A Manual of

PAPER CHROMATOGRAPHY

and

PAPER ELECTROPHORESIS

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Paper Chromatography

Paper Electrophoresis

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PREFACE TO PART I, SECOND EDITION

The object of the first section of this monograph is to provide a readily accessible source for some of the many uses of paper chromatography that have appeared since the publication by Consden, Gordon, and Martin approximately fifteen years ago. No attempt has been made to list all the references in which paper chromatography has been used but rather an effort has been made to write a practical manual in which tried and proved procedures, employing relatively simple equipment and available reagents, are summarized. It is hoped that sufficient detailed information is provided, in the majority of instances, so that the reader will be able to apply the technique of paper chromatography to his particular problem without recourse to an extensive search of the literature.

The authors will appreciate the corrections of any erroneous statements and hope that readers will be so kind as to call to our attention important papers which may have been omitted.

We wish to thank many workers in the field of paper chromatography who contributed numerous photographs and drawings.

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PREFACE TO PART II, SECOND EDITION

A number of changes have been incorporated in the present edition, particularly in Chapter XIX. A classified bibliography of more than 1800 articles on paper electrophoresis has been added.

The author particularly wishes to thank Dr. Henry Kunkel and Dr. Rodes Trautman for valuable suggestions relating to the treatment of mobility which appear in Chapter XV. Mr. F. G. Williams has afforded great assistance, particularly in connection with revisions of the chapter relating to quantitative considerations. I also wish to thank Miss Ernestine Hutchins and Mrs. Eve Lier for their services in connection with the organization and classification of the bibliography.

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PAPER CHROMATOGRAPHY

Chapter I

INTRODUCTION

"By chromatography is meant those processes which allow the resolution of mixtures by effecting separation of some or all their components in concentrated zones on or in phases different from those in which they are originally present, irrespective of the nature of the force or forces causing the substances to remove from one phase to another" (T. I. Williams, 1952).

Paper chromatography may have had its origin with the description by Pliny (23–79 A.D.) of the use of papyrus impregnated with an extract of gall nuts for the detection of ferrous sulfate. Or it may have had its origin with the studies of "Kapillaranalyse" by Runge, Schönbein, and Goppelsroeder[1] in the period 1850 to 1910. (See also Weil, 1950, 1951, 1953*a*, *b*.)

There is little doubt that M. S. Tswett should be given credit for discovering the principle of preferential adsorption (adsorption chromatography) of plant pigments on a large variety of adsorbents packed in a glass tube. Tswett reported his findings to the Biological Section of the Warsaw Society of Natural Sciences in 1903. (See Hesse and Weil, 1954, for a translation of Tswett's first paper on chromatography.) It is not within the scope of this book to cover the field of column chromatography.

The great popularity of the present-day paper chromatography is due, in the authors' opinion, to A. J. P. Martin of Cambridge and London and his coworkers, R. Consden, A. H. Gordon, and R. L. M. Synge. In 1938 Neuberger was interested in separating the neutral amino acids and observed that the partition coefficients of acetylated amino acids between water and an immiscible organic solvent differed for the various amino acids. This observation was extended by Martin and Synge (1941*a*), who built a rather complicated forty-plate apparatus for the continuous separation of acetylated amino acids. In order to simplify the equipment and to enhance the separation of substances with very similar partition coefficients by increasing the number of theoretical plates, Martin and Synge (1941*b*) decided to use an inert support (e.g., silica gel) to hold one of the phases (water) and to pass the immiscible solvent through a bed of the water-containing silica gel. The ideal conditions for this type of chromatogram employing two liquid phases were, first, that the solute is not absorbed by the supporting material and, second, that its distribution between the two liquid phases is not influenced by its concentration or by the presence of closely related solutes. These ideal conditions were approached experimentally by Mar-

[1] Cf. W. G. Brown (1939) for an adaptation of capillary analysis.

tin and Synge and by a number of other investigators. The procedure was, however, difficult and tedious, and the preparation of the inert support left much to be desired.

In order to eliminate the preparation of silica gel and to reduce the quantity of materials needed, Martin *et al.* (Consden, 1944) replaced silica gel by filter paper as the inert support. With the use of filter paper the acetylation of the amino acids was no longer necessary, and they could now be detected directly on the paper by treatment with ninhydrin (triketohydrindene hydrate).

