked on all sheets; the paper was washed, dried, and stored (1).

Equipment

(*a*) Thomas-Mitchell chromatographic tank assembly with glass rods, troughs, and continuous technique accessories[3] was used with 8 × 8-in. sheets. The slotted cover and clips were coated with Teflon (1). (*b*) Thomas-Kolb chromatographic jar[3] was used with 8 × 11 1/4-in. sheets. A double-slotted cover, similar to the above accessory, was made for the jar and was coated with Teflon. (*c*) Two Pyrex 3-quart utility baking dishes,[4] each 9 1/4 × 14

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[1] Rohm and Haas Amberlite MB-1, analytical grade, indicator-free was satisfactory.

[2] Marketed by E. I. du Pont de Nemours & Co., Inc. Wilmington, Del.
[3] Arthur H. Thomas Co., Philadelphia, Pa.
[4] Most department stores.