TECHNIQUES IN PROTEIN CHEMISTRY

J. LEGGETT BAILEY

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by

J. LEGGETT BAILEY, Ph.D., F.R.I.C., F.L.S.
Twyford Laboratories,
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PREFACE

In writing this book I have been guided by the needs of biochemists who have had limited practical experience of the chemistry of proteins and who may wish to try their hand at a tentative characterization and structural examination of the protein or peptide part of their particular experimental system.

Some technical aspects of the subject have become rather complicated by numerous modifications in procedure and at times, it is difficult even for the specialist worker to decide on the respective merits of all the variants. My aim therefore has been to compile a practical handbook in which established analytical methods are discussed in detail, recent promising techniques are touched on briefly, and in which theoretical considerations are introduced only where they have a direct bearing on practice.

A recurring difficulty that has to be faced by all who work with proteins is the lack of a simple criterion of purity that might be applied to purified preparations. Crystallinity has long been abandoned as a criterion of homogeneity and quantitative solubility tests have been applied in very few cases. The minimum requirement generally acceptable is evidence of homogeneity from data provided by column chromatography, electrophoresis under different conditions of pH, and sedimentation in the ultracentrifuge. Two chapters in this book are devoted to column chromatography and electrophoresis of proteins. A discussion of the ultracentrifuge is not included since the information is available in special operational manuals. In the interests of brevity accounts of the more complicated physical equipment, *e.g.* counter-current distribution apparatus, are also omitted.

The final criterion for a single molecular species is the demonstration of a unique amino acid sequence and hence the major part of this book is concerned with methods for sequence determination, selective cleavage of peptide chains, fractionation

of mixtures of peptides, amino acid analysis and other techniques required in structural work on proteins. The general pattern of presentation is such that each chapter deals with a particular topic and is more or less self-contained. There may be some advantage in this over the alternative plan of a stage by stage examination of the methods used in the elucidation of the complete structure of a particular protein.

Few biochemists will wish to proceed with a complete structural investigation of the protein molecules that interest them — at least not using the methods presently available. Great advances have been made in fields such as enzymology, intermediary metabolism and photosynthesis where little is known of the chemical nature of the protein enzymes involved. However, sooner or later all biochemical events must be explained at the molecular level in terms of organic and physical chemistry and a knowledge of the complete structure of proteins, nucleic acids and other large molecules will be required.

The brilliant studies of Sanger and his collaborators on insulin will ever remain a landmark in the development of modern protein chemistry. It may be that the methods employed by Sanger will not be applied again *in toto* for the structural investigation of other proteins, but it is to be hoped that the basically simple approach manifest throughout the insulin work will be maintained in future fashioning of methods.

It is generally recognized among teachers that biochemical concepts are difficult to communicate to young people at the pre-university level. I would suggest to any science masters among my readers that some of the simpler experimental methods outlined in this book might be adapted to fire the imagination of senior pupils. With guidance some original research contributions might even result.

London, July 1962 J.L.B.

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J.L.B.

CONTENTS

CHAPTER 1

PAPER CHROMATOGRAPHY OF AMINO ACIDS AND PEPTIDES

GENERAL CONSIDERATIONS

Since the classical work of Consden, Gordon and Martin[1], and later that of Dent[2], an overwhelming number of systems have been devised for the separation of amino acids by paper chromatography. Some workers have concentrated on the separation of certain groups of amino acids, others have sought the maximum resolution of a large number of components. Many have achieved their end in an overnight run whereas a whole week has been needed for more ambitious separations. Kowkabany and Cassidy[3] have examined 22 grades of filter paper and 5 different solvent systems and 53 other types of paper with two different solvent systems. Following on the earlier study of the effects of inorganic salts on paper chromatograms by Consden and Gordon[4], Westall[5] and others, a very detailed study of the influence of buffers over a wide pH range on the migration of amino acids was made by McFarren[6]. Between the years 1944–1954 over 1200 major contributions to paper chromatography were published in addition to many more short notes on the subject. The literature today is not only bewildering to any newcomer to the field but also presents a problem to the reviewer who is required to be brief.

