THIN-LAYER CHROMATOGRAPHY OF PROTEINS ON SEPHADEX G-100 AND G-200

C. J. O. R. MORRIS

Department of Experimental Biochemistry, The London Hospital Medical College, London (Great Britain)

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Gel filtration[1] and thin-layer chromatography[2] are now both established analytical techniques. The advantages of thin-layer chromatography, speed and adaptability to very small samples, make it very attractive for the fractionation of proteins on the ultramicro scale. HOFMANN[3] has described the chromatography of several proteins on thin layers of hydroxyl-apatite, while DETERMANN[4] and JOHANSSON AND RYMO[5] have described thin-layer methods using the cross-linked dextran gel filtration medium Sephadex (Pharmacia, Uppsala, Sweden). The method of DETERMANN[4] was developed for the tightly cross-linked dextran gel Sephadex G-25, and was not suitable for the chromatographic separation of proteins. The method of JOHANSSON AND RYMO[5] was used with both Sephadex G-25 and G-75, and in the latter case could be used for the chromatography of small proteins.

The availability of the loosely cross-linked bead-form materials Sephadex G-100 and G-200 has extended the upper molecular weight limit for the successful chromatography of proteins up to at least 180,000. The application of these materials in the thin-layer chromatography of proteins has necessitated the development of a special technique described in detail in this paper.

EXPERIMENTAL

Materials

Sephadex G-100 (water regain 10, batch No. TO 1992) and G-200 (water regain 20, batch No. 224C) both with particle size $<$ 400 mesh were obtained from Pharmacia A.B., Uppsala, Sweden through the courtesy of Dr. B. GELOTTE.

Proteins

Bovine serum albumin (BSA)	Crystalline, Armour & Co. England.
Bovine γ-globulin (γ)	Armour & Co. England.
α-Chymotrypsin (Chy)	3 $\times$ crystallized, Seravac Laboratories, Colnbrook, England.
Cytochrome *c* (Cyt)	Salt-free, Seravac Laboratories.
Haemoglobin (Hb)	Human carboxy-, prepared from washed red cells, stored at —15°.
Lysozyme (Lys)	4 $\times$ crystallized, gift from Dr. J. R. MARRACK.
Myoglobin (Myo)	Crystalline, whale, Seravac Laboratories.
Ovalbumin (Ova)	Crystalline, Light & Co. Colnbrook, England.

Ovomucoid (Ovm)	Crystalline, Light & Co.
Pepsin (Pep)	2 × crystallized, Armour & Co.
Ribonuclease (Rib)	Crystalline, B. D. H. Ltd., Poole, England.
Thyroglobulin (Thy)	Light & Co.
Trypsin (Try)	2 × crystallized, Seravac Laboratories.

Method

Gel suspensions were prepared by thoroughly mixing 6 g of Sephadex G-100 or 4 g of Sephadex G-200 with 100 ml of the solvent in a beaker. The concentrations given are critical, and should be strictly adhered to, although it is possible that different optimum concentrations may be necessary with different batches of Sephadex. Care should be taken to ensure that no aggregates are present in the final gel suspension. The quantities given are sufficient for six 10 × 20 cm plates. The gel suspension should be stored in the covered beaker for at least 48 h to allow swelling of the dextran gel to proceed to completion. 10 × 20 cm glass plates are thoroughly cleaned with detergent and distilled water before use, and the dry plates are coated with a 0.9 mm thick layer of Sephadex by means of a Camag thin-layer spreader (Camag A.G., Muttenz, Switzerland). The plates are immediately transferred to a closed vessel containing a dish of the solvent, and stored in the horizontal position for at least 18 h before use. This pre-equilibration process markedly improves the reproducibility of the results. The plates are then mounted horizontally, preferably with illumination of the translucent gel layer from underneath, and 0.5–1.0 μl of the test solution containing 1–20 μg of protein applied as a series of spots about 1.5 cm apart in a line 3 cm from a short edge of the plate. A micro-pipette or preferably a micrometer syringe may be used for the application. The size of the sample zone should not exceed 3 mm, and especial care should be taken to ensure that the soft gel layer is not damaged during the application.

