CHROM. 3724

THIN-LAYER GEL FILTRATION OF PROTEINS

I. METHOD

BERTOLD J. RADOLA
Institute of Radiation Technology, Federal Research Centre for Food Preservation, 75 Karlsruhe (Germany)

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SUMMARY

Several aspects of thin-layer gel filtration have been re-examined. By the use of standard densitometric equipment, a quantitative evaluation of the thin-layer gel filtration pattern has been made possible. The recovery of proteins by the print technique has been studied using a number of proteins of different molecular weights.

For three Sephadex gels, G-75, G-100 and G-200, a linear correlation was found between the R_M value (defined as the ratio of the migration distance of the tested protein to that of myoglobin, which was used as a standard) and the molecular weight or the molecular radius of a number of globular proteins.

Fibrinogen fitted well into the straight line of the R_M–log molecular radius relationship, but not into that of the R_M–log molecular weight relationship. Apoferritin did not fit into the R_M–log molecular weight relationship and its molecular radius of 80 Å is in conflict with values found by other methods.

On Bio-Gel P-300 ferritin was separated into three fractions which on disc electrophoresis were found to differ in the content of the electrophoretically distinct α, β and γ-components.

The use of thin-layer filtration for the separation of proteins has been described in many papers[1–21]. For a number of proteins a correlation has been obtained between migration, referred to a standard protein, and molecular weight[3–5,7–10]. However, despite its simplicity and potential usefulness for the determination of molecular weights of proteins on the microgram scale, the method has not yet found wide application, especially when compared with the column technique of gel filtration and with other thin-layer chromatographic methods. This appears to be due to technical limitations concerning reproducibility of results, a lower accuracy, as compared with the column technique for determination of molecular weight, and the lack of a quantitative approach in the evaluation of the gel filtration pattern.

Currently, two different types of apparatus are generally used for thin-layer gel filtration. Usually the plates coated with the gel are placed in a moist chamber at angles differing for each particular gel and chosen so as to obtain the optimal flow

rate[2,4]. Alternatively, the sandwich chamber may be used[5]. The separation usually requires several hours depending on the gel used, the length of the plate and the angle at which the plate is inclined. Subsequently, the proteins are detected either directly on the plate[1,3,10] or by taking a print with filter paper which can be dried and stained by any of the dyes found convenient in paper electrophoresis[2,4,5]. In most of the thin-layer gel filtration techniques reported, the proteins are applied as spots. While localization of the spots can easily be achieved, densitometry has not been attempted and cannot be readily accomplished because of the irregular geometry of the spots. When using the spot application technique in studies with heterogeneous systems, the components present in smaller amounts are not easily detected.

An attempt has been made, therefore, to improve thin-layer gel filtration with the aim of getting more reproducible results. Standard densitometric equipment has been used for the quantitative evaluation of the results. Several aspects of the method have been re-examined, including preparation of the plates, application of the sample and detection of the proteins. Three Sephadex gels, G-75, G-100 and G-200, were employed, and in addition, two Bio-Gel preparations, P-60 and P-300, were tested for their suitability for thin-layer gel filtration. Quantitative relationships were established between the migration rates referred to a standard protein and the logarithms of the molecular weight or the Stokes radius of a number of well defined proteins. The extension of the fractionation range by the use of Bio-Gel P-300 is demonstrated by the separation of ferritin.

EXPERIMENTAL

Materials

Sephadex gels G-75, G-100 and G-200, all "superfine", were obtained from Pharmacia, Uppsala, Sweden. Several batches of each gel were used. Polyacrylamide gels, Bio-Gel P-60 and P-300, both with particle size 400 mesh, were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Bovine serum albumin, human serum albumin and bovine γ-globulin were obtained from Behringwerke, Marburg, Germany, while catalase, creatinephosphokinase, peroxidase and cytochrome c (horse heart) came from Boehringer und Söhne, Mannheim, Germany. Human ceruloplasmin and transferrin were supplied by KABI, Stockholm, Sweden, sperm whale myoglobin, ferritin (2 × crystallized) and protamine sulfate (salmon and herring) came from Koch and Light, Colnbrook, England, while α-chymotrypsin, haemoglobin, β-lactoglobulin, ovalbumin (5 × cryst.) and ribonuclease (5 × cryst.) were from Serva, Heidelberg, Germany.

Methods

Preparation of the gel plates: A gel suspension was prepared by mixing 8 g of Sephadex G-75, 6 g of the G-100 and 4 g of G-200 or 5.4 g of Bio-Gel P-60 and 3.5 g of P-300 with 100 ml of 0.5 *M* NaCl containing 0.02 *M* phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 7.2–7.4. The gel suspension was kept at 4° for two to three days and was evacuated briefly with a water pump before coating the plates. Two thin-layer spreaders were tried out, one from Camag A.G., Muttenz, Switzerland and the other from Desaga, Heidelberg, Germany. The latter was found more suitable for obtaining evenly coated plates. Glass plates 20 × 20 cm in size were used, except for a few

experiments in which 40 × 20 cm plates were employed. A 0.5 mm layer was used throughout, since a 0.3 mm layer was found unsuitable due to rapid evaporation at the edges. A slight excess of solvent was used in order to obtain a smooth surface on spreading. The plates were either allowed to dry at room temperature for 5–15 min and then transferred to a moist chamber where they could be stored for about two weeks, or they were used immediately after appropriate pre-equilibration as described below.

The apparatus consisted of a perspex chamber, 50 × 25 × 10 cm, with a glass plate cover. The arrangement of the plates was essentially as described by MORRIS[4]. Two 20 × 20 cm plates or one 40 × 20 cm plate could be used per chamber; the angle of the gel plates was fixed by a number of 20 × 5 cm glass plates at the lower end of the gel plates. A 20 × 6 cm Whatman No. 3 paper strip was used to establish contact between the buffered solution and the gel layer. At the lower end of the gel plate another 20 × 2 cm Whatman No. 3 strip was placed to prevent the collection of drops at the end of the plate and thereby to obtain a more even flow. Before each run, the gel plates were mounted in the apparatus in the evening and a flow of solvent through the gel was established during the night for pre-equilibration of the gel. This was found to be a necessary step for obtaining reproducible results.