It is worth re-stating the more generally accepted basic facts about paper chromatography before dealing with particular systems. Three phases are involved in the sealed chromatographic unit; a moving liquid phase, a stationary phase at the surface of the cellulose fibres and a vapour phase which may or may not be in equilibrium with the liquid phase. The rate of migration of the moving liquid phase is a function in the first place of the physical properties of the solvent. Important factors are the

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viscosity, surface tension and density, all of which are dependent on temperature. Secondly, the rate of migration is a function of the pore size of the paper; a medium possessing very fine channels or capillaries will exert a greater suction force on the liquid than one in which wide channels predominate. Paper usually differs in structure along the two dimensions of its surface and solvent will usually rise faster up a strip cut in the machine direction. Slightly damp paper tends to curl with the axis of curl parallel to this direction. Heterogeneity in the paper can lead to different flow rates along either dimension resulting in lack of reproducibility. The migration of the boundary between dry and moist paper in descending chromatography is more nearly uniform than is the case in ascending chromatography. Even for downward flow, however, solvent near the solvent source is behaving differently to that at the advancing front. At the commencement of a chromatogram, during the initial surge, solvent is flowing over as well as through the saturated area. At the solvent front liquid creeps forward through the finest capillaries.

The rates of migration of solutes are governed by specific partition between the moving liquid phase and the stationary gel-like phase which is usually considered to be located in the amorphous regions of the cellulose. Good paper is at least 96% α-cellulose (insoluble in strong NaOH) and about 40% is in the amorphous state. Most of the water is held by the amorphous regions; chemically bound water exists in native cellulose to the extent of 5.9%. Consden, Gordon and Martin[1] have likened the stationary phase to a strong solution of soluble polysaccharide. Rates of migration of selected amino acids come close to the theory for simple liquid–liquid distribution but in view of the strong affinity of the basic amino acids for cellulose specific adsorption or ion-exchange effects appear to operate. It is usual to express migration rates according to the definition

$$R_F = \frac{\text{distance travelled by solute zone}}{\text{distance travelled by solvent front}}$$

In systems where the solvent is allowed to flow off the paper the R_F value is compared to that of a convenient reference substance *e.g.* leucine. As a physical constant depending on the partitioning effect the R_F value is greatly affected by temperature variations. Non-uniform operating conditions can easily arise from interactions between the vapour and liquid phases resulting from temperature variations during a run.

In a very fine exposition of the subject Hanes and co-workers[7–12] discuss most of the factors governing the operating chromatogram and show how apparent weaknesses in the technique, after proper study, may be exploited to most useful ends. The 1961 series of papers deserve a wide reading, as the conclusions apply equally well to the separation of families of compounds other than amino acids.

TWO-DIMENSIONAL SEPARATIONS

The classical procedure of Consden, Gordon and Martin[1] has been widely used in laboratories all over the world. Sheets of Whatman No. 1 paper are developed downwards with phenol–ammonia in one dimension for 24–72 h and after drying developed with collidine in the other dimension for 24–48 h. This phenol–ammonia collidine combination proved to be a most useful system but many workers found the published R_F values of limited use owing to variations in isomer composition of the collidine. A comprehensive diagram constructed by Dent showing the distribution of amino acids and other substances in phenol and 2,4-lutidine + 2,4,6-collidine (1 : 1 by vol) is shown in Fig. 1.

