CHROMATOGRAPHIC TECHNIQUES

Clinical and Biochemical Applications

Edited by

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FOREWORD

WE are in the midst of the feverish activity which has been stirred up in this new chromatographic era. During the last ten years relatively simple techniques have been developed which enable the identification to be made of microgram quantities of complex materials without any need for prior purification or isolation. A new world of scientific investigation has been opened up on which we can now stake claims and then explore and exploit. It is possible for any or all of us to participate in this new gold rush and to note with awe the bewildering profusion of new as well as of well-known compounds which can be revealed in almost any biological material. The literature cannot keep up with the current rapid expansion of knowledge and of improvements in techniques. Those who are already experienced in one particular application but who must at times wander into new directions soon become painfully aware of their inadequacy, and of that of the literature in question. For those who begin the problem is worse.

It is against this background that I view this excellent production by Dr. I. Smith and his many colleagues. Thank goodness somebody competent has felt the urge to lay aside for the moment his other more exciting work and to stimulate and organize the production of an excellent and concise statement of the present position with regard to the major present uses of chromatography. They have concentrated especially on the clinical and biochemical applications of qualitative paper chromatography, which must always be the ideal technique for beginning any particular investigation. They have described many of the more important methods in critical and concise fashion with key references for those who are more serious. In addition an attempt has been made to help with the interpretation of results for those especially concerned in the investigation of human disease. I think this book will remain useful for a longer time than do most books written about rapidly evolving subjects, and I am most grateful to them for having undertaken it.

C. E. DENT

London, May, 1957.

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PREFACE

THE newcomer to paper chromatography is confronted with an enormous number of books, scientific papers and catalogues describing a bewildering variety of apparatus much of which is of value only for particular purposes. Thus paper sheets of every possible size and shape, held up by cotton, staples, glass or metal or polythene frames and troughs in apparatus containing a variety of subsidiary devices for establishing equilibrium have been used. I therefore set myself the task, with the aid of J. B. Jepson, of designing a "Universal Chromatographic Apparatus" which would be of value for research, routine and teaching purposes and it appears that this has now been accomplished.

The main apparatus and the associated methods and techniques were demonstrated at two scientific meetings (Discussion on "Paper Chromatography and Electrophoresis" arranged by the Fine Chemicals Group of the Society of Chemical Industry and held at King's College, London in January 1954; Midlands Society of Analytical Chemistry annual general meeting, held at the University of Birmingham, summer 1954) and the interest it evoked led first to a stream of enquiries as to its availability and adaptability and later to its use in many fields of biochemical investigation. Further, a unique opportunity arose for testing the apparatus and methods on over two hundred post-graduate and post-doctoral students at a series of Practical Courses in Paper Chromatography which I held at the Technical College, Acton, and where the apparatus and techniques used were solely those described here. The results were highly successful and illustrated that, even with no previous experience in this field, it was possible for the students to produce satisfactory chromatograms. It is in response to many requests from these students and colleagues that this book has been written, but it is hoped that it will also be of value particularly to clinical biochemists as well as to all those who use chromatography in organic, agricultural and biochemistry.

As well as this apparatus there is also described a very simple but equally satisfactory apparatus in which identical chromatographic separations can be obtained. This is of value not only for the smaller laboratory, or when only a few samples have to be run, but also because many of the methods are now brought within the reach of the family doctor and I am already aware of a number of such doctors who use chromatography as an adjunct to diagnosis and as a means of carrying out research on their own patients. The cost of this latter apparatus, with the necessary chemicals and solvents, is very small and, if used locally, might well take a great portion of the screening work off the hospital service. The use of rapid routine screening by chromatographic methods may well save weeks in the diagnosis of certain diseases and the application of such methods to the study of genetically determined variations in metabolism has hardly been appreciated so far. Although the original aim of describing one major apparatus of general application

has been accomplished I felt that in the case of the steroids the original apparatus and technique has proved of such value that it was worth retaining in its entirety.

Briefly the purpose of this work is, therefore, to bring together in concise form tried and tested methods and techniques which have been used by clinical and other biochemists to solve some of their problems. Its function is not to discuss the history and theories of chromatography—all of which has been done admirably elsewhere. References to other chromatographic methods have thus been kept to a minimum whereas references to applications have been made as full as possible in a book of this size.

