The Gel-Filtration Behaviour of Proteins Related to their Molecular Weights over a Wide Range

By P. ANDREWS
National Institute for Research in Dairying, Shinfield, nr. Reading, Berks.

(*Received* 21 *December* 1964)

1. Correlation between elution volume, V_e, and molecular weight was investigated for gel filtration of proteins of molecular weights ranging from 3500 (glucagon) to 820000 (α-crystallin) on Sephadex G-200 columns at pH 7·5. 2. Allowing for uncertainties in the molecular weights, the results for most of the carbohydrate-free globular proteins fitted a smooth V_e–log(mol.wt.) curve. In the lower part of the molecular-weight range the results were similar to those obtained with Sephadex G-75 and G-100 gels. 3. V_e–log(mol.wt.) curves based on results with the three gels are taken to represent the behaviour of 'typical' globular proteins, and are proposed as standard data for the uniform interpretation of gel-filtration experiments. 4. Some glycoproteins, including γ-globulins and fibrinogen, do not conform to the standard relationship. The effect of shape and carbohydrate content on the gel-filtration behaviour of proteins is discussed. 5. As predicted by the theoretical studies of other authors, correlation exists between the gel-filtration behaviour and diffusion coefficients of proteins. 6. The lower molecular-weight limit for complete exclusion of typical globular proteins from Sephadex G-200 varies with the swelling of the gel, but is usually $>10^6$. 7. The concentration-dependent dissociation of glutamate dehydrogenase was observed in experiments with Sephadex G-200, and the sub-unit molecular weight estimated as 250000. The free sub-units readily lose enzymic activity. 8. Recognition of the atypical gel-filtration behaviour of γ-globulins necessitates an alteration to several molecular weights previously estimated with Sephadex G-100 (Andrews, 1964). New values are: yeast glucose 6-phosphate dehydrogenase, 128000; bovine intestinal alkaline phosphatase, 130000; *Aerobacter aerogenes* glycerol dehydrogenase, 140000; milk alkaline phosphatase, 180000.

The proposal, arising from experiments with agar-gel columns, that gel filtration can be used to estimate the molecular weights of proteins (Andrews, 1962) has been supported by further reports of correlation between the molecular weights of globular proteins and their behaviour on Sephadex or agar-gel columns (Wieland, Duesberg & Determann, 1963; Whitaker, 1963; Iwatsubo & Curdel, 1963; Largier & Polson, 1964; Andrews, 1964; Andrews, Bray, Edwards & Shooter, 1964). The need for caution in interpreting the gel-filtration behaviour of proteins in terms of molecular weights has often been emphasized. Whilst this will always be true, it also reflects the existing lack of information on the gel-filtration behaviour of well-characterized proteins, particularly those of high molecular weight and of different structural types. The present investigation is concerned with these points, and with finding a relationship between the molecular weights and gel-filtration behaviour of 'typical' globular proteins which could be used as standard data for interpreting gel-filtration results.

MATERIALS AND METHODS

Proteins. Glucagon, cytochrome *c*, myoglobin, chymotrypsinogen, ovalbumin, serum albumin monomer and dimer, *Escherichia coli* phosphatase, alcohol dehydrogenase, human γ-globulins and thyroglobulin were of the same quality and from the same suppliers as those used in previous gel-filtration experiments (Andrews, 1964). Of the remainder, crystalline aldolase, catalase, fumarase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase were obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany, purified malate dehydrogenase from Servac Laboratories (Pty.) Ltd., Holyport, Maidenhead, Berks., bovine γ-globulins (fraction II) from Armour Laboratories, Eastbourne, Sussex, purified bovine transferrin from Mann Research Laboratories, Inc., New York, N.Y., U.S.A., and urease ('urease-soluble', 1500 Sumner units/g.) from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Fibrinogen was prepared from

bovine fibrinogen (fraction I, type I; Sigma Chemical Co., St Louis, Mo., U.S.A.) by precipitation with $(NH_4)_2SO_4$ as described by Laki (1951).

Crystalline or highly purified samples of human caeruloplasmin, *Escherichia coli* β-galactosidase, α-conarachin, ferritin and apoferritin, α-crystallin, ovomucoid, lactoperoxidase, R-phycoerythrin and fetuin were generously provided by Professor H. F. Deutsch, Professor K. Wallenfels, Dr J. M. Dechary, Dr Pauline M. Harrison, Dr T. C. Laurent, Dr M. D. Melamed, Mr A. Pickering, Dr J. Porath and Dr R. G. Spiro respectively. 'Blue dextran' (dextran with a blue dye chemically bound to it), weight-average mol. wt. $\sim 2 \times 10^6$, was kindly supplied by Dr B. Gelotte.