The method of paper partition chromatography consists in applying a *small* drop of the solution containing the substances to be separated to a strip of filter paper a short distance from one end. The drop is allowed to dry, and the end of the paper nearest to the spot is placed in the developing solution, usually a water-containing organic solvent, so that the solvent flows past the "spot" by capillary action and on down the length of the paper.

Although it was originally believed that the paper functioned solely as an inert support for the aqueous portion of the developing solvent, hence the original name paper partition chromatography, it is now generally recognized that, although paper chromatography may function in some cases purely by partition, it more commonly acts by a combination of partition, adsorption, and ion exchange. Regardless of the mode of action, the modifications, or the extensions of the method of paper chromatography, there has seldom been a technical development so thoroughly described and so adequately presented. In spite of hundreds of studies with this procedure, no major improvements or changes have been made since the original publication of "Qualitative Analysis of Proteins: A Partition Chromatographic Method Using Paper" by R. Consden, A. H. Gordon, and A. J. P. Martin in 1944.

The impetus given by Martin *et al.* has encouraged other investigators to apply the procedures of paper partition chromatography, capillary analysis (Goppelsroeder, 1899), and combinations of these methods to a host of substances, natural and synthetic, organic and inorganic, with striking success. The object of this manual is to present some of the results of these numerous investigations on paper chromatography so that the student may have a sufficient idea of past studies in order to allow him to choose the method which appears to be the most promising for the solution of his particular problem. Although experiments on both qualitative and quantitative paper chromatography are described, the quantitative aspects are stressed where possible.

Because this manual is of a practical nature, the theoretical aspects of

chromatography are minimized, although it will be seen from the results presented that certain simple rules should be employed:

1. The composition of the flowing solvent should be kept constant throughout the development. This is done by keeping the chromatogram in an enclosed chamber, the space of which is saturated with the developing solvents at constant temperature.

2. The developing solvent should move at a relatively slow rate (*ca.* 2–3 cm./hr.). The rate of solvent flow is dependent on the type of paper used, on the ratio of the width of the "wick" to that of the paper chromatogram, on the composition of the solvent, and on the temperature of the chromatogram chamber.

3. The choice of a solvent should be one in which the components to be separated have a small but definite solubility. If the substances are too soluble, they will appear at or near the solvent "front" of the chromatogram. If they are too insoluble in the solvent, they will remain at the point of application. If the factors of adsorption and ion exchange are neglected, the movement of a substance in a paper chromatogram is a function of its solubility in the developing solvent. Thus, solvents for water-soluble substances are usually water-containing organic compounds, whereas solvents for substances soluble in organic solvents but insoluble in water are often aqueous solutions of organic solvents.

Chapter II

THEORY OF PAPER CHROMATOGRAPHY

The resolution of mixtures of solutes on filter paper may depend on surface adsorption, on ion exchange, or on partition between solvents. Goppelsroeder's investigations of the capillary ascent of organic and inorganic solutes into strips of paper were examples of adsorption chromatography. Substances adsorbed to filter paper were separated by passing through the paper a solvent which would preferentially elute each substance in the mixture. The separation of materials on strips of filter paper impregnated with alumina is another example of adsorption phenomena (Flood, 1949).

Ion exchange may have an effect on the separation of substances on paper. In the resolution of mixtures of ions, some exchange must occur with polar constituents of the cellulose and with impurities present in the paper.

Although adsorption and ion exchange must be present to some extent in all chromatographic work on filter paper, the predominant factor is usually that of partition between two immiscible phases. In the early work on separation of mixtures of amino acids, Consden (1944) found that excellent separations were obtained with solvents that were only partially miscible with water. After equilibration of the paper with the vapor of a solvent saturated with water, solvent development produced separations.

The movement of a solute zone was explained conveniently as follows: The cellulose fibers have a strong affinity for the water present in the solvent phase but very little for the organic liquid. The paper itself is thought of as an inert support holding a stationary aqueous phase. As solvent flows through a section of the paper containing the solute, a partition of this compound occurs between the mobile organic phase and the stationary water phase. Thus, some of the solute leaves the paper and enters the organic phase. When the mobile liquid reaches a section of the paper containing no solute, partition again occurs. This time, solute is transferred from the organic phase to the paper phase. With continuous flow of solvent, the effect of this partition between the two phases is the transfer of a solute from the point of its application to the paper to a point some distance along the paper in the direction of solvent flow.

