ESTIMATION OF MOLECULAR WEIGHTS OF PROTEINS BY BIO-GEL P GEL FILTRATION

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Molecular sieve chromatography, or gel filtration, is a very well known technique for fractionation, isolation, analysis and purification of proteins, enzymes, hormones, nucleic acids, etc. It was suggested by LATHE AND RUTHVEN[1] that the dimensions of proteins and polysaccharide molecules might be estimated by this technique; although restricted to certain homologous series of macromolecules, correlation between molecular weight and size in gel filtration has been demonstrated by GRANATH AND FLODIN[2]. ANDREWS[3] showed by experiments with agar gel columns that this technique can be used as a comparative method to estimate molecular weights of proteins. This has been suggested by further reports about correlation between molecular weights of globular proteins and their behaviour on Sephadex or agar gel columns[4–11] or polyacrylamide columns[12].

Bio-Gel P is a copolymer of acrylamide and methylenebisacrylamide; the present report is concerned with finding a relationship between the molecular weights and the gel filtration behaviour of some globular proteins. Experiments are described in connection with three of the Bio-Gel P gel filtration materials (P-100, P-200 and P-300).

MATERIAL AND METHODS

Proteins

The proteins used in this study are listed in Table I. Coproporphyrinogenase was purified as described by BATLLE, BENSON AND RIMINGTON[25] and purified δ-aminolaevulinic acid dehydrase was prepared in this laboratory from cow liver (unpublished work).

Preparation and use of gel columns

Columns were packed in vertical glass tubes (30 cm long and either 1.6 or 1.8 cm I.D.), equipped with capillary outlets. A small glass wool plug and glass beads were added to fill the bottom of the tube to obtain a small mixing chamber. It is advisable to coat the column before use to avoid distortion of the bands which is due to the tendency of the buffer to flow at a faster rate near the glass wall. Desicote (Beckman) used diluted, or a solution of 1 % dichlorodimethylsilane in benzene can be

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used. The solution is heated to about 60°, poured into the column, allowed to stand for 15–20 min, it is then decanted and the column dried in an oven. The procedure is repeated to give a double coat and the column can then be used for several months. Bio-Gel P-100 (Control No. 2880, particle size 50–150 mesh, U.S. standard), Bio-Gel P-200 (Control No. 2810 and No. 2759, particle size 50–150 mesh, U.S. standard) and Bio-Gel P-300 (Control No. 2859 and No. 2760, particle size 50–150 mesh, U.S. standard), were generous gifts from Bio Rad Laboratories, Richmond, Calif., U.S.A.

The dry gel was added slowly, with constant stirring, to the buffer to be used for elution and allowed to swell for 24 h. Each column was packed with the swollen gel, previously deaerated under reduced pressure, as described for Sephadex columns by ANDREWS[9,11]. When packing was completed, the upper surface of the gel was covered with a 1 cm layer of Bio-Gel P-30 and a well fitting filter paper disc (Whatman No. 1). Columns were operated at a flow rate of 0.17 ml/min to 0.5 ml/min.

Except where otherwise stated columns were equilibrated either with 0.05 *M* Tris–HCl buffer, pH 7.4, containing 0.1 *M* KCl or with 0.067 *M* phosphate buffer, pH 6.8, containing 0.1 *M* KCl.

All experiments were done at room temperature. Proteins were dissolved in the equilibration solution (2–4 ml) and applied to the top of the column by layering under the solution already present (the density of the protein solution was increased by the addition of sucrose, when necessary).

Column effluents were collected with an automatic fraction collector (The Locarte Co., London, S.W. 7).

Examination of the column effluents

Proteins were estimated either spectrophotometrically at appropriate wavelengths or by suitable enzyme assays (Table I), in a Beckman D.U. Spectrophotometer. Urease was measured by titration, with 0.01 *N* HCl, of the ammonia it liberated from a solution containing urea[26]. Sucrose was estimated by a colorimetric method[27].

RESULTS

Behaviour of proteins of known molecular weight in columns of different size

Two sets of small columns were used. The results shown in Fig. 1 were obtained with 1.6 × 30 cm columns (60 ml bed volume) and those of Fig. 2 with 1.8 × 30 cm columns (76 ml bed volume). Columns of these sizes do not give the best resolution possible; nevertheless the results obtained with the 1.8 × 30 cm columns are good enough for our purpose.

The proteins shown in Table I were used alone and in several combinations, together with sucrose to determine their elution volumes from the Bio-Gel columns. In most experiments cytochrome *c* was also added to the mixture of proteins.