In the two general chapters there are instructions on the use of the chromatographic and desalting apparatus described and this is followed by a full discussion on the preparation and use of solvents and location reagents and the factors which affect the movement of a substance on the chromatogram; incorporated in this discussion are answers to many of the questions raised by newcomers to the field. I have felt that it is far more useful to describe and discuss in detail one method of preparing a reagent, e.g. ninhydrin, which has been thoroughly tested than to list the hundred or so ways in which others have used it with varying results and sensitivities. A feature of this book is, therefore, that many of the methods have been used successfully over a number of years and that each method and reagent is considered critically with respect to its value, reactivity, and limitations. However, where the interest in a family of compounds is only very recent, e.g. the phenolic acids, then the methods have not been able to stand the test of time; nevertheless these methods should be of value to those about to enter into a study of such new and interesting compounds.

I believe that experiments using—and not simply illustrating—chromatographic techniques should now form part of the training of all science and preclinical students and so a number of simple experiments suitable for undergraduates have been incorporated into one chapter at the end of the book. This chapter has been written in such a way that, apart from references to pictures of apparatus in other parts of the book, it can be taken as a separate entity and used by the undergraduate as an introduction to the subject. Some of the experiments are taken from the course for preclinical students at the Middlesex Hospital Medical School and others from the post-graduate courses previously mentioned.

Recently, and again in conjunction with J. B. Jepson, I made a teaching film entitled "Principles of Chromatography" (16 mm. sound, colour; available from "Educational Foundation for Visual Aids," 33 Queen Anne Street, London, W.1), which illustrates the techniques and apparatus described here and in which many of the student experiments described in Chapter 17 are carried out. The film has been found most valuable as an introduction to the subject for all grades of student unfamiliar with this technique.

Much of the work described here was originally intended for publication in the usual scientific journals, but the authors decided to incorporate this unpublished material with their other published results in

order to present a more complete review of their subject. In other cases, authors have incorporated results which were already in the press and which will have appeared only a short while before this monograph. Finally, some workers have allowed us to make use of their results although they do not figure personally as authors. To all of these colleagues I am grateful, as much of the merit this book may have is due to them.

I am grateful to many workers, now far too numerous to mention, who donated authentic compounds for the determination of R_f values and colour reactions, or urine specimens; to B. J. Stevens who built my first electrolytic desalter and carried out much of the early experimental work on that apparatus; to J. J. Loughlin, A.I.S.T., chief technician at the Courtauld Institute, and members of his staff who built much of the apparatus described here before it became commercially available; to my wife who carried out much of the routine practical work; to the Middlesex Hospital Photographic Department for photographing all the chromatograms and apparatus shown here; to J. B. Jepson who read and criticized the whole manuscript, made many valuable suggestions now incorporated into the text, drew the standard maps and gave continuous help and encouragement to me; and to Sir Charles Dodds for permission to carry out all the experimental work in his laboratory.

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May, 1957.

All of the apparatus described in this book is available from: Aimer Products Ltd., 56–8 Rochester Place, Camden Town, London, N.W.1.

CONTENTS

CHAPTER 1

INTRODUCTION

A PAPER chromatogram is prepared in the following way. A drop of the solution containing the compounds to be separated is placed near the end of a strip of paper and allowed to dry. The strip is then placed so that a few millimetres of it dips into a solvent but it is essential that the dried spot is not immersed in this—otherwise it will dissolve off into the solvent and be lost. Solvent then commences to flow along the paper, over the spot and towards the far end of the paper and when it reaches this end, or when some convenient time has elapsed, the strip is withdrawn and rapidly dried. In order to prevent evaporation from the surface of the paper the operation is conducted in an air-tight container. Thus a one-way or one dimensional strip chromatogram has been prepared. If the paper acted merely as an inert support, separation of the components of the mixture would be caused exclusively by continuous partition between the solvent flowing along the paper and the water held in the paper—filter paper contains about 15 per cent of its weight of water—and this may be the case in some circumstances. More usually the paper does affect the separation process in a number of ways: it acts as an adsorbent in a similar manner to the alumina column; it has a strong affinity for polar molecules which are held by hydrogen bonding and van der Waals forces; it functions as an ion exchange material due to its content of carboxyl groups. Which of these forces predominates in any particular situation depends on the type of compounds being separated and the solvents used. In all cases, however, the result is the same, namely, the compounds present in the original mixture become distributed along the length of the strip and a partial or complete separation is achieved. For convenience, the position of a substance on a chromatogram is specified by its "R_f," which is defined as the distance the material has moved from the original point of application divided by the distance the solvent front has travelled from that point (see Fig. 1.1).