Development is carried out in the apparatus shown in Fig. 1.

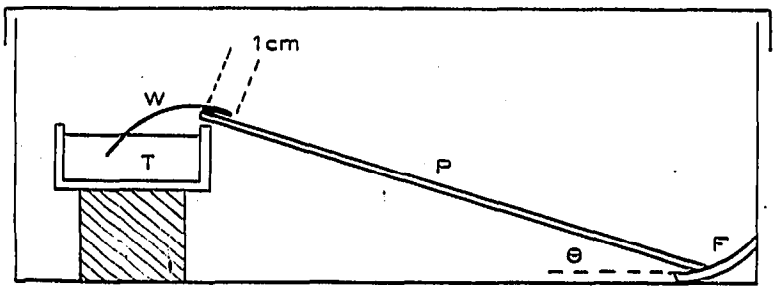

Fig. 1. Apparatus for thin-layer chromatography of proteins.

Solvent (0.5 *M* NaCl in the studies reported here) is led to the plate P by means of the Whatman No. 3 MM filter paper wick W. Excess liquid is prevented from accumulating at the bottom of the plate by the filter paper pad F which is moistened to ensure good contact with the gel layer. The wick W should overlap about 1 cm onto the gel layer and the gel surface at the upper edge of the plate should be 1–1.5 cm above the level of the solvent in the trough T. The angle θ should be about 10° for Sephadex G-100 plates and about 20° for G-200 plates. Human CO-haemoglobin should migrate about 70 mm in 4–5 h under optimum operating conditions. Excessively fast develop-

ment leads to streaking of the zones, but development can be continued up to 8 h or more without apparent loss in resolution. Excessively slow migration rates are however usually an indication of incorrectly prepared plates.

When development is considered to be complete (haemoglobin, cytochrome *c* or myoglobin may be used as markers), the plate is removed from the development chamber, and supported horizontally after careful removal of the wick W. A 10 × 20 cm piece of Whatman No. 3 MM filter paper is then applied progressively to the gel surface, starting near the line of initial zones and proceeding to the other end of the plate. Care must be taken to avoid trapping air bubbles between the gel and the paper. This is facilitated if the smoother side of the paper is downwards, and if the paper is rolled on to the gel layer. Schleicher & Schüll No. 2043b paper appears to give slightly better prints with Sephadex G-100 plates but is unsuitable for G-200 plates owing to its lower water capacity. The position of the line of initial zones is then marked on the paper and the covered plate transferred to an oven for drying at 80–90° for 30 min.

The protein zones may be located on the dried plate by any appropriate staining technique. Staining in 0.2 % Ponceau S in 10 % aqueous acetic acid for 30 min, followed by extensive washing with water to remove excess dye, has been used in most of the present work. 1 % Naphthalene Black 12B (E. Gurr & Co., London, England) in methanol–water–glacial acetic acid (50:40:10), or 0.01 % Nigrosine (E. Gurr & Co.) in the same solvent, followed by washing with the mixed solvent, have been found to be useful where a greater sensitivity is required. During the early stages of the washing process the paper prints become detached from the gel plates so that they may finally be washed and dried separately.

Column chromatography

96 × 1.1 cm columns of Sephadex G-100 (batch No. TO 33) or Sephadex G-200 (batch No. TO 41) (both 100–400 mesh), were packed by the sedimentation method recommended by the manufacturers. 2–8 mg samples of the test proteins in 0.5 ml of 0.5 *M* NaCl were applied to the column and development continued with the same solvent at a flow rate of 2–2.5 ml/h/cm^2. The column effluent was monitored at 257 mμ by means of an LKB Uvicord recording absorptiometer, and the retention volumes (V_e) of the test proteins obtained directly from the positions of the recorded zone maxima. The value of the gel internal volume (V_i) was obtained from the retention volume of water, the electrical conductance of the column effluent being monitored with a Radiometer type CDM 2 conductance meter and an LKB type 6520 A recorder. The column void volume (V_0) was estimated from the retention volumes of α-macroglobulin or thyroglobulin, which were identical on both the Sephadex G-100 and G-200 columns. Partition coefficients (K_d) were calculated from the retention:

$$K_d = (V_e - V_0)/V_i \qquad (1)$$

RESULTS

Typical thin-layer chromatograms of cytochrome *c*, ovalbumin and thyroglobulin on Sephadex G-100, and lysozyme, haemoglobin and γ-globulin on Sephadex G-200, individually and in mixtures are shown in Figs. 2 and 3 respectively. Fig. 4 shows a thin-layer chromatogram of haemoglobin, bovine serum albumin, γ-globulin and a