Phenol can be purified by steam distillation; details of a purification procedure have been described by Draper and Pollard[13]. The usual mixture is 900 g detached crystals to 100 g deionized water. To prepare a saturated solution this stock solution is shaken with excess water and the lower layer run off for use. The aqueous layer can be used in the bottom of a tank for the purpose of equilibration. Just before use 1% of concentrated ammonium hydroxide solution is added together with a few

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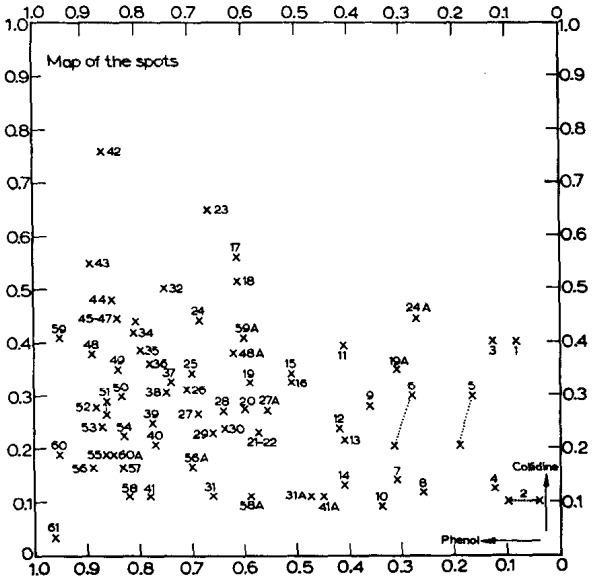

Fig. 1. Average positions of spots taken up on phenol–collidine two dimensional chromatograms. Phenol first solvent (from Dent[2]).

(For further explanatory text see next page.)

crystals of potassium cyanide to prevent formation of oxidation products catalyzed by traces of copper.

The following is a purification procedure for pyridine bases devised by A.J.P. Martin (see Ref.[14]). Bromine (5–10 ml) is added gradually with cooling and shaking to a litre of the base. The mixture is filtered through glass wool and excess bromine removed by the addition of a few crystals of sodium thiosulphate. The filtered liquid is allowed to stand over solid NaOH, filtered again and distilled.

The high toxicity and evil smell of collidine have tended to make this solvent less popular and *n*-butanol–acetic acid–water

α-Alanine (20)
β-Alanine (29)
Allothreonine (15)
α-Amino-*n*-butyric acid (26)
α-Aminoisobutyric acid (37)
γ-Aminobutyric acid (40)
ε-Aminohexanoic acid (55)
α-Amino-ε-hydroxycaproic acid (38)
α-Amino octanoic acid (43)
δ-Aminopentanoic acid (57)
α-Aminophenylacetic acid (33)
Arginine (56)
Asparagine (13)
Aspartic acid (5)
Carnosine (54)
Citrulline (30)
Cystathionine (7)
Cysteic acid (1)
Diiodotyrosine (17)
Djenkolic acid (14)
Ethanolamine (48)
Ethanolamine–phosphoric acid (10)
Glucosamine (24)
Glutamic acid (6)
Glutamine (21)
Glutathione (4)
Glycine (12)
Histamine (59)
Histidine (27)
Homocysteic acid (3)
Hydroxylysine (31)
Hydroxyproline (28)
Lanthionine (8)
Leucine (45)
Isoleucine (46)
Lysine (58)
Methionine (34)
Methionine sulphone (25)
Methionine sulphoxide (39)
α-Methyl-α-amino-*n*-butyric acid (49)
Methyl histidine (51)
Monoiodotyrosine (23)
Norleucine (47)
Norvaline (35)
Ornithine (41)
Phenylalanine (44)
Proline (52)
Serine (9)
Serine-phosphoric acid (2)
Taurine (11)
β,β,β¹,β¹-Tetramethylcystine (19)
Threonine (16)
Thyroxine (42)
Tryptophan (32)
Tyrosine (18)
Valine (36)

(4 : 1 : 5) is often used instead (Partridge[14]). A much used system in which collidine is replaced by butanol–acetic acid is the method of Levy and Chung[15].

Method of Levy and Chung

Following McFarren's idea of using aqueous buffers[6] these authors employed *m*-cresol–phenol (1 : 1), pH 9.3 borate buffer, and in view of the high pH an acid-washed paper, Whatman No. 52, was used. The butanol–acetic acid–water (4 : 1 : 5) irrigation is carried out first on a sheet of dimensions 22.5 × 18 in.