Paper chromatography as practised today derives from the work of Consden *et al.*[1] They described an apparatus in which the strip was suspended in a trough to which solvent was then added. This solvent flowed down the paper, by gravity, to produce a one-way descending paper chromatogram, the chromatogram being removed and dried after the solvent had flowed a sufficient distance; an example of the type of apparatus in current use is that shown in Figs. 2.6 and 2.5 pp. 15, 13.

Because of the original difficulties in constructing satisfactory apparatus, Williams and Kirby[2] modified the technique so that the solvent flowed up the strip instead of down. The strip, prepared as before, was suspended vertically so that one end just dipped below the surface of the solvent which then flowed upwards, by capillary action,

over the spot and towards the far end of the paper which was removed and dried after the required length or time of flow; in this way an ascending strip chromatogram was prepared. Apparatus useful for this technique is shown in Figs. 2.3 and 2.1 p. 10, 8. Strip chromatograms prepared by either the ascending or descending technique yield separations similar enough to be compared.

Other apparatus has been described[3] in which the strip is held horizontally and solvent is fed to it by means of a short wick of paper or a glass capillary dipping into a small beaker of solvent just below the strip. Solvent travels up the wick and then moves horizontally along the paper to produce a horizontal (or radial or circular) chromatogram; these cannot be compared with ascending or descending chromatograms.

Where a mixture contains many components it will be obvious that complete separation of all of these on a strip of finite length may not occur, and many examples are known where two substances run to the same position on the chromatogram. In order to overcome this difficulty recourse has been made to the use of a number of different solvents with different properties, so that components running together in one solvent will probably separate in another solvent. Although many one-way chromatograms, each in a different solvent, could be compared, a great deal more information is obtained if two solvents are used in conjunction to prepare a two-way chromatogram than if the two are used to prepare two separate one-way papers (see Fig. 1.1). A two-way chromatogram is prepared by placing a drop of the mixture near a corner of a square or rectangular sheet of paper. Solvent is then allowed to travel up the whole sheet with the result that a one-way separation is obtained and then, after drying completely, the paper is turned at right-angles and run in a second solvent which performs a further separation and causes the components to be distributed on the paper in two dimensions instead of the previous one dimension. Similar two-way separations or "sheet" chromatograms are obtained by the ascending or descending techniques; only one-way separations are possible with the radial method which is thus the most limited method of the three. Whichever method of chromatography is used it is valuable to prepare standard "maps" by chromatographing mixtures of pure authentic compounds and these can be kept as permanent records either by tracing round the spots obtained or by photographing the chromatogram; with aminoacids, for example, a mixture of those aminoacids usually found in protein hydrolysates (or a hydrolysate from a purified protein of established composition such as egg albumen) should be chromatographed. With solutions of biological origin such as urine, it is valuable to chromatograph a number of normals so that the "pattern" may impress itself on the memory and later enable unusual or abnormal spots to be detected rapidly.

Using the techniques described above it is possible to obtain a satisfactory one or two-way paper chromatogram of a single mixture. However, in all research and control work it is usually essential to be able to prepare many chromatograms under the same conditions, often simultaneously, and much labour has been expended on the design of a simple apparatus in which this may be accomplished. In my experience