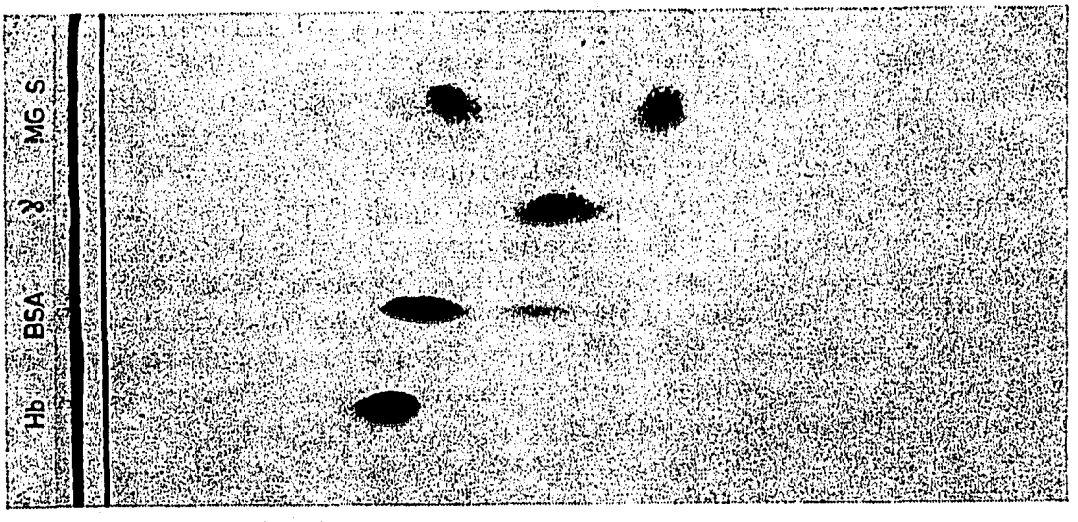

Fig. 4. Thin-layer chromatogram of haemoglobin (Hb), bovine serum albumin (BSA), γ-globulin (γ) and a macroglobulinaemia serum (MG S) on Sephadex G-200 –0.5*M* NaCl.

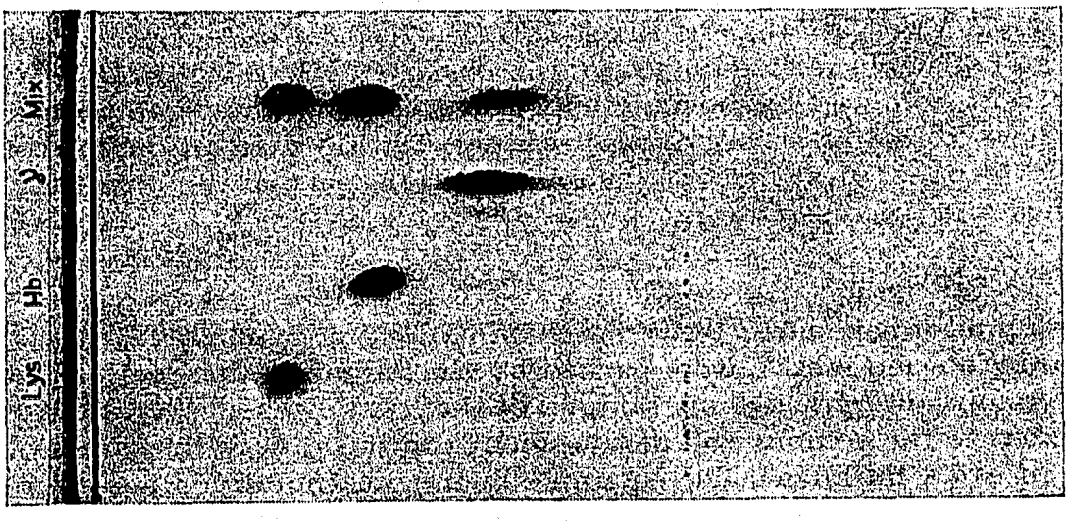

Fig. 3. Thin-layer chromatogram of lysozyme (Lys), haemoglobin (Hb) and γ-globulin (γ) on Sephadex G-200–0.5 *M* NaCl.