Preparation and use of gel columns. Sephadex G-200 gel-filtration medium (lots no. To 46 and To 3016) was sieved, and material of 200–300 mesh particle size added to dilute KCl solution and allowed to swell for at least 5 days before use. Columns (50 cm. × 2·5 cm. diam.; bed vol. ~ 240 ml.) with satisfactory flow rates (15–18 ml./hr.; 3·3–4·0 ml./cm.^{2}/hr.) were packed under light pressure in the cold (2–5°) with the swollen gel, previously deaerated under reduced pressure, by a modification of Flodin's (1961) method. The gel, admixed with saline or buffer and initially contained in a reservoir placed above a vertical glass chromatographic column, itself filled with saline or buffer, gradually accumulated in the column as liquid percolated through. To regulate the pressure, a second reservoir, containing only saline or buffer, was attached to the first one by a flexible tube and its height adjusted until the liquid level in it was only about 20 cm. above the column outlet. All experiments were carried out in the cold. Lowering the temperature of a column results in a decrease in flow-rate, and raising its temperature by more than a few degrees usually results in the appearance in it of large air-bubbles.

Columns were equilibrated either with 0·05 M-tris–HCl buffer, pH 7·5, containing KCl (0·1 M), or with 0·15 M-KCl. Samples were dissolved in the equilibration solution (2 ml.) and applied to the tops of the columns by layering under solution already present. Column effluents were collected in 3 ml. fractions with a collector (Aimer Products Ltd., London) fitted with a siphon. For further details of procedure, see Andrews (1964).

Estimation of proteins, blue dextran and sucrose in column effluents. Proteins were estimated either by light-extinction measurements at appropriate wavelengths or by suitable enzyme assays (Table 1). Urease was estimated by continuous titration with 0·01 N-HCl, with a Radiometer titrator type TTT1c coupled to a Radiometer titrigraph type SBR2c (Radiometer, Copenhagen, Denmark), of the ammonia it liberated from a solution (4 ml.) of urea (160 μ-moles), EDTA (5 μmoles) and KCl (160 μmoles) at pH 7·8 and 25°. A stream of CO_2-free N_2 (200 ml./min.) was maintained through the reaction mixture. The records of acid addition became linear 2–3 min. after the addition of enzyme solution (0·1–1 ml.) to the substrate solution, and rates (0·05–0·5 μequiv. of acid added/min.) measured in this way were proportional to the amounts of enzyme added. Glutamate dehydrogenase was assayed by measurement of the oxidation of NADH in the presence of excess of NH_4^+ and α-oxoglutarate (Olson & Anfinsen, 1952).

Blue dextran and sucrose in column effluents were estimated by extinction at 625 mμ and by a colorimetric method with anthrone (Trevelyan & Harrison, 1952) respectively.

The effluent volume corresponding to maximum concentration of a solute (elution volume, V_e) was estimated to the nearest 1 ml. from an elution diagram by extrapolating both sides of the solute peak to an apex.

RESULTS

Gel-filtration behaviour of proteins of known molecular weight. Determination of the gel-filtration behaviour of all the proteins listed in Table 1 on the same Sephadex G-200 column was impracticable. Instead, cytochrome *c*, serum albumin, lactate dehydrogenase, human γ-globulins and β-galactosidase were chosen as reference proteins and their elution volumes measured for each of the columns used. In addition, the void volume (V_0) of each column (elution volume of substances completely excluded from the gel pores) was measured in experiments with blue dextran plus reference proteins. Then the other proteins were run in admixture with reference proteins, and the observed separations in the molecular weight range covered by the reference proteins were converted by simple proportionation (where necessary) into separations attainable with one column. Results were cross-checked in experiments with combinations of proteins which did not include the reference ones. Admixture of small amounts of enzymes, having relatively negligible absorption at 230 and 280 mμ, with larger amounts of other proteins, and their estimation by enzymic assay and by extinction measurements at 230 or 280 mμ respectively, was frequently advantageous in correlating the gel-filtration behaviour of various proteins. The behaviour of most of the proteins listed in Table 1 was observed for at least two concentrations which in most cases differed by three-fold or more (Table 1), but except with chymotrypsinogen (cf. Andrews, 1964) the concentration changes had no significant effect on the elution volumes. Most of the experiments were carried out with columns equilibrated with tris–potassium chloride, but columns equilibrated only with 0·15 M-potassium chloride were used for experiments with caeruloplasmin, which undergoes changes in colour and sedimentation behaviour in the presence of tris (Kasper & Deutsch, 1963), and with urease, to avoid the presence of buffer in the enzyme assay. Proteins examined under both conditions showed no difference in behaviour as between one medium and the other.