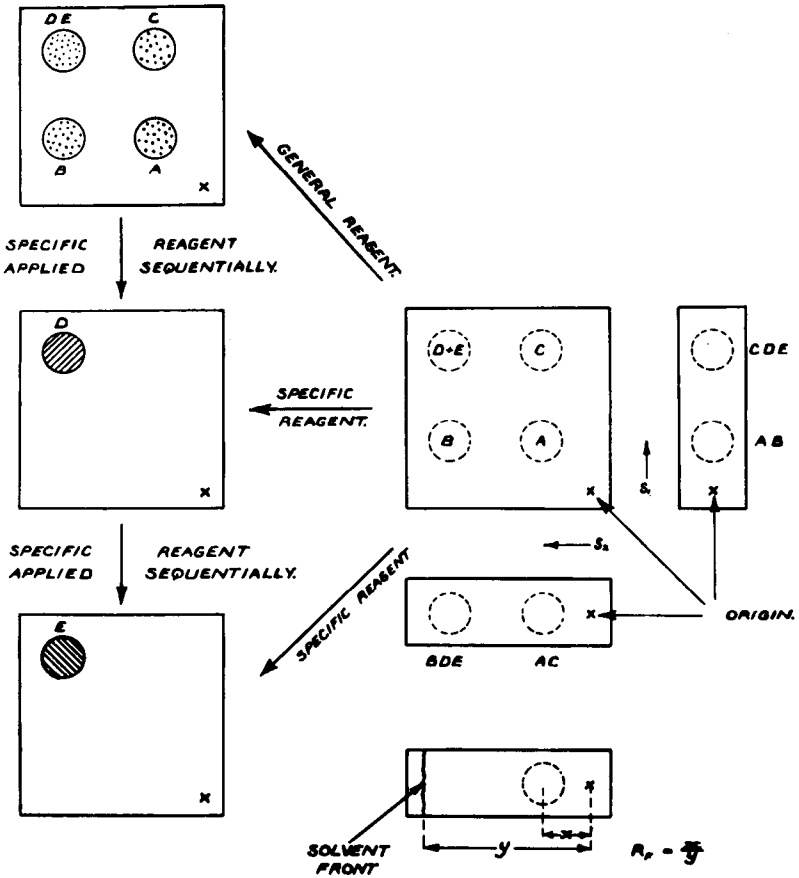

FIG. 1.1. Diagram illustrating one- and two-dimensional paper chromatography, the use of general and specific reagents and sequential application of reagents (multiple dipping). ABCDE are five components present in a mixture. In solvent S_1 the R_f values of A and B are equal; those of C, D and E are also equal. In solvent S_2 the R_f values of A and C are equal; those of B, D and E are also equal. A one-way chromatogram using either solvent therefore shows only two spots on location with a general reagent. A two-way chromatogram shows four spots on location with a general reagent, but by the application of specific reagents to duplicate chromatograms one of the spots is shown to be a mixture of two components D and E having the same R_f in both solvents. When the reagents can be applied sequentially only one chromatogram need be prepared, otherwise three separate chromatograms are necessary.

the most valuable apparatus so far described is the ascending frame of Datta *et al.*[4] and a slightly modified version of their apparatus has been used as the basic apparatus (see Fig. 2.1 p. 8) for all work described in this book except that on the steroids. Their apparatus consisted of a simple frame on which papers, 8 in. square, were held and with which many one-way or two-way chromatograms could be prepared with a minimum of time, labour, space, and chemicals. The dimensions were increased to 10 in. square as interest originally centred around the aminoacids, and this was the distance that the phenolic solvent ascended in an overnight run; experience has since shown that the size is admirable for the separation of many families of compounds, including a number not mentioned here. The 10 in. square papers used here enable ten one-way chromatograms or one two-way chromatogram to be run on each sheet so that a total of 50 one-way or 5 two-way separations can be obtained on the one frame in a single tank. The most important point about this apparatus, however, is the fact that chromatograms of a very high degree of reproducibility can be obtained and that actual papers, prepared on many different occasions, can be directly compared; thus the standard aminoacid mixtures which we have used for our work in this field have yielded practically identical maps whenever they have been used for both one- and two-way chromatography. In general, the ascending technique is completely satisfactory for the investigation of almost all problems but as there are certain cases where the descending technique has advantages (see later) the apparatus has been so designed that it may be easily and quickly converted to one in which descending chromatograms may be prepared (see Fig. 2.5 p. 13).