Sometimes differences of 2 or 3 ml were obtained in elution volumes for some proteins in separate experiments with the same column. This may be due to temperature changes or changes in density of the column packing, though swelling of the gel packed in a column was not observed over a period of some weeks. For individual proteins V_e also varied as much as 4 ml to 6 ml from one column to another, which were identical in size but packed with gel from different batches; this is probably due to differences in the degree of cross-linking from batch to batch.

TABLE I

PROTEINS EMPLOYED IN THE STUDY

Protein	*Source*	*Mol.wt.* $\times 10^{-3}$	*Reference*	*Amounts used (mg)*	*Method of estimation*
Thyroglobulin (bovine), purified	Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.	670	EDELHOCH[13]	1–2	*E* at 230 mμ
Urease, "urease soluble"	British Drug Houses Ltd., Poole, Dorset, England	470–510	SHULMAN[14], GORIN *et al.*[15]	2–6 (crude)	See the text
Edestin	D.Th. Guchardt G.M.B.H., Chemische Fabrik Görlitz	310	FRUTON AND SIMMONDS[16]	2	*E* at 230 or 280 mμ
Catalase (from ox liver)	Boehringer & Soehne G.M.B.H., Mannheim, Germany	230–250	SAMEJIMA AND YANG[17]	0.04–0.1	Disappearance of H_2O_2 followed at 230 mμ (TOHUHIKO AND PETERS[21])
Serum albumin dimer (present in bovine serum albumin)		134	(2 × mol. weight of serum albumin)	~0.4–0.5	*E* at 220 mμ
Serum albumin bovine; fraction V from bovine plasma	Armour Pharmaceutical Co. Ltd., Chicago, Ill., U.S.A.	67	PHELPS AND PUTNAM[18]	1–8	*E* at 230 or 280 mμ
Haemoglobin	Sigma Chemical Co., St. Louis, Mo., U.S.A.	64.5	HILL *et al.*[19]	2–10	*E* at 410 mμ
Ovalbumin	Schering-Kahlbaum A.G., Berlin	45	WARNER[20]	1–2	*E* at 230 mμ
Pepsin	British Drug Houses Ltd., Poole, Dorset, England	35	FRUTON AND SIMMONDS[16]	2	*E* at 230 or 280 mμ
Chymotrypsinogen, purified	Worthington Biochem. Corp., Freehold, N.J., U.S.A.	25	HARTLEY[21]	1	*E* at 220 or 230 mμ
Ribonuclease, purified	Worthington Biochem. Corp., Freehold, N.J., U.S.A.	13.7	HIRS *et al.*[22]	0.5–2	*E* at 220 mμ
Cytochrome *c* from horse heart, type II	Sigma Chemical Co., St. Louis, Mo., U.S.A.	12.4	MARGOLIASH[23]	0.4–2	*E* at 412 mμ
Insulin, purified	Eli Lily & Co., Liverpool, England	6	FRUTON AND SIMMONDS[16]	1	*E* at 230 or 280 mμ
Sucrose	British Drug Houses Ltd., Poole, Dorset, England	0.348		4–8	See the text

The elution volume of many of the proteins listed in Table I was observed for at least two concentrations, which usually differed by three or four fold; except with haemoglobin, no significant effect on the elution volumes was shown over the range of concentrations studied. The smallest quantity that can be used is 0.2 to 0.3 mg, or less with purified enzymes which are generally used in microgram amounts and can be estimated by specific and sensitive assay methods.

Elution volumes for haemoglobin at pH 6.8 and 7.4, showed variation with protein concentration, being lower than expected from their molecular weight when small amounts of protein were used and suggesting some degree of dissociation of the molecules into subunits under these conditions. ANDREWS[9,11] has already found the same behaviour for haemoglobin, β-lactoglobin and glutamate dehydrogenase with his Sephadex columns, which makes proteins such as haemoglobin, unsuitable as reference substances in gel filtration.

Most experiments were carried out with columns equilibrated with potassium phosphate buffer pH 6.8 or Tris–potassium chloride buffer pH 7.4, but for some experiments with urease, columns equilibrated with 0.15 *M* potassium chloride were used; urease examined under both conditions showed similar behaviour.

Relationship between elution volume and molecular weight

Figs. 1 and 2 show plots of elution volumes (V_e) from Bio-Gel P-100, P-200 and P-300 columns against log molecular weight, for proteins listed in Table I.