For application of the substance, the pre-equilibrated plates were removed from the chamber and mounted horizontally. A line was marked on the glass plate beneath with a felt-marker at a distance of 3 cm from the upper end. The substance was applied as a streak: 10–20 μl of a 0.2–2% protein solution was placed on the edge of a microscopic cover slide (18 × 18 mm) and held against the gel surface. In a few seconds the solution had soaked into the gel, most rapidly with Sephadex G-75, slower with Sephadex G-200 and slowest with Bio-Gel P-300. Care had to be taken to touch the gel surface with the whole length of the edge of the cover slide, otherwise a drop forms giving rise to an irregular starting zone. To avoid displacement of the samples during application, all samples were first prepared on the cover slides and then quickly applied. On a 20 cm broad plate five to six samples can be run simultaneously. When applied at a distance of 2–2.5 cm from the edge, no distortion of the zones due to an edge effect was noted. Myoglobin was included as a reference protein in each run and was placed always on the middle of the plate. The plates, after application of the samples, were replaced in the apparatus and the paper wick was pushed slightly downwards to ensure good contact with the gel layer. With a particular gel the flow rate depends on the angle at which the plate is inclined and the level of the solvent in the reservoir vessel. The liquid level was kept constant at 1 cm from the upper end of the vessel. The angles were $\sim 5°$ and 10° for the G-75 and G-200 Sephadex gels, respectively. The optimal angle had been found empirically, so as to obtain a flow at which the myoglobin migrates at a rate of 1 cm per hour. The runs were usually complete in 4–7 h.

After completion of the run, the plates were taken from the chamber, and the contact paper wicks and the paper strip at the lower end of the plate were removed. Filter paper Whatman No. 3 was then used to take a print from the gel; Whatman paper No. 1 could be used for Sephadex G-75 and Bio-Gel P-60. A piece of filter paper, 20 × 17 cm, was rolled onto the gel layer from the edge near the starting line, which was marked on the paper with a felt-marker by viewing against light. The liquid phase was soaked off from the gel layer by the paper, which after 30–60 sec

was stripped away and dried in an oven at 110° for 15 min. Drying the paper on the glass plate has been found to distort the zones strongly due to uneven evaporation of water from the plate.

Staining

Proteins were stained either with Amido Black 10B or with Coomassie Brilliant Blue R250, which was the dye of choice when sensitivity[22] was important. A saturated solution of Amido Black 10B (E. Merck AG, Darmstadt, Germany) in methanol–glacial acetic acid (9 : 1, v/v) was used; the staining time was 5–10 min. For destaining, two volumes of the 9 : 1 methanol–acetic acid solution were mixed with one volume of water. The first two washing solutions were rejected; the subsequent ones were decolorized with charcoal and then re-used. Coomassie Blue (Serva, Entwicklungslabor, Heidelberg, Germany) was used as a 0.25% solution in methanol–acetic acid (9 : 1, v/v). After staining for 10 min, the paper was destained first for a few minutes with tap water until no dye could be washed off, then with a mixture of methanol–acetic acid and water (50 : 10 : 50, v/v). The first washing solution of the latter was rejected, and the subsequent ones were decolorized with charcoal.

Densitometry was accomplished with the Chromoscan recording and integrating densitometer (Joyce, Loebl & Comp. Ltd., Gateshead, England) with the thin-layer scanning attachment. The instrument was operated in reflectance with 10 × 1 mm apertures. The 595 or 620 filters were used with Amido Black staining and Coomassie Blue staining.

For molecular weight determinations, the distance from the starting line to the middle of each zone was measured with an accuracy of 0.05 mm either directly on the print or on the densitogram. The results were expressed by the R_M value defined as the ratio of the migration distance of the tested protein d_P to that of myoglobin d_M, which was used as the reference protein:

$$R_M = \frac{d_p}{d_M}$$

Calculations of the constants of the equations describing the relationship between the R_M value and the molecular weight or Stokes radius were performed with an IBM computer.

Gel electrophoresis

Electrophoresis of ferritin in polyacrylamide gel was performed as described by CLARKE[23]. A Tris–glycine buffer pH 8.2–8.3 was employed. Two gel layers, 5% (6 cm) and 2.5% (TEMED free, 0.5 cm), were used. A 0.1% solution of $K_4Fe(CN)_6$ in 0.1 *M* HCl was employed for staining ferritin. The stained gel was kept in 10% acetic acid.

RESULTS

The separation of two protein mixtures on Sephadex G-200, as shown in Fig. 1, illustrates the efficiency of the method. Two mixtures containing sperm whale myoglobin, bovine serum albumin, bovine γ-globulin and horse spleen ferritin (mixture I) and cytochrome c, ovalbumin and bovine serum albumin (mixture II) were resolved on a 20 × 20 cm plate in a run of 6.5 h. The mixtures contained 1% of each of the

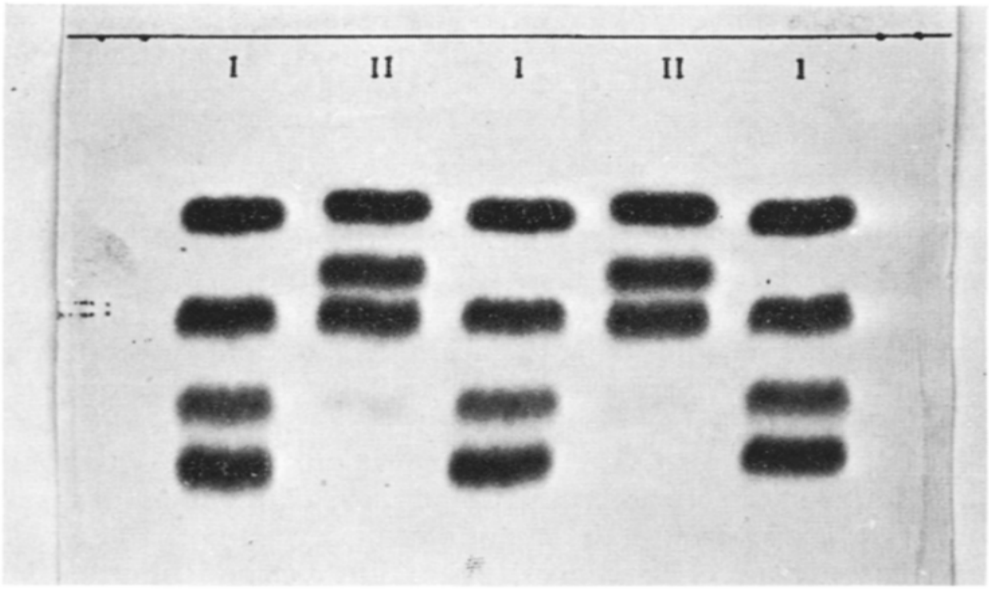


Fig. 1. Thin-layer gel filtration on Sephadex G-200. Proteins (from top to bottom): I = myoglobin, bovine serum albumin, bovine γ-globulin and ferritin; II = cytochrome c, ovalbumin and bovine serum albumin. Sample: 10 μl of the protein mixtures containing 1% of each protein. Plate: 20 × 20 cm. Separation time: 6.5 h. Staining with Amino Black 10B.

proteins, 10 μl being applied on the plate. The corresponding densitometric curves are shown on Fig. 2. Identical migration distances, with reference to the starting line, of the protein common to both mixtures, namely bovine serum albumin, can be clearly seen both on the plates and on the densitograms. Distinct differences in the migration of cytochrome c and myoglobin can also be observed.