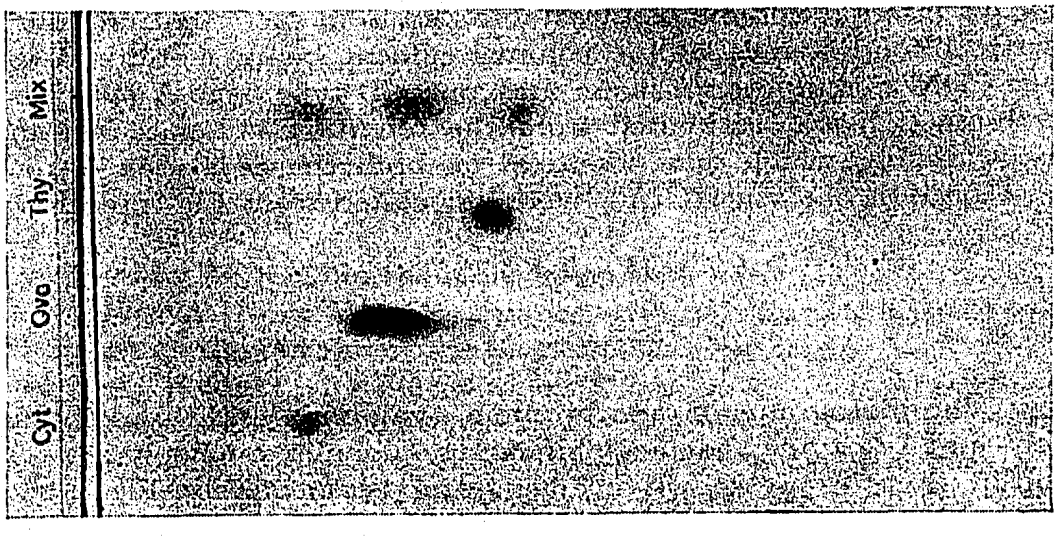

Fig. 2. Thin-layer chromatogram of cytochrome *c* (Cyt), ovalbumin (Ova) and thyroglobulin (Thy) on Sephadex G-100–0.5 *M* NaCl.

macroglobulinaemia serum on Sephadex G-200. The fast-moving abnormal macroglobulin component is clearly evident (see FLODIN[6]), which suggests that the thin-layer method may be of diagnostic value in this condition.

Since the position of the liquid front cannot be located in the present method, the migration velocities of the various test proteins have been compared with that of haemoglobin on thin-layer plates of Sephadex G-100 and G-200. An R_{Hb} value is then defined by:

$$R_{Hb} = d_p/d_{Hb} \qquad (2)$$

where d_P and d_{Hb} are the distances traversed by the test protein and by haemoglobin respectively during the development of the chromatogram. R_{Hb} values for twelve proteins on G-100 and G-200 based on the mean of 9–13 determinations, are collected in Table I, together with the molecular weights of the proteins.

TABLE I

R_{Hb} VALUES OF PROTEINS IN THE SYSTEMS SEPHADEX G-100–0.5 M NaCl AND SEPHADEX G-200–0.5 M NaCl

Protein	*Molecular weight* $\times 10^{-3}$	R_{Hb}	
		G-100	*G-200*
Cytochrome *c*	13.0	0.68	0.74
Ribonuclease	13.6	0.68	0.74
Lysozyme	14.5	0.65	0.70
Myoglobin	16.9	0.79	0.80
α-Chymotrypsin	22.5	0.87	0.87
Trypsin	23.8	0.83	0.86
Ovomucoid	27.0	0.94	1.03
Pepsin	35.0	0.99	1.04
Ovalbumin	45.0	1.03	1.04
Haemoglobin	68.0	1.00	1.00
Bovine serum albumin	65.0	1.14	1.22
Bovine γ-globulin	180.0	1.28	1.54
Thyroglobulin	650.0	1.33	1.83
Macroglobulins	1,000	—	1.86