The results plotted in Fig. 1, were obtained with small columns and except for Bio-Gel P-100, the relationship between V_e and log (mol.wt.) is not completely linear;

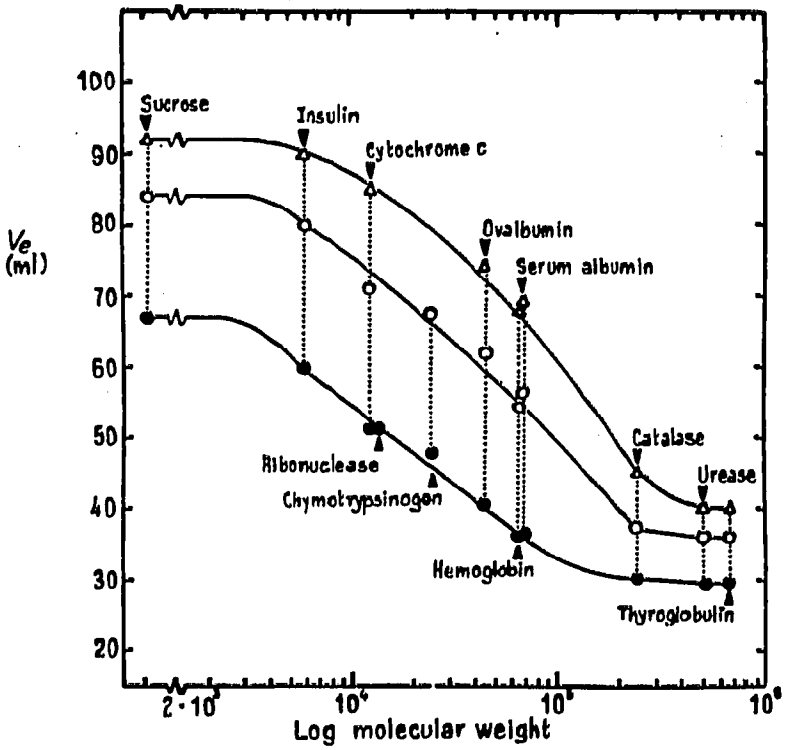

Fig. 1. Plots of elution volume (V_e) against log(mol.wt.), for proteins on Bio-Gel P-100 (●——●), P-200 (○——○) and P-300 (△——△). Columns (1.6 cm × 30 cm), equilibrated with 0.067 *M* potassium phosphate buffer, pH 6.8, containing 0.1 *M* potassium chloride.

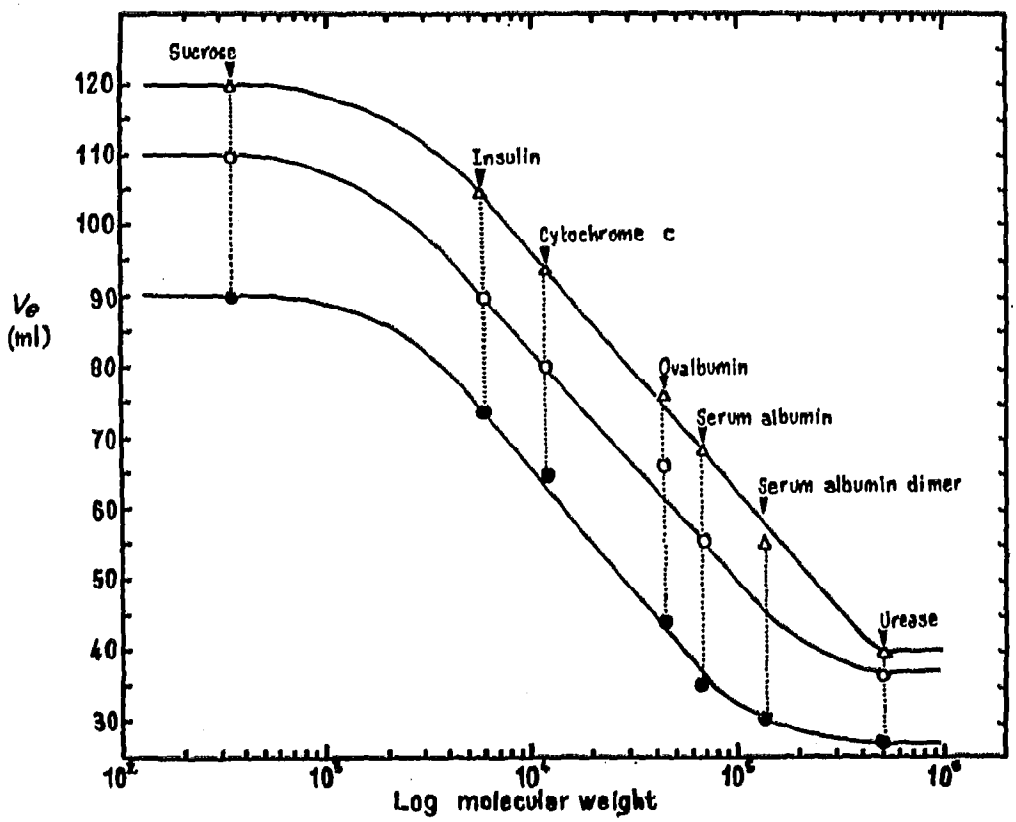

Fig. 2. Plots of elution volume (V_e) against log (mol.wt.), for proteins on Bio-Gel P-100 (●——●), P-200 (○——○) and P-300 (△——△). Columns (1.8 cm × 30 cm) equilibrated with 0.067 *M* potassium phosphate buffer, pH 6.8, containing 0.1 *M* potassium chloride.