A number of factors were found to influence zone size, *viz.* flow rate, protein concentration and sample volume. A flow rate of 1 cm per hour for myoglobin was optimal and yielded well defined zones without the distortion which was frequently observed when the flow rate was doubled. At the same angle of the plates, there was a decreasing flow rate for the three Sephadex gels studied, G-75, G-100 and G-200. With Bio-Gel P-60, the flow rate was comparable to that of Sephadex G-75. With the other polyacrylamide gel tested, P-300, the flow rates were extremely slow and could not be improved even by increasing the angle of the plates up to 40–50°. This is in contrast to the Sephadex gels examined, which all showed a marked dependence of the flow rate on the inclination angle. Improved flow rates could be obtained with

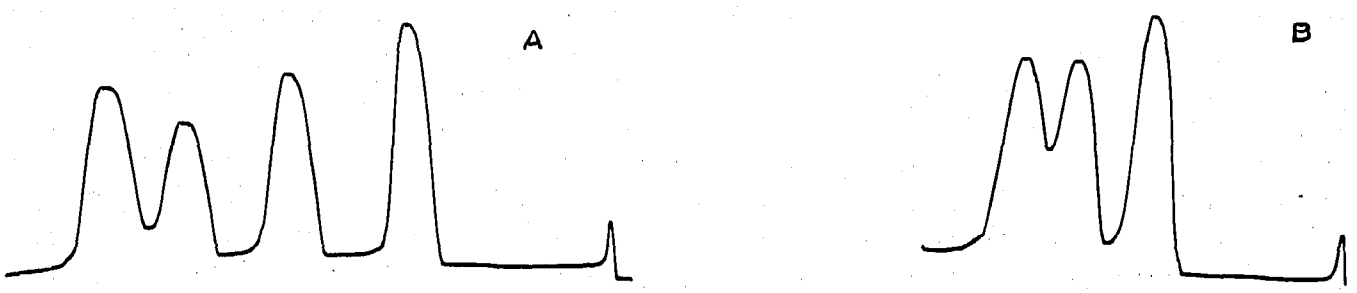


Fig. 2. Densitograms of the two protein mixtures of Fig. 1. A = Mixture I; B = mixture II. The small peak on the right corresponds to the starting line.

Bio-Gel P-300, however, when the plates were coated with suspensions swollen for about ten to fourteen days at 4°, instead of for only two to three days, which was the standard procedure for other gels. Even when gels of P-300 were treated in this manner, the running time had to be increased two to three times to obtain a migration distance of myoglobin comparable to that on Sephadex G-200.

The effect of protein concentration and sample volume on zone size was investigated using sperm whale myoglobin and bovine serum albumin solutions. With 1% protein solutions applied in 10 μl quantities with a cover slide (18 mm edge), as described under *Methods*, the zones obtained after migrating 5–12 cm were usually 8–10 mm wide and 22–23 mm long (Fig. 1). The effect of concentration on the width of the zones was studied on Sephadex G-75 and G-200 with myoglobin solutions of 0.5, 1.0, 2.0 and 3.0%. All solutions were applied in a volume of 10 μl. After gel filtration for 6–7 h with a flow rate of about 1 cm per hour, prints were taken and stained with Amido Black. A 1% solution was run on each plate; its width was 1.0. With the 0.5% solution, a narrowing of the zones was observed by a factor of 0.5–0.6 of the control. With solutions of higher concentration a broadening of the zone width by a factor of 1.15–1.2 for the 2% and 1.3–1.5 for the 3% solution, respectively, was obtained.

Similarly zone size was found to depend on the volume in which the sample was applied. A 1.5 increase of zone width was observed when the sample volume was increased from 10 μl to 40 μl. There was only a small variation in the length of the zone by a factor of no more than 1.1–1.2 of the control, when either protein concentration or sample volume were increased up to 3% or 40 μl.

Besides these differences in the size of the zones, the highest protein concentration (3%) and sample volume (40 μl) caused a slight decrease in the R_M value in experiments with sperm whale myoglobin and bovine serum albumin on two of the Sephadex gels studied, G-75 and G-200. The effect of protein concentration on the R_M value was also studied with haemoglobin and β-lactoglobulin, because both of these proteins may be eluted from gel columns with elution volumes which are concentration-dependent[3]. With thin-layer gel filtration on Sephadex G-200, the R_M value obtained for haemoglobin was constant (1.31) for a 1–2% solution. For β-lactoglobulin there was no difference in the R_M value (1.26) in the 0.5–2% range, while a slight decrease (1.23) was observed with a 3% solution.

The print technique was used throughout this work. Preliminary experiments had shown that much better results were obtained when the paper print was removed after a brief contact with the gel layer (10–30 sec being sufficient) than when the paper was dried on the glass plate in contact with gel, a procedure employed by a number of workers. In order to estimate the amount of protein removed by the print technique, different mixtures of proteins containing 1% of each of the protein were run on Sephadex G-75 and G-200 gel plates. After completion of the run, the first print was taken either after a contact of 30 sec or after 10 min contact. Immediately after removal of the first print, a second print was taken, the paper being kept in contact with the gel for an additional 5 min. All the prints were dried as usual, stained with Amido Black and evaluated densitometrically. Since no additional material could be removed by a third print, the area of the curves of these two prints was taken as 100%.