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in the long direction by the descending method. After about 16 h the paper is dried at 40°C, trimmed top and bottom, the area occupied by the amino acids covered with a glass sheet and the rest of the paper sprayed with pH 9.3 borate buffer (200 ml of 0.1 *M* boric acid + 113.5 ml of 0.1 *N* NaOH). The dried paper is then run for 16 h in the other dimension with cresol–phenol (25 g of phenol, 25 g of *m*-cresol and 7 ml of pH 9.3 buffer). Fig. 2 shows a typical chromatogram. If a No. 1 paper is to be

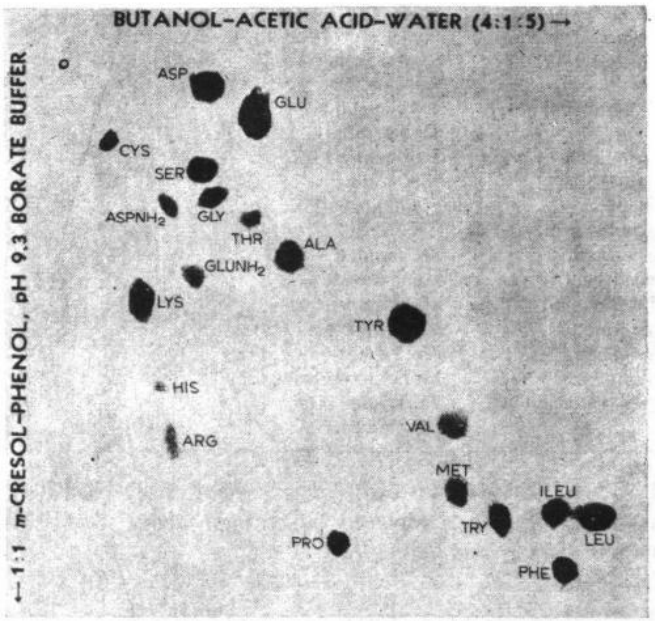

Fig. 2. Two-dimensional chromatogram by method of Levy and Chung[15] (ASP, aspartic acid; ALA, alanine; ASP.NH_2, asparagine; ARG, arginine; CI, citrulline; CYS. AC, cysteic acid; CYS, cystine; GLY, glycine; GLU, glutamic acid; GLU.NH_2, glutamine; HIS, histidine; HY.PRO, hydroxyproline; ILEU, isoleucine; LEU, leucine; LYS, lysine; MET, methionine; OR, ornithine; PHE, phenylalanine; PRO, proline; SER, serine; THR, threonine; TRY, tryptophan; TYR, tyrosine; VAL, valine).

used the recommended mobile phase has the composition 30 g of *m*-cresol, 15 g of phenol and 7.5 ml of pH 8.3 borate buffer (300 ml of 0.1 *M* boric acid + 60 ml of 0.1 *N* NaOH). The use of buffered phenol usually leads to a clear chromatogram with the minimum of discoloration.

Method of Rockland and Underwood

A map constructed by Rockland and Underwood[16] illustrating a technique suitable for rapid routine screening of a large number of samples is shown in Fig. 3. Separations are carried out by ascending chromatography on 5 × 5 in. squares of Schleicher and Schüll 589 Blue Ribbon filter paper (Whatman equivalent

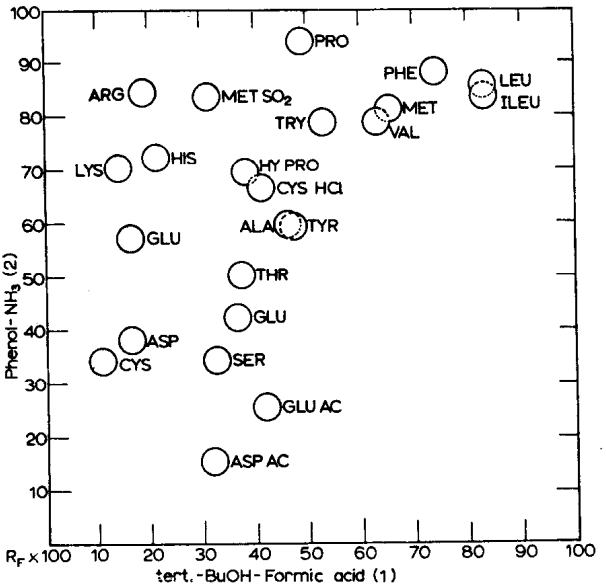

Fig. 3. Two-dimensional chromatogram by method of Rockland and Underwood[16].