DISCUSSION

The chromatogram prints illustrated in Figs. 2 and 3 show the method gives compact, well-defined symmetrical zones with relatively little tailing. Investigation of a series of concentrations of a single protein have shown that R_{Hb} values are concentration independent. The resolving power of the method is remarkably high, and provided that the load is restricted to about 10 μg of any individual protein, substances differing in R_{Hb} by about 0.1 can be completely resolved in a 10 cm migration. The resolving power of the 20 cm plate is thus at least equal to that of a 120 cm column (compare WHITAKER[7]). This high resolving power is undoubtedly due to the small particle size (< 400 mesh) and to the low linear flow rates (1.4–1.6 cm/h) used. Attempts to use the ordinary chromatographic grades of Sephadex (100–400 mesh) gave inferior results, probably due to the greater size heterogeneity of the gel particles. Similarly higher flow rates gave impaired resolution. Since diffusion of the solute zones does not appear to be a limiting factor, plates 50–100 cm long could probably

be used with advantage for difficult separations. The maximum load which can be used without zone distortion appears to be about 20–30 μg of most proteins. The minimum is set only by the limitations of the methods of zone detection.

The relations between R_{Hb} and K_d values for the test proteins on Sephadex G-100 and G-200 are shown in Figs. 5 and 6 respectively. It can be seen that for both systems linear relations exist, so that for Sephadex G-100.

$$K_d^{G100} = 1.35 - 1.0\ R_{Hb}^{G100} \quad (3)$$

while for Sephadex G-200:

$$K_d^{G200} = 1.35 - 0.73\ R_{Hb}^{G200} \quad (4)$$

These equations allow the K_d values for proteins on preparative scale gel filtration columns to be predicted from preliminary experiments with 2–10 μg of material taking about 4 h for completion. Equations (3) and (4) are of course only strictly valid for the solvent used in these experiments.

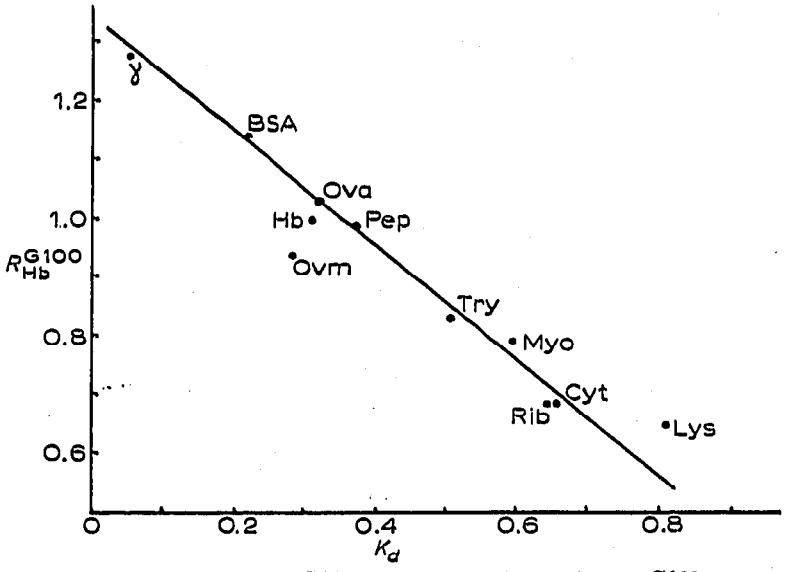

Fig. 5. K_d^{G100} as a function of R_{Hb}^{G100}.

Lysozyme, haemoglobin and ovomucoid depart from the linear relations on both Sephadex G-100 and G-200, although the deviations are less in the latter case. These proteins also show abnormal K_d values in relation to their molecular weights (see below), and their departure from equations (3) and (4) are probably due to minor differences in the Sephadex preparations used for the column and for the thin-layer experiments.