The amount of protein removed did not appreciably depend on the time of

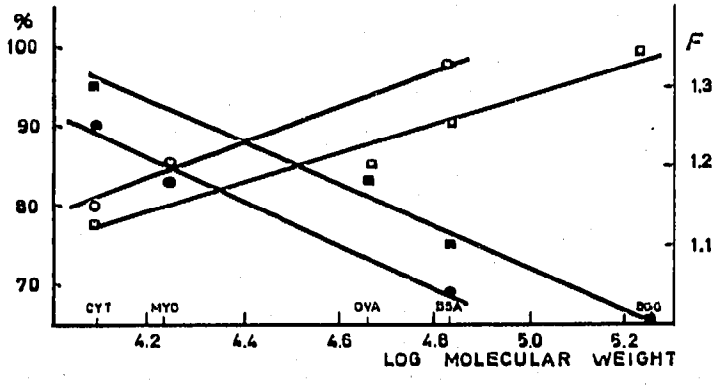
TABLE I

RECOVERY OF PROTEINS (%) BY THE PRINT TECHNIQUE ON SEPHADEX G-75 AND G-200[a]

Protein	*Sephadex G-75*		*Sephadex G-200*	
	First print	*Second print*	*First print*	*Second print*
Protamine	53	47	54	46
Cytochrome c	80	20	77	23
Myoglobin	85	15	—	—
Ovalbumin	—	—	85	15
Serum albumin (bovine)	96	4	90	10
γ globulin (bovine)	—	—	100	0
Ferritin	—	—	100	0

[a] Print taken with filter paper Whatman No. 3. Contacts with the gel layer: first print, 30 sec.; second print, 5 min. Staining with Amido Black 10B, followed by planimetric evaluation of the densitogram.

contact of the paper with the gel; a short contact of 30 sec was almost as effective as a contact of 10 min. The amount of protein removed did, however, depend on its molecular weight. The results of these experiments are presented in Table I. On Sephadex G-200 ferritin and γ-globulin were removed completely with the first print by a short contact, while with all the other proteins an additional amount could be removed by a second print. Both on Sephadex G-75 and G-200, the quantity removed by this print was highest with the three low molecular weight proteins myoglobin, cytochrome c and protamine. With cytochrome c and myoglobin there was again little difference in the recovery of the protein resulting from short or long contact of the first print with the gel. Only with protamine, a strongly basic protein, could a 10–20% increase of the recovery be obtained with 10 min contact.



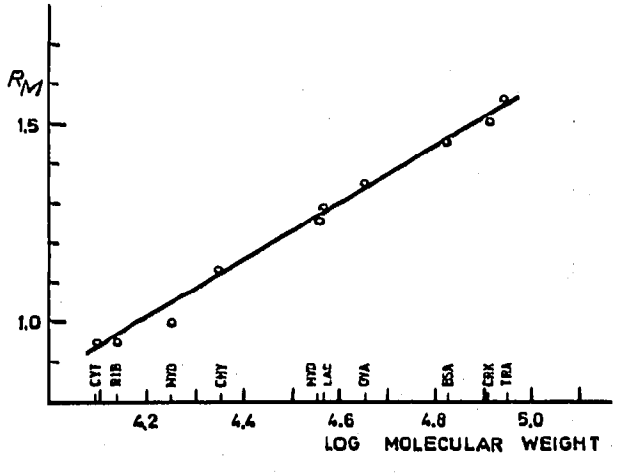


Fig. 3. Recovery of proteins by the print technique. (○——○) Sephadex G-75; (□——□) Sephadex G-200; F = factor correcting recovery to 100%. (●——●) Sephadex G-75; (■——■) Sephadex G-200.

Fig. 4. R_M as a function of the logarithm of molecular weight for thin-layer gel filtration on Sephadex G-75.

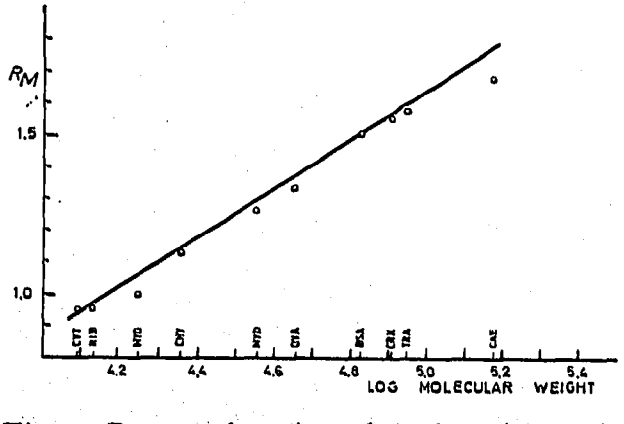
In Fig. 3 the recovery of proteins differing in molecular weight removed by the first print on Sephadex G-75 and G-200 is shown together with the curves for the correction factors calculated to obtain a 100% recovery.

The R_M value was not found to depend on the swelling time of the suspension. This was proved for ferritin and fibrinogen, both proteins of high molecular weight, for which differences would be expected on the basis of experiments with column gel filtration[24]. Plates coated with Sephadex G-200 suspensions kept before spreading for three days at 4°, which was the standard procedure, or for three weeks under the same conditions resulted in identical R_M values for both proteins.

On the basis of these experiments, the following operational parameters were found to yield optimal results: (a) with single proteins, 0.2–2% protein solutions should be used, while in mixtures of proteins the concentration of the individual components should also be of the same order; (b) a sample volume of 10–20 μl per 18–20 mm (cover slide edge); (c) a flow rate of 1 cm per hour and a final migration distance of 5–6 cm for sperm whale myoglobin; (d) prints taken after a short contact of 30–60 sec with the gel.

Relationship between molecular weight or molecular radius and R_M value

For a number of proteins of known molecular weight, as listed in Table II, the R_M values were determined. 1% solutions of a single protein, or mixtures of several proteins containing 1% of each, were prepared in 0.5 M NaCl containing 0.02 M phosphate buffer. Of these solutions, 10 μl were applied to the plates. Five samples were run on each plate with sperm whale myoglobin in the middle. At least twelve samples were run for each individual protein. The arithmetic mean, the standard deviation and the standard error are given in Table II. A good linear relationship between R_M value and logarithm of the molecular weight was obtained for all the Sephadex gels studied. The slopes and the intercepts of the regression lines were calculated by the method of least squares. For the proteins used in these calculations,