Paper chromatography is a technique for effecting the separation of closely related substances but, as the majority of compounds of biological importance are colourless and so not visible on the paper, the problem of locating these still remains. The most widely used method is that of applying to the dry chromatogram a chemical reagent which will react with some or all of the compounds of interest to produce a colour—this being done by dipping the chromatogram through a bath of the reagent (see Fig. 2.9 p. 25) followed by some appropriate treatment such as allowing to dry in air or heating until the colours appear. The location reagent may be a general one, that is one which will locate all the components of interest, or a more specific one, that is one which reacts with only one component in the original mixture. Until recently it was necessary to prepare a separate chromatogram for each reagent but by use of the "multiple-dipping" technique of Jepson and Smith[5] it is now possible to apply a sequence of three or four reagents to a single chromatogram with the result that a great deal more information can be obtained with very little extra labour (see discussion p. 26). In fact it may be considered that this multiple procedure lends a "third dimension" to paper chromatography.

It cannot be too often stressed that with the enormous number of naturally occurring substances which will react with any given reagent it may be essential to prepare two-way chromatograms—possibly with different pairs of solvents—and to make use of the maximum number of location reagents. All of these points are illustrated in Fig. 1.1.

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Books

(*a*) Introductory Books

Elements of Chromatography. Williams, T. I. Blackie, London, 1954.
A good general survey of Paper and Column Chromatography.

Introductory Notes on Chromatography. British Drug Houses, Poole, 1948.
Deals exclusively with column chromatography. Free pamphlet.

(*b*) Comprehensive Monographs

Principles and Practice of Chromatography and *Progress in Chromatography*. Zechmeister, Chapman & Hall, 1950.
The above two books deal almost exclusively with adsorption columns.

A Manual of Paper Chromatography and Paper Electrophoresis. Block, R. J., Durrum, E. L., and Zweig, G. Academic Press, New York. 2nd Edn., 1955.
Very comprehensive but not very critical. Recommended.

Paper Chromatography. Cramer, F. 2nd Edn. English Translation. Macmillan, London, 1954.
Very good but not as comprehensive as Block's book.

A Guide to Filter Paper and Cellulose Powder Chromatography. Balston & Talbot. Reeve Angel, London, 1952.
An excellent review of the literature up to 1952. It will not be reprinted.

Modern Methods of Plant Analysis. Edited by Paech & Tracey. Springer, Berlin, 1955.
This work contains much critical information on the chromatographic investigation of plant materials.

Chromatography. British Medical Bulletin, 1954, volume 10, No. 3.
This contains some brilliant chapters by specialists in different fields of chromatography applied to medicine and biochemistry. Most chapters contain chromatographic details followed by interpretation of results.

Spot Tests in Organic Analysis. F. Feigl. Elsevier Publishing Co. 5th Edn., 1956.
This outstanding book lists an enormous number of spot tests which can be carried out in solution. It is usually a simple matter to modify these for use as chromatographic location reagents.

CHAPTER 2

CHROMATOGRAPHIC APPARATUS AND TECHNIQUES

The Chromatographic System. The fundamental measurement in chromatography is that of R_f, which is defined as follows:

$$R_f = \frac{\text{distance substance travels from the origin}}{\text{distance solvent front travels from the origin}}.$$

This value, which is a physical constant of the substance concerned, should therefore be reproducible. However, it is commonplace that published R_f values vary, to a greater or lesser extent, from apparatus to apparatus. The reason for this is, of course, that it is seldom the case that the chromatographic system is fully described and so, in effect, every worker is using a different system. Such a description should include the following:

1. The dimensions of the apparatus.
2. The grade of paper.
3. Ascending or descending solvent flow and length of flow.
4. The volume used and composition of the solvent travelling along the paper.
5. Any other liquid or vapours incorporated for special purposes.
6. Equilibration time (if any).
7. The temperature.
8. The nature of the mixture to be chromatographed and previous mode of treatment, e.g. type of desalting.

Any variant in any of the above conditions will affect the R_f value, although in many cases the effect will not be great enough to be appreciable in a solvent flow of 10–20 in. More useful is the fact that relative R_f values are usually much more constant and consequently the "pattern" or "map" soon becomes recognizable. Particularly when a number of spots appear in both normal and abnormal conditions, e.g. glutamine, glycine, and taurine in urines, it is possible to use these as reference positions for other spots which may occasionally be present. In what follows, all of the above points will be discussed.