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No. 541) during about 12 h at 19°C. The first solvent is *tert.*-butyl alcohol–water–formic acid prepared from 10 ml formic acid, 295 ml of deionized water and 695 ml of *tert.*-butyl alcohol. This solvent is allowed to migrate to within $\frac{1}{4}$ in. of the upper edge of the paper. The second solvent is undersaturated phenol prepared from 775 g phenol and 215 ml deionized water. Immediately before use 94 ml of the phenol solution is mixed with 1 ml concentrated ammonium hydroxide.

Amino acid solutions for spotting

For spotting purposes it is useful to assay a given sample of a peptide or protein hydrolysate first by the quantitative ninhydrin method of Moore and Stein (see p. 73). A suitable load for application to paper is one micromole of total amino nitrogen in a volume of 5 to 10 μl. A standard mixture of amino acids is conveniently stored in 10% isopropanol in a concentration of 500 μg of α-amino nitrogen per ml. Alternatively 0.02 *M* solutions of amino acids in 0.1 *N* hydrochloric acid may be prepared and an aliquot neutralized with 1 *N* sodium hydroxide to bromothymol blue before application to paper. Micropipettes suitable for the application of solutions to paper are shown in Fig. 4; A is a commercial type and B is a simple pipette made from a drawn out capillary tube. The latter type is calibrated by filling the pipette several times with 6 *N* HCl and emptying on to a small piece of filter paper. The filter paper is then dropped into some water and titrated with standard alkali.

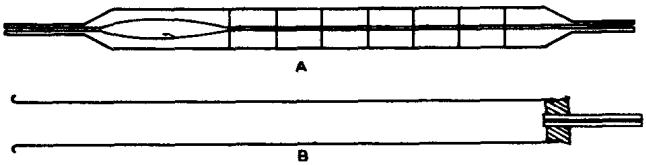

Fig. 4. Micropipettes for spotting purposes. A, commercial type; B, simple capillary type.

ONE-DIMENSIONAL SEPARATIONS

In recent years considerable attention has been given to the problem of maximum resolution of amino acids by one-dimensional chromatography. The main drawbacks of two-dimensional chromatography are the following:

1. Only one sample can be separated for each run.
2. The amount of sample is limited as overloading leads to distortion of zones.
3. Heterogeneity in the paper and other defects give rise to irregular shaped diffuse spots.
4. The method is not ideal for quantitative work.

Among the very many systems proposed for one-dimensional chromatography the following are capable of near-perfect separations of the common amino acids.

1. Buffered phenols and alcohols (McFarren[6]).
2. Use of five different solvent systems involving 5 to 6 days' irrigation (Redfield and Guzman Barron[17]).
3. Use of three different solvent systems and irrigation periods of 5 days (Fowden[18,19]).
4. Two solvent systems to resolve 16 amino acids sufficiently well for quantitative analysis (Roland and Gross[20]).
5. Repeated irrigation with the same solvent and intermediate drying of the paper. Separation of 13 amino acids and two pairs (Keil[21], Dubrovskaia *et al.*[22]).
6. Separation of 13–15 from a mixture of 18 amino acids over a period of 40 h using miscible buffered solvents, followed by rapid resolution of two pairs by simple reversed chromatography (Hanes and co-workers[7–12]).

Two of these systems have been developed for quantitative work and will be described in detail. A useful diagram illustrating the patterns of separation of a number of published systems is shown in Fig. 5.

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