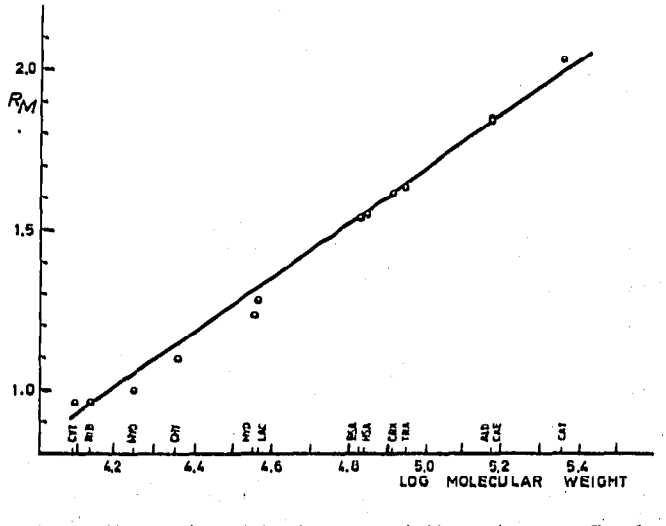
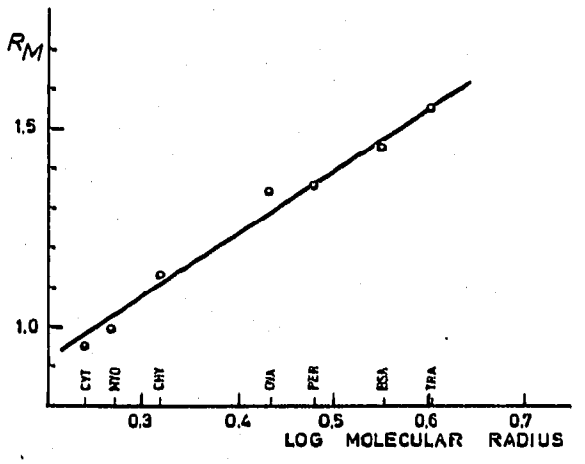


Fig. 5. R_M as a function of the logarithm of molecular weight for thin-layer gel filtration on Sephadex G-100.

Fig. 6. R_M as a function of the logarithm of molecular weight for thin-layer gel filtration on Sephadex G-200.



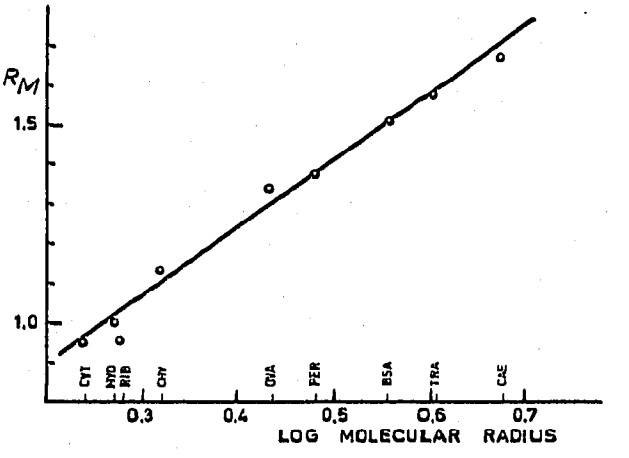


Fig. 7. R_M as a function of the logarithm of molecular radius for thin-layer gel filtration on Sephadex G-75.

Fig. 8. R_M as a function of the logarithm of molecular radius for thin-layer gel filtration on Sephadex G-100.

the results are graphically presented for Sephadex G-75, G-100 and G-200 in Figs. 4, 5, and 6, respectively. With Sephadex G-75, the upper limit for linearity was 90 000. Creatinekinase and transferrin still fitted into the straight line. With Sephadex G-100, the same upper limit for linearity was found. On Sephadex G-200 linearity could be obtained up to a molecular weight of 240 000.

The equations[8] describing the relationship between the logarithm of the molecular weight and the R_M value, calculated according to the method of least squares, were found to be:

$$\text{G- 75:} \quad \log M = 1.402\, R_M + 2.776 \tag{1}$$

$$\text{G-100:} \quad \log M = 1.281\, R_M + 2.896 \tag{2}$$

$$\text{G-200:} \quad \log M = 1.172\, R_M + 3.015 \tag{3}$$

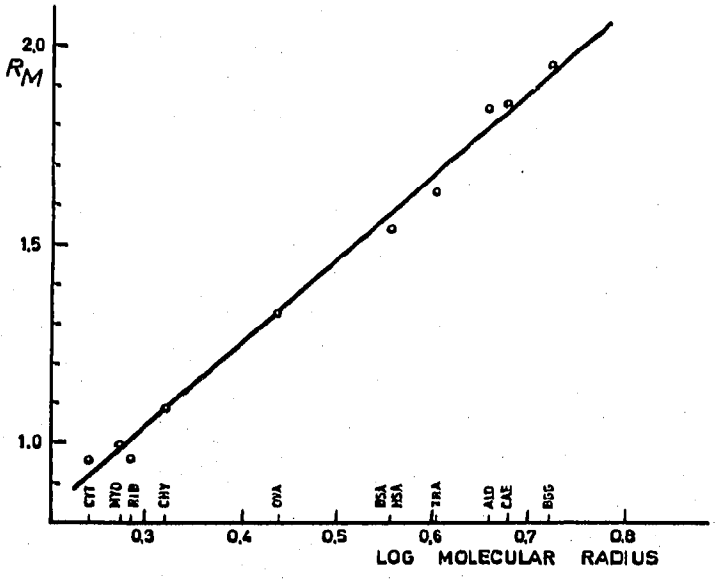


Fig. 9. R_M as a function of the logarithm of molecular radius for thin-layer gel filtration on Sephadex G-200.

TABLE II

R_M VALUES, STANDARD DEVIATIONS AND STANDARD ERRORS OF PROTEINS IN THIN-LAYER GEL FILTRATION ON SEPHADEX G-75, G-100 AND G-200