UNIVERSAL APPARATUS FOR ASCENDING AND DESCENDING PAPER CHROMATOGRAPHY

The Tank. The tank is an all-glass 12 in. cube with rounded internal corners. The top edges are ground flat and the tank is closed by a heavy glass plate with a ground contact surface. Air-tightness can be completed, if necessary, by means of a layer of glycerol along the top edges but, in this case, it is necessary to weight down the top plate.

Tanks may be made from 12 in. cube aquarium tanks and are quite suitable provided that all metal and putty surfaces are coated with paraffin wax.

The Internal Fitments. All other parts of the apparatus (the frame, solvent tray, solvent troughs, suspension rods, etc.) can be constructed of metal or polythene.

Apparatus constructed completely of metal, such as aluminium-silicon alloy No. 2 or dural, has been found resistant to the solvents described elsewhere in this book and has been used to prepare the chromatograms shown. Such apparatus is, therefore, resistant to solvents containing phenol, acetic and formic acids, concentrated ammonia and aromatic liquids, but cannot be used when the solvent incorporates mineral acid. Stainless steel has also been used.

Polythene apparatus is equally suitable for most solvents but cannot be used with benzene or other solvents which swell or otherwise affect it; however, it is quite unattacked by mineral acid. Polythene or polythene coated-metal dip trays are also quite resistant to all the location reagents described later.

Metal and polythene apparatus is both unbreakable and readily cleaned and so is preferable to glass fitments and wooden tanks.

Technique for Ascending Paper Chromatography

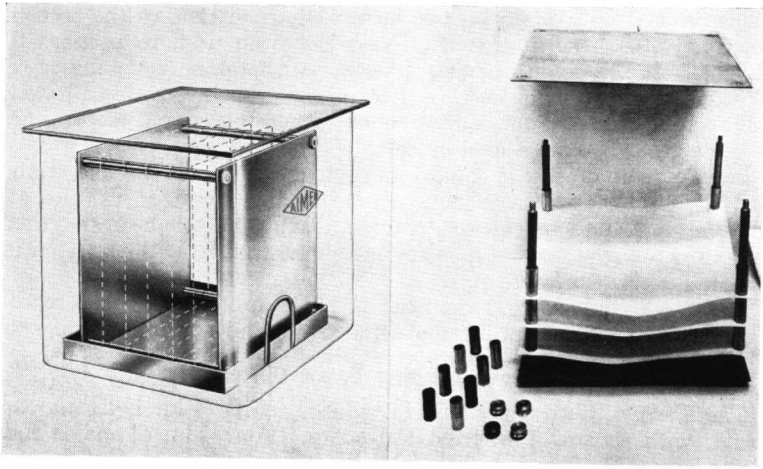
The Frame. Two plates, 10 in. square, carry $\frac{1}{4}$ in. holes at each corner with centres $\frac{1}{2}$ in. from the edges. Four $\frac{3}{8}$ in. diameter rods, $8\frac{1}{4}$ in. long, are threaded with $\frac{1}{4}$ in. Whitworth threads, giving a rod length between the shoulders of $7\frac{1}{2}$ in. The threaded parts of the rods fit loosely through the plate holes and are held on knurled lock-nuts, thus forming a frame to support the papers. Each rod carries six loosely fitting collars, $1\frac{1}{2}$ in. long and $\frac{13}{32}$ in. internal diameter which act as spacers for the papers. A tray, $10\frac{1}{2}$ in. $\times$ 9 in. $\times$ 1 in., with handles and supporting bars (under the tray) rests squarely on the bottom of the tank, and the frame sits in this. [1 in. = 2·54 cm.]

Preparation of the Papers. The papers, 10 in. square and already punched, are removed from the packet as required, and placed on a clean surface.