WHITAKER[7] has shown that V_e/V_0 on Sephadex G-100 columns is directly proportional to the logarithms of the molecular weights (M) for a number of proteins, including several of those used in the present work. A similar relation between R_{Hb} and log M should also hold in view of equations (3) and (4). The plot of R_{Hb}^{G100} values against log M was in fact non-linear, although all the experimental points lay on a smooth curve. The plot of R_{Hb}^{G200} against log M was however linear, obeying equation (5):

$$\log M = 1.47\ R_{Hb}^{G200} + 3.0 \quad (5)$$

and is shown in Fig. 7. The reason for this discrepancy is unknown.

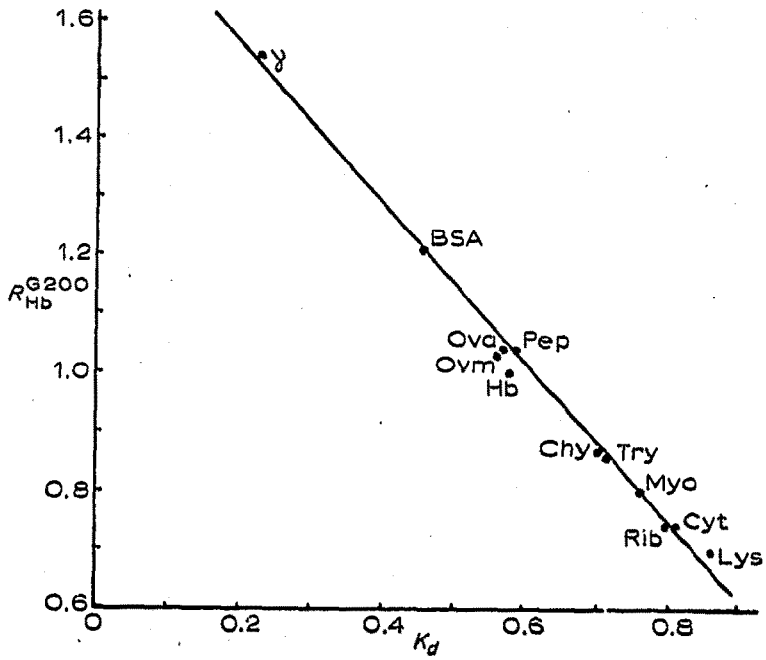

Fig. 6. K_d^{G200} as a function of R_{Hb}^{G200}.

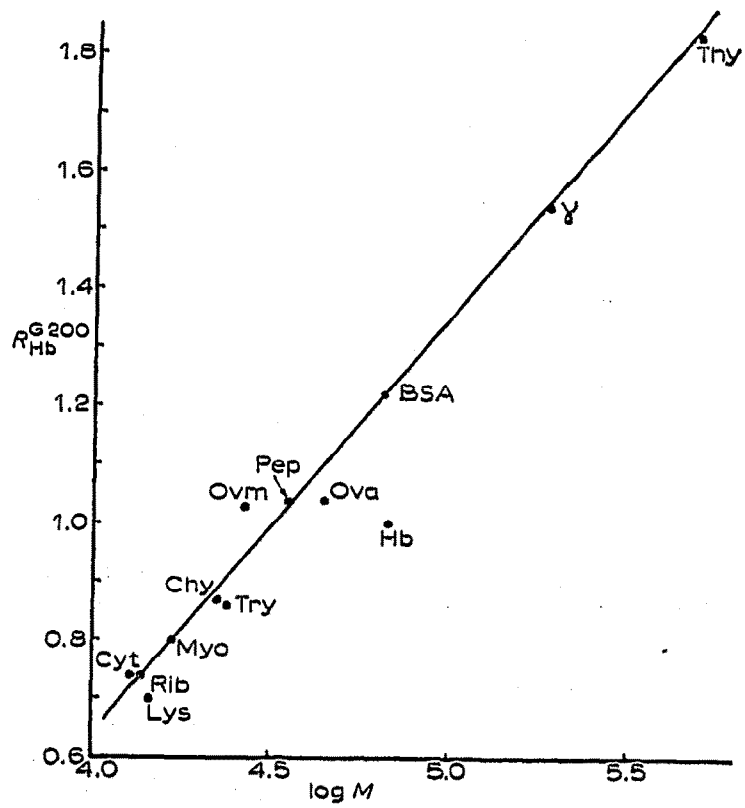

Fig. 7. Log M as a function of R_{Hb}^{G200}.