Protein	*Code*	*Molecular weight*[a] $\times 10^{-3}$	*Molecular radius*[a] *(mμ)*	*G-75*			*G-100*			*G-200*		
				R_M	σ	$\sigma/\sqrt{n}$	R_M	σ	$\sigma/\sqrt{n}$	R_M	σ	$\sigma/\sqrt{n}$
Cytochrome c	CYT	12.4	1.74	0.9522	0.0470	0.0100	0.9515	0.0184	0.0042	0.9597	0.0299	0.0049
Ribonuclease	RIB	13.7	1.92	0.9529	0.0248	0.0045	0.9590	0.0214	0.0064	0.9642	0.0205	0.0044
Myoglobin	MYO	17.8	1.88	1.0			1.0			1.0		
α-Chymotrypsin	CHY	22.5	2.09	1.1323	0.0298	0.0082	1.1366	0.0210	0.0040	1.0952	0.0188	0.0043
Myoglobin dimer	MYD	35.6		1.2549	0.0014	0.0004	1.2628	0.0007	0.0002	1.2337	0.0013	0.0004
β-Lactoglobulin	LAC	36.5		1.2908	0.0118	0.0034				1.2821	0.0369	0.0084
Peroxidase	PER	40.0	3.02	1.3599	0.0286	0.0065	1.3766	0.0244	0.0081	1.3241	0.0326	0.0094
Ovalbumin	OVA	45.0	2.73	1.3467	0.0615	0.0123	1.3390	0.0294	0.0064	1.3318	0.0092	0.0023
Serum albumin (bovine)	BSA	67.0	3.60	1.4547	0.0923	0.0201	1.5090	0.0293	0.0088	1.5434	0.0400	0.0078
Serum albumin (human)	HSA	69.0	3.61							1.5457	0.0573	0.0153
Creatinekinase	CRK	81.0		1.5027	0.0420	0.0126	1.5508	0.0117	0.0033	1.6185	0.0696	0.0186
Transferrin (human)	TRA	89.0	4.02	1.5572	0.0828	0.0249	1.5781	0.0047	0.0014	1.6358	0.0305	0.0088
Alcohol dehydrogenase (yeast)	ALD	150.0	4.55							1.8459	0.0524	0.0165
Caeruloplasmin	CAE	150.0	4.73				1.6719	0.0205	0.0065	1.8574	0.0525	0.0131
γ-Globulin (bovine)	BGG	169.0	5.25							1.9552	0.1564	0.0326
Catalase	CAT	240.0	5.22							2.0362	0.0566	0.0141
Fibrinogen	FIB	330.0	10.07							2.4850	0.0593	0.0182
Apoferritin	APF	465.0	6.00							2.3373	0.0510	0.0117

[a] Molecular weights and radii were taken from refs. 8, 24, 31, 34, 39, 40 and 41.

Sephadex G-75 and G-100 gave a better linearity for the proteins of lower molecular weight than the G-200 gel.

A linear correlation was also obtained between the Stokes radii and the R_M values. In Figs. 7, 8 and 9 the results are shown for Sephadex G-75, G-100 and G-200. The corresponding equations relating the R_M value and the logarithm of the molecular radius calculated for the proteins shown in Figs. 7–9 were:

$$\text{G- 75:} \quad \log r = 0.627\, R_M - 0.376 \tag{4}$$
$$\text{G-100:} \quad \log r = 0.588\, R_M - 0.329 \tag{5}$$
$$\text{G-200:} \quad \log r = 0.479\, R_M - 0.202 \tag{6}$$

Comparison of the separation efficiency of Sephadex G-200 and Bio-Gel P-300

Experiments with ferritin have demonstrated that with Bio-Gel P-300 the fractionation range can be considerably extended. When crystalline ferritin was separated on Sephadex G-200, a single zone was obtained which, on densitometry, showed a slight asymmetry in the leading part (Fig. 10). On Bio-Gel P-300 three zones were detected, which could be densitometrically evaluated without staining the ferritin due to its intense brown colour. They were found to amount to 80%, 16% and 4% of the total material in the order of increasing migration distance. These figures, compared with those obtained by other workers on gel electrophoresis[25–27],

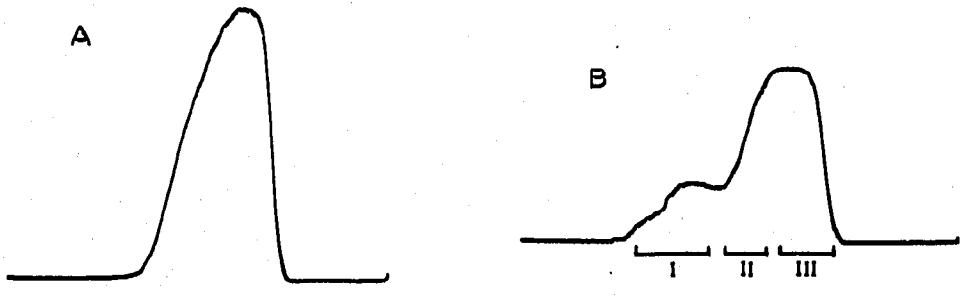


Fig. 10. Thin-layer gel filtration of ferritin on (A) Sephadex G-200 and (B) Bio-Gel P-300. Fractions I, II and III were isolated by preparative thin-layer gel filtration and used for electrophoresis in polyacrylamide gel.

strongly suggested that the fractions obtained on thin-layer gel filtration correspond to the ferritin components found on gel electrophoresis in polyacrylamide and starch.

A preparative thin-layer gel filtration method followed by polyacrylamide gel electrophoresis was employed to test this hypothesis. 120 μl of a 10% ferritin solution were applied by means of a 12 cm long glass plate to a standard 20 × 20 cm glass plate with a 0.5 mm layer of Bio-Gel P-300. An angle of 20° was used. The separation could be followed easily because of the intense colour of ferritin. After a run of 24 h, a print was taken with filter paper Whatman No. 3. Three strips were cut perpendicularly to the direction of separation (as indicated in Fig. 10) and were eluted with distilled water in a moist chamber by placing one end of the strip in a beaker filled with distilled water. The eluates were dialyzed and concentrated by vacuum evaporation to 1–2% protein concentration. The result of gel electrophoresis of these fractions is shown in Fig. 11. Distinct differences in the separation of the α, β and γ-

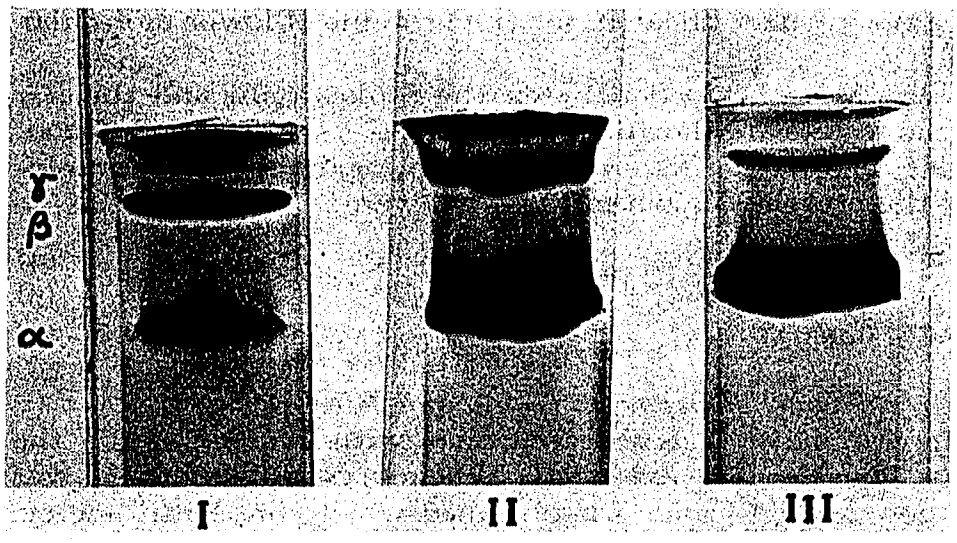


Fig. 11. Disc electrophoresis of ferritin fractions isolated by preparative thin-layer gel filtration on Bio-Gel P-300. For description of fractions I, II and III, see Fig. 10.

ferritin in the thin-layer fractions can be seen. The most rapid fraction (FI) on thin-layer gel filtration consists predominantly of β and γ-ferritin with some α-ferritin. Fractions FII and FIII are composed of α-ferritin with only small amounts of the electrophoretically slower β and γ-ferritins.