For one-way chromatograms, a pencil line is drawn 2·5 cm. up from the lower edge and the points of application (origins) are marked with a cross or dot. The outside origins should be at least 2·5 cm. from the edges, the others should be evenly spaced not less than 2 cm. apart giving a maximum of eleven origins per sheet, (if many one-way chromatograms have to be marked then it is convenient to construct a perspex template with V-shaped nicks cut into it and spaced at the distances usually required, and if many papers have to be marked simultaneously then the pencil may be pressed heavily on the top sheet when the marks will show through on to the next sheet and on pencilling in these indentations a third paper is marked and so on). If the paper is to be cut into strips for testing with various location reagents, then the number of origins should be reduced and spaced further apart; for example with a total of six origins on one sheet these should be spaced 4 cm. apart. Thus up to 55 one-way chromatograms can be run on the

frame at any one time although, of course, a lesser number can be run. For two-way chromatograms, a single origin is marked in the bottom right-hand corner 3 cm. from each edge. Thus five two-way chromatograms can be run on the same frame.

The name or code of each solution should be noted beneath each origin, with the volume to be applied. Only lead or liquid lead pencil



a b

FIG. 2.1. (*a*) The Universal Apparatus is assembled for ascending chromatography. The frame, holding from one to five papers, sits in the solvent in the bottom of the tray.

FIG. 2.1. (*b*) The frame is being assembled. The papers are placed horizontally on the frame and are held apart by collars; finally the end plates are placed in position and held by the nuts. The solutions to be chromatographed are applied to the origins with the frame still in the horizontal position shown.

should be used to write on paper chromatograms as ink of all types runs in the solvents (see Chapter 17).

The four long rods are then placed through the holes in the end plates and the nuts screwed on. Then one collar is placed over each rod, and the first paper is threaded over the rods and pushed down into place. Another four collars are placed on the frame and followed by another paper, and so on until all the papers are in place. The last set of collars are placed in position, the other end plate is put on the frame and the remaining four nuts are threaded on to the projecting threads. Only at this stage are the solutions to be chromatographed applied to the origins (see p. 15).

Final Assembly of the Apparatus. The tray is placed firmly at the bottom of the tank and the required volume of solvent is placed in the tray. The frame is placed squarely in the tray and, after ensuring that all papers are dipping into the solvent, the glass top-plate is placed in position. The solvent should be seen to rise evenly up each paper.

Drying the Papers. After the solvent has ascended for the required time or height, the frame is removed from the tank and stood for a few seconds on a sheet of absorbent paper to blot off liquid retained on the bottom edge. It is then dried by placing in a fume cupboard with the corners raised on two or four corks and with a fan blowing across the

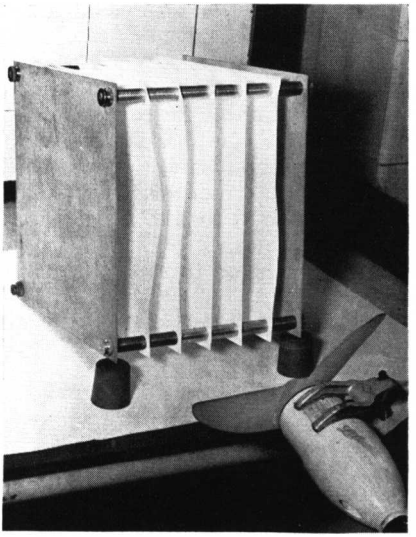


FIG. 2.2. Drying the frame. The frame is placed in a fume cupboard and stood with its corners raised on two or four corks to allow free circulation of air. The draught from a simple fan is sufficient to dry the papers rapidly.

papers. Drying is often hastened if the nuts and collars are loosened and the frame turned round at intervals.

When dry, the frame is ready for the second run in the right angles direction in the case of two-way chromatography, or the papers are removed and the separated substances can then be located as described later.

Cleaning the Apparatus. The metal nuts, collars, and rods are easily cleaned by placing them in a tall sink tidy held in a stream of hot water but traces of residual solvent may be rinsed off with acetone. The plates are similarly washed with a scrubbing brush and warm water. Occasionally traces of white powder are seen on the plates after the chromatographic solvent has been dried off but this never interferes with the separation on paper and is easily washed off. If the solvent is no longer required it should be poured away and the tray washed and dried, but it is quite unnecessary to wash out the tank between runs unless a different solvent is to be used in it. Polythene or polythene-coated materials should only be washed with cold water, and this is quite sufficient for cleaning.