Lysozyme, haemoglobin and ovomucoid again behave anomalously. The relatively slow migration of lysozyme, probably due to an ion-exchange retardation of this very basic protein has been observed repeatedly[7–9]. Haemoglobin behaves as though it had a molecular weight of 33,000, approximately one-half of the value obtained by sedimentation–diffusion in solution and from X-ray crystallographic data. This anomaly had also been observed previously by ANDREWS[10], on agar, by WHITAKER[7] on Sephadex G-100, and by FAWCETT AND MORRIS[11] on polyacrylamide gel columns. These observations on different gel media make it unlikely that the anomaly is due to ion-exchange or adsorption retardation, while the magnitude of the effect suggests that under certain conditions haemoglobin may dissociate in solution into subunits comprised of a single α and a single β chain with a molecular weight of about 34,000. Alternatively the anomaly may be due to an equilibrium in solution between the 68,000 molecule and the α and β chains, as in the case of α-chymotrypsin investigated by WINZOR AND SCHERAGA[12]. The anomalous behaviour of ovomucoid which has an R_{Hb} value corresponding to a molecular weight of 37,000 has also been observed by WHITAKER[7].

The general validity of equation (5) however allows an estimate of the molecular weight of a protein to be made with an amount of the order of 1 μg by thin-layer chromatography on Sephadex G-200. It may be possible to lower this limit by an order of magnitude if more sensitive methods of zone location can be devised. Preliminary experiments in this laboratory have shown that the Sephadex gel layer can be overlaid with a thin uniform agar layer so that it may be possible to employ the techniques of immuno-diffusion[13] or specific enzymic reaction[14] for the location of protein zones with a great increase both in sensitivity and specificity.

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SUMMARY

1. A detailed description is given of a technique for the thin-layer chromatography of 1–20 μg amounts of proteins on the cross-linked gel filtration media Sephadex G-100 and G-200.

2. A close correlation has been obtained between the results of the chromatography of twelve test proteins on chromatographic columns and on thin-layer plates.

3. The relation between the molecular weights of proteins and their behaviour on Sephadex gels is discussed and a method for the estimation of protein molecular weights from the results of thin-layer chromatography is derived.

NOTE ADDED IN PROOF

Since this paper was submitted for publication, ANDREWS[15] and JOHANSSON AND RYMO[16] have described methods for thin-layer chromatography of proteins on Sephadex G-100 and G-200.

REFERENCES

1 J. PORATH AND P. FLODIN, *Nature*, 183 (1959) 1657.
2 E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin, 1962.
3 A. F. HOFMANN, *Biochim. Biophys. Acta*, 60 (1962) 458.
4 H. DETERMANN, *Experientia*, 18 (1962) 430.
5 B. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 16 (1962) 2067.
6 P. FLODIN, *Dextran Gels and Their Application in Gel Filtration*, Pharmacia, Uppsala, 1962.
7 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
8 J. PORATH, *Biochim. Biophys. Acta*, 39 (1960) 193.
9 F. MIRANDA, H. ROCHAT AND S. LISSITZKY, *J. Chromatog.*, 7 (1962) 142.
10 P. ANDREWS, *Nature*, 196 (1962) 36.
11 J. S. FAWCETT AND C. J. O. R. MORRIS, to be published.
12 D. J. WINZOR AND H. A. SCHERAGA, *Biochemistry*, 2 (1963) 1263.
13 O. OUCHTERLONY, *Acta Pathol. Microbiol. Scand.*, 26 (1949) 507.
14 R. L. HUNTER AND C. L. MARKERT, *J. Histochem. Cytochem.*, 7 (1959) 42.
15 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
16 B. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 18 (1964) 217.