DISCUSSION

Many of the claims for the advantages of thin-layer gel filtration over the column technique are fully justified. Simplicity and speed, need of a minimal amount of material in the microgram range, high resolution and good reproducibility are among the features of the method. Application of the substance as a streak has made possible subsequent densitometric evaluation of the pattern thus providing a means for a quantitative approach. The possibility of running different samples simultaneously on the same plate is a useful property of the thin-layer technique, which should prove of value in the analysis of heterogeneous systems and fractions isolated during preparative work. Thin-layer gel filtration is basically an analytical method; however, preparative separations with amounts up to 10 mg of proteins can be easily accomplished on standard 20 × 20 cm plates with a 0.5 mm layer and the protein load could probably be increased on plates with thicker layers. Besides this, in preparative work thin-layer gel filtration presents a useful supplementation of the column gel filtration technique for establishing optimum separation parameters (gel type and solvent).

The possibility of taking a print after completion of the run is a valuable characteristic of thin-layer gel filtration. Thus a permanent record is obtained which can be handled as easily as paper chromatograms and electropherograms and which can be evaluated by standard densitometric equipment. Protein detection by the print technique has many advantages over direct detection on the glass plate. While incomplete removal of the proteins of low molecular weight appears to be a drawback of the print technique (Table I), a complete removal is not a critical factor in many

experiments. When quantitative evaluation of systems containing proteins of different molecular weight is attempted, incomplete removal will introduce an error for the components of low molecular weight which can however be compensated for either by applying a correction factor (Fig. 3) or by taking two consecutive prints.

Thin-layer gel filtration has proved to be equally well suitable for a qualitative analysis of homogeneous proteins and of heterogeneous systems, as well as for the determination of the molecular weight of proteins. While thin-layer and column gel filtration differ in many respects, a comparison of the results obtained by both techniques demonstrates that they are basically identical.

The suitability of gel filtration for the determination of molecular weight of globular proteins has been fairly well established[3,8,24,28,29]. Good correlations were found between molecular weight and gel filtration behaviour for a number of globular, carbohydrate-free proteins on a variety of different gels. With more information on the gel filtration behaviour of different proteins, some of the limitations of the method are being better realized[24,30]. Simultaneously, new potentialities of gel filtration for determination of other molecular parameters are being recognized[24,31,32].

While most of the results on the determination of molecular weight by gel filtration have been obtained with the column technique, there are already a few reports that a good correlation exists also between gel filtration behaviour and molecular weight on thin layers[3-5,7-10]. For all the Sephadex gels studied, G-75, G-100 and G-200, a good linear relationship between R_M value and molecular weight was obtained (Figs. 4–6). The upper limit for the linear correlation for Sephadex G-75 was about 90 000, a higher value than that calculated for the exclusion limit of this gel in column gel filtration[8]. For the G-100 gel also 90 000 was found as an upper limit for the linear correlation. Caeruloplasmin and yeast alcohol dehydrogenase, both with a molecular weight of 150 000, did not fit well into the straight line of the R_M–log molecular weight relationship. However, no proteins with an intermediate molecular weight between 90 000 and 150 000 were tested. For the G-100 gel an extension of the upper limit above the value of 90 000 appears to be possible. For the G-200 gel the upper limit for the linear correlation was 240 000. This value is also higher than that found in column gel filtration, though linearity up to a range of 500 000 and even higher has been claimed for the G-200 gel in columns depending on the gel batch and the swelling time[24].

No evidence for linearity was obtained in thin-layer gel filtration on Sephadex G-200 in the range above 240 000. None of the three proteins tested, with molecular weights higher than that of catalase (240 000), namely fibrinogen, apoferritin and serum macroglobulins, fitted into the straight line of the R_M–log molecular weight relationship. The anomalous gel filtration behaviour of fibrinogen can be explained in terms of its strong molecular asymmetry. The molecular weight found for apoferritin was 575 000, a value much higher than those found by other methods. Some of the possible reasons for the deviation of apoferritin will be discussed later. For the serum macroglobulins an R_M value of 2.35 was obtained[33] with a corresponding molecular weight of 600 000, which is much lower than the accepted value of 820 000–1 000 000 (see ref. 34).

There is only very limited information on the gel filtration behaviour of proteins with molecular weights higher than 240 000 on Sephadex G-200 thin layers. In the range above 240 000, linearity of a relationship between the logarithm of the molecu-

lar weight and the migration rate was obtained with thyroglobulin (mol.wt. 650 000) in one laboratory[4], whereas a deviation from linearity was observed for the same protein in another laboratory[10]. One possible explanation for these conflicting results may be differences in the technique employed for detecting the proteins. Drying the paper print in contact with the gel layer on the glass plate, a technique used by MORRIS[4] has been found in this laboratory to cause a displacement of the zones strongly affecting the zones located at the ends of the plates. In addition, thyroglobulin has been shown to be heterogeneous[24,35], another possible reason for irregular gel filtration behaviour.

Obviously further work is needed with well defined proteins of molecular weights higher than 240 000, to establish the quantitative relationship in this range. Non-linearity of the relationship between the logarithm of the molecular weight and the migration rate in this range would not present a limiting factor for molecular weight or Stokes radii determinations since it has been already demonstrated in column gel filtration that the non-linear part of the calibration curves (reduced elution volume and log molecular weight) may also provide a useful basis for these determinations[24].