however, data are reproducible. The relationship between V_e and log (mol.wt.) for bigger columns is shown in Fig. 2. For Bio-Gel P-100 the experimental points lie close to a straight line in the molecular weight range 4,000–80,000, for Bio-Gel P-200 the range is 4,000–150,000, and for Bio-Gel P-300 the range is 5,000–400,000. However, the useful working range of P-100 and P-200 extends above these values; but it must be noted here that the upper limit for these gels depends upon the extent to which the gel has swollen. As with Sephadex gels[9,11] the exclusion limit, in terms of molecular weight, is greater than that specified by the manufacturer.

Experiments with insulin, cytochrome *c*, ovalbumin, serum albumin and pepsin, using Bio-Gel P-100 and P-300 columns (1.8 × 30 cm), were carried out at pH 4.0, 5.0, 6.0, 6.8, 7.4 and 8.0. Plots of V_e against log (mol.wt.) at each pH were similar to those at pH 7.4 (Fig. 2), plots at pH 6.8 and 7.4 fitted identical curves; but at lower pH's, the slope of the linear parts of the graphs slightly increased as the pH decreased.

Experiments with several proteins indicate that quantitative recovery is obtained (in all cases greater than 90 %) when using these Bio-Gel columns.

Molecular weight estimations

Elution volumes of coproporphyrinogenase and δ-aminolaevulinic acid dehydrase were determined on calibrated Bio-Gel P-200 columns, and their molecular weights were estimated from calibration curves. Each enzyme was put through the columns alone, and they were also run in admixture with cytochrome *c*. For

coproporphyrinogenase a value of 85,000 was found, while 80,000 was obtained using Sephadex columns[25]. The molecular weight of δ-aminolaevulinic acid dehydrase was estimated to be 140,000.

DISCUSSION

The relationship between the molecular weight of proteins used in these experiments and their elution volumes from Bio-Gel columns (Fig. 2) is similar to that obtained for Sephadex columns[9,11].

The linear parts of the curves shown in Fig. 2, indicate the molecular weights between which each gel gives optimum separation of proteins, but the useful parts of the curves extend beyond the linear stretches. However, the 30 cm columns used in this study did not give the best resolution obtainable with these gels; better results can be attained by using longer columns.

The amount of any protein necessary for a molecular weight estimation depends on the method used to measure its concentration in the column effluents; but generalizing, 0.2 mg of material is sufficient for such an estimation.

As has already been found by ANDREWS[9], dissociation of haemoglobin occurs while it passes through the column, therefore haemoglobin is not convenient as a protein standard.

Because of the anomalous behaviour of glycoproteins in gel filtration[11] only globular proteins were used for calibration of Bio-Gel columns, as most of the enzymes belong to this group of proteins the method seems to be useful.

Estimates of the molecular weights of simple proteins with Bio-Gel P-100, P-200 and P-300 (Fig. 2) are accurate to within 12 %, though estimates for other types of protein may be less accurate. On the whole, molecular weight estimations by Bio-Gel are, however, fairly reliable and the absence of ionic groups on the inert matrix of polyacrylamide diminishes the undesirable adsorption effects to a minimum.

The use of Bio-Gel offers advantages over other gel filtration materials. It swells more rapidly in various solvents; as stated above, it has an inert matrix which minimises adsorption; the Bio-Gel P series covers a wide range of molecular weights from 200–> 400,000 and its cost is less than similar materials.

In spite of the possible causes of error, also found with other gel filtration media, the method proves to be very useful and practical.

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SUMMARY

Correlation between elution volumes, V_e, and molecular weight was investigated by Bio-Gel filtration of proteins of known molecular weight on Bio-Gel P-100, P-200 and P-300 columns at pH 6.8 and 7.4. The relationship between V_e and molecular weight was similar over the pH range 4.0 to 8.0.

The molecular weights of two enzymes, coproporphyrinogenase and δ-amino-

laevulinic acid dehydrase, were estimated on calibrated Bio-Gel P-200 columns; their respective values were in agreement with those obtained by using calibrated Sephadex columns.

The uncertainty in values estimated by this procedure was put at 12 %.

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