The processes which occur during the chromatographic analysis on filter paper may be compared with the techniques of fractional distillation and continuous liquid-liquid extraction. Martin and Synge (1941*a*) first experi-

mented with a stage continuous liquid-liquid extraction train for the separation of amino acids. This laborious procedure was simplified and improved by immobilizing one phase on a mechanical support known to have weak adsorptive properties, such as silica, starch, or paper.

These investigators have worked out a theory of chromatography based on its similarity to distillation with fractionating columns. This analogy gives a picture of the concentration of solute at any time and place in a chromatographic column and of the way in which the resolution of a mixture depends on the length of the column. (Martin and Synge developed this treatment of chromatography in connection with separations on columns of silica gel, but it is also applicable to partition chromatography on paper.)

The chromatographic column is regarded as being divided into successive layers of such thickness that the solution issuing from each is in equilibrium with the mean concentration of solute in the non-mobile (silica) phase throughout the layer. The thickness of such a layer is termed the H.E.T.P. (height equivalent to one theoretical plate). For the equations to be manageable, certain simplifying assumptions are made. It is assumed that diffusion from one plate to another is negligible and that the partition of solute between the two phases is independent of its concentration and of the presence of other solutes. The following symbols are used.

h = H.E.T.P.
A = Area of cross section of the column.
A_s = Area of cross section of the non-mobile phase.
A_1 = Area of cross section of the mobile phase.
A_I = Area of cross section of the inert solid; *i.e.*, $A_s + A_1 + A_I = A$.
v = Volume of solvent used to develop the chromatogram.
α = Partition coefficient = grams solute per milliliter of non-mobile phase per gram solute per milliliter of mobile phase at equilibrium.
V = $h\,(A_1 + \alpha A_s)$.
R = $\dfrac{\text{Movement of position of maximum concentration of solute}}{\text{Simultaneous movement of surface of developing fluid in the empty part of the tube above the chromatographic column.}}$
r = Serial number of "plate" measured from top of column.
Q_r = Total quantity of solute in plate r.

Suppose that a unit mass of a single solute is put into the first plate and is followed by pure solvent; it is possible to calculate the amount of solute in each plate after infinitesimal volumes, δv, of the mobile phase have passed (see Table I). The quantity of solute in each plate can be expressed as a term of the binomial expansion of $[(1 - \delta v/V) + \delta v\ V]^n$. When n

TABLE I

Volume of solvent passed	Serial number of plate, r			
$x\,v$	1	2	3	4
0	1	0	0	0
1	$(1-\delta v/V)$	$\delta v/V$	0	0
2	$(1-\delta v/V)^2$	$2(1-\delta v/V)\delta v/V$	$(\delta v/V)^2$	0
3	$(1-\delta v/V)^3$	$3(1-\delta v/V)^2\delta v/V$	$3(1-\delta v/V)(\delta v/V)^2$	$(\delta v/V)^3$
4	$(1-\delta v/V)^4$	$4(1-\delta v/V)^3\delta v/V$	$6(1-\delta v/V)^2(\delta v/V)^2$	$4(1-\delta v/V)(\delta v/V)$

successive volumes of solvent δv have passed,

$$Q_{R+1} = \frac{n!(1-\delta v/V)^{n-r}(\delta v/V)^r}{r!(n-r)!}. \tag{1}$$

When n is large, this becomes

$$Q_{r+1} = \frac{1}{r!}\left(\frac{n\,\delta v}{V}\right)^r e^{-n\delta v/V}. \tag{2}$$

But $n\,\delta v = v$ = volume of solvent used to develop the chromatogram. Therefore,

$$\frac{Q_{r+1}}{z} = \frac{1}{r!}\left(\frac{v}{V}\right)^r e^{-v/V}. \tag{3}$$

By Stirling's approximation this becomes, when r is large,

$$Q_{r+1} = \frac{1}{(2\pi r)^{\frac{1}{2}}}\left(\frac{v}{Vr}\right)^r e^{r-v/V}. \tag{4}$$

When $v/rV = 1$, Q_{r+1} is a maximum and has the value $(2\pi r)^{-\frac{1}{2}}$. If r is the number of the plate containing the maximum concentration of solute, its distance from the top of the column is rh. But

$$rh = \frac{hv}{V}, \tag{5}$$

i.e., the position of maximum concentration has moved a distance hv/V directly proportional to the volume of solvent used to develop the chromatogram. If

$$R = \frac{\text{Movement of zone}}{\text{Movement of surface of liquid}}, \tag{6}$$