Although it has been repeatedly stated that the accuracy of the molecular weight determinations by the thin-layer method is lower than that of the column technique, the results reported in this paper indicate that this is not necessarily so. Although it has not been possible to eliminate fluctuations in migration of the same protein on the same plate, statistical evaluation of several runs (10–15) of a single protein or a protein mixture which can easily be obtained on two to three plates of the standard size shows good reproducibility of the R_M values. An accuracy of $\pm$ 10% for the determination of molecular weight was calculated for the Sephadex G-200 gel. A value of 10% for the uncertainty in molecular weight determinations of carbohydrate-free, globular proteins by the column gel filtration technique has been considered to be appropriate[24]. The effect of fluctuations in migration on thin-layer gel filtration can be reduced by using an internal standard whereby the reference protein is run in a mixture with the unknown protein, provided that there is a sufficient difference in molecular weight for the distinct separation of both and that there is no interaction between them. The early claims for differences in standard curves for thin-layer molecular weight estimations with various batches of Sephadex[9] could not be confirmed. Identical R_M values were obtained with different batches of Sephadex G-75, G-100 and G-200.

All the proteins proposed for calibration of gel filtration columns proved to be suitable for establishing the quantitative relationship between migration rate and molecular weight on thin layers. Globular proteins, which on column gel filtration yield values consistent with those obtained by other methods, gave similar values also on thin-layer gel filtration, while carbohydrate-rich proteins, known to deviate on column gel filtration[3,24,28,36], migrate anomalously also on thin layers.

For some of the proteins a different behaviour was observed with different gel types. Thus peroxidase, a carbohydrate-containing enzyme[37,38], with a molecular weight of 40 000 was found to have a molecular weight of 48 000 and 46 000 by thin-layer gel filtration on Sephadex G-75 and G-100, respectively. By thin-layer gel filtration on Sephadex G-200, however, a value of 41 000 was obtained for the molecular weight. Ovalbumin, another glycoprotein, fitted well into the straight-line correlation between the molecular weight and the R_M value for the G-75 and G-100 gel;

it was however repeatedly found to deviate from the straight line on Sephadex G-200. The reason for this anomalous behaviour on certain gel types is not known.

The molecular weight obtained for bovine γ-globulin was 200 000, which is very close to the value obtained on column gel filtration[24], but is considerably higher than the accepted molecular weight of 169 000 (see ref. 39). Haemoglobin has been shown to dissociate on column gel filtration[3,28] and this dissociation has also been observed in thin-layer gel filtration[4]. The R_M value obtained for a 1–2% protein concentration corresponds to a molecular weight of 35 600, a value very close to one-half of that obtained by sedimentation and X-ray crystallographic data. The concentration-dependent dissociation of β-lactoglobulin, found on column gel filtration[3], however, was not observed when 0.5–3% solutions of this protein were separated by thin-layer gel filtration of Sephadex G-75 and G-200.

While there is general agreement that the gel filtration behaviour of proteins is correlated to molecular size, there is some uncertainty as to which size parameter is decisive. In a theoretical treatment of the gel filtration process a relationship between the gel filtration behaviour of proteins and their Stokes radii has been deduced[31,40]. For most proteins studied, useful empirical correlations have been obtained between gel filtration behaviour and molecular weight or gel filtration behaviour and Stokes radii or diffusion coefficients. The basis for this is both the similar frictional ratios and the partial specific volumes of the proteins used[3,24,28,29,31,37]. For ferritin, urease and fibrinogen, it has been claimed that the gel filtration behaviour is a function not of the molecular weight but of the Stokes radius[32]. This claim may be criticized because there is no general agreement on the molecular weight of ferritin or urease and because fibrinogen belongs to the group of carbohydrate-containing proteins for which a deviation from normal behaviour on gel filtration has been observed[8].

When ferritin and fibrinogen with myoglobin added as an internal standard were run on the same plates of Sephadex G-200, a higher R_M value was found for the latter. With an R_M value of 2.485 fibrinogen does not fit into the linear relationship between the R_M values and the molecular weight, but it correlates fairly well with the R_M–Stokes radius relationship (Fig. 9, eqn. (6)). Ferritin and apoferritin, prepared from it by reduction with sodium dithionite, gave identical R_M values when run on the same plates with myoglobin added as an internal standard. Since the molecular weight of ferritin depends on the iron content, its iron-free protein component, apoferritin, with a defined molecular weight of 465 000 and the same molecular diameter as ferritin, is much better suited for determining if it is the molecular weight or the Stokes radius which determines gel filtration behaviour. With an R_M value of 2.34, apoferritin did not fit into the straight line of the R_M value–molecular weight relationship (Fig. 6, eqn. (3)). The Stokes radius obtained for this value corresponded to 80 Å, a value very close to that calculated in one of the papers postulating gel filtration behaviour to be a function of the Stokes radius[32]. This value, however, is in conflict with the available data on the diameter of apoferritin, derived from X-ray diffraction measurements on wet crystals, by which a diameter of 122 ± 6 Å was obtained[41]. A diameter of about 100 Å was determined by electron microscopy[42–44]. The difference may be due to molecular shrinkage caused by loss of water. Thus the results obtained with ferritin, apoferritin and fibrinogen are not consistent. A possible reason for the deviation observed for apoferritin appears to be the heterogeneity of ferritin and the apoprotein derived from it, which has been well established in gel

electrophoretic experiments and has also been confirmed in this paper by thin-layer gel filtration on Bio-Gel P-300.

On starch gel and polyacrylamide gel electrophoresis, crystalline ferritin and apoferritin have been shown to occur as several distinct components, termed α, β and γ, according to decreasing electrophoretic mobility[25–27,45]. By a number of methods, including column gel filtration on Sephadex G-200, an enrichment of some of the components has been achieved[45,46]. Ultracentrifugal analysis provided evidence that the electrophoretically distinct apoferritin components differ in molecular size and probably correspond to the dimer, trimer and even higher polymers of a monomeric unit with the molecular weight of 465 000 (see refs. 45 and 47). Whereas crystalline ferritin gave on thin-layer gel filtration with Sephadex G-200 only a single zone, with Bio-Gel P-300 three zones were detected (Fig. 10), which on densitometry were found to amount in the order of increasing migration rate, to 80%, 16% and 4% of the total material, respectively. These figures compare well with those obtained on gel electrophoresis[27], where 75–85% of α-ferritin, 10–15% of β-ferritin and about 10% of γ-ferritin have been found. Thin-layer gel filtration on Sephadex G-200 of the three ferritin fractions isolated by the preparative method on Bio-Gel P-300 yielded different R_M values; the highest R_M value was obtained for Fraction I, the lowest for Fraction III.

The results obtained with the three fractions, isolated after preparative thin-layer gel filtration, indicate that a separation of the electrophoretically distinct components of ferritin can also be achieved by means of gel filtration, provided a suitable gel is chosen. It is an unsolved question whether the small amounts of α-ferritin present in Fraction I and the β- and γ-ferritin present in Fractions II and III are due to overlapping or whether they reflect association–dissociation phenomena[48].

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