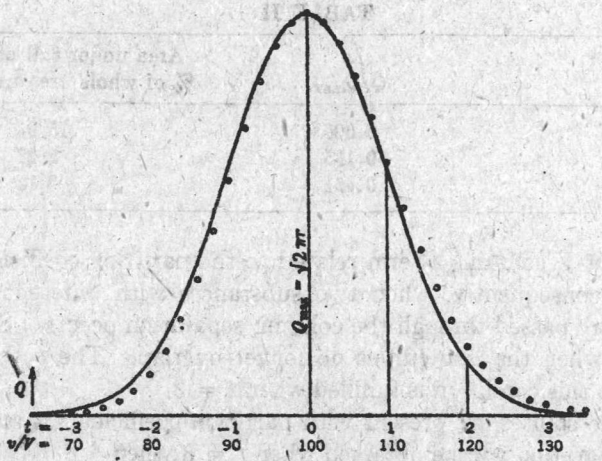

FIG. 1. The points represent the relation between Q and v/V for $r = 100$. The full line is the normal curve of error with abscissa t, *i.e.*, $Q/Q_{max} = \exp(-\frac{1}{2}t^2)$.

then

$$R = \frac{vh/V}{v/A} = \frac{Ah}{V} = \frac{A}{A_1 + \alpha A_s} \tag{7}$$

Therefore,

$$\alpha = \frac{A}{RA_s} - \frac{A_1}{A_s}. \tag{8}$$

If the concentration of solute in plate $(r + 1)$ is plotted against v/V using equation 4, the curve shown in Fig. 1 is obtained. This curve is plotted for $r = 100$. When r becomes infinite, this curve becomes identical with the normal curve of error and can be expressed in the form

$$\frac{Q}{Q_{max}} = e^{-\frac{1}{2}t^2}. \tag{9}$$

v/V and t are related by the equation

$$\frac{v}{V} = r + t(r)^{\frac{1}{2}} + \frac{t^2}{3}. \tag{10}$$

Similarly, the area under the curve can be expressed as

$$\frac{v}{V} = r + t(r)^{\frac{1}{2}} + \frac{t^2}{4}. \tag{11}$$

The values of Q/Q_{max} for various values of t are shown in Table II.

For a given solute the position of maximum concentration is determined

TABLE II

t	Q/Q_{max}	Area under tail of curve as % of whole area under curve
1	0.606	15.9
2	0.135	2.27
3	0.011	0.13

by v/V. But V contains a term related to the partition coefficient of the solute, and consequently, when two substances with different partition coefficients are passed through the column, separation occurs. Separation is complete when the two curves no longer overlap. The figures shown indicate that this condition is fulfilled when $t = 3$.

Now if two solutes are present with partition coefficients α and β, then complete separation will be obtained when $t = 3$; then

$$\frac{A_1 + \alpha A_s}{A_1 + \beta A_s} = \frac{r - 3(r)^{\frac{1}{2}} + 2.25}{r + 3(r)^{\frac{1}{2}} + 2.25}, \tag{12}$$

since less than 0.2% of the solute with partition coefficient β has passed the $(r + 1)$th plate and more than 99.8% of the solute with partition coefficient α is passed the same plate.

In practice, the whole of the solute will not be initially concentrated in the first plate but will be spread over a number of plates. The effect of this will be for a number of chromatograms to be successively started, and the concentration in any plate at a given time will be the sum of the concentrations due to each chromatogram. Thus the region of maximum concentration will be broadened, and the total width of the band will be greater than that shown in Fig. 1 approximately by the initial width of the band before development by solvent was begun. The number of plates required for resolution will be correspondingly increased.

The separations obtainable in practice are less than the theory predicts for two principal reasons. First, the partition coefficient is seldom a constant, usually decreasing as the solution becomes stronger. This results in the front of the band becoming sharper and the rear more diffuse and wider, since the concentrated part moves faster than the more dilute part.

An increase in separation over the theoretical may be produced by interaction between two solutes. The more strongly adsorbed solute may displace the weakly adsorbed one and form a sharp boundary between the two.

The conclusion that R and α are related was checked by Martin and Synge in an actual experiment. They passed a solution of 2 mg. each of