Figs. 1 and 2 are plots of elution volumes (V_e) from Sephadex G-200 columns against log (mol. wt.) for all the proteins listed in Table 1. The elution volumes used in Fig. 1 for sucrose, blue dextran, the reference proteins, glucagon, ferritin, fibrinogen, α-crystallin and about half the other proteins were obtained in experiments with a column packed with gel which had been allowed to swell for 5 days before

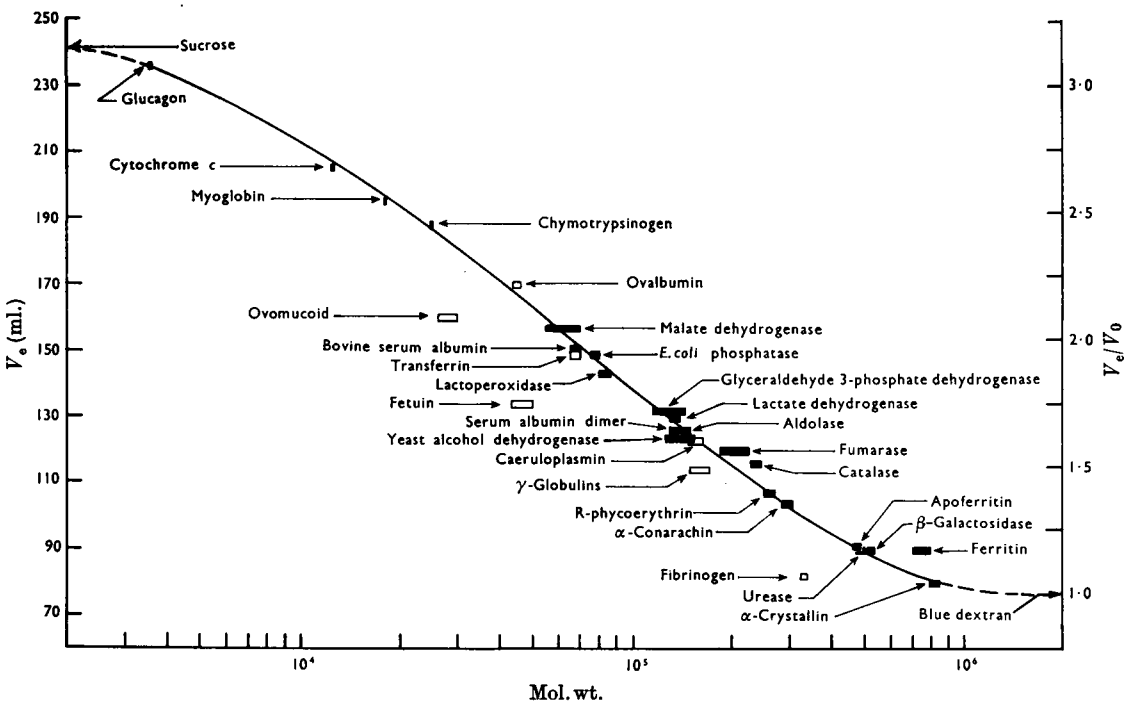

Fig. 1. Plot of elution volume, V_e, against log (mol. wt.) for proteins on a Sephadex G-200 column (2·5 cm. × 50 cm.) at pH 7·5. Experimental details are given in the text. Open bars represent glycoproteins. The lengths of bars indicate uncertainties in molecular weights (Table 1) and the widths indicate uncertainties of ±1 ml. in V_e.

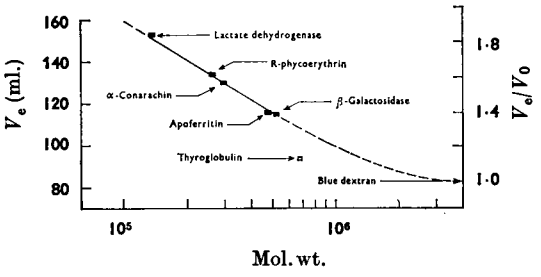

Fig. 2. Plot of elution volume, V_e, against log (mol. wt.) for proteins on a column (2·5 cm. × 50 cm.) of more expanded Sephadex G-200 than that used to obtain the results in Fig. 1. Experimental details are given in the text. The method of plotting the data is given in the legend to Fig. 1.

use, whereas values for the remaining proteins were calculated from the results of experiments with columns packed with gel which had been swelling for longer periods. Swelling of the gel packed in a column was not evident over periods of several weeks, for neither an expansion of the gel bed nor a change in the separations obtainable with the column was observed; nevertheless the gel from a column that had been kept for such a time was invariably more than sufficient to pack another column of the same size. Separations obtained with gels swollen to various extents by storage in dilute salt solution were very similar in the molecular-weight range 25 000–500 000, but the more swollen gels effected rather poorer separation below this range, and wider separations above it, than are indicated by Fig. 1. However, separation between β-galactosidase and blue dextran obtained with Sephadex G-200, lot no. To 46, after it had undergone swelling in buffer solution in the cold for about 3 months (Fig. 2), was nearly twice that obtained with lot no. To 3016 after similar treatment. Presumably such differences are due to differences in the degrees of cross-linking, and hence in water-regain values, of the two gels. The manufacturer's specification for water regain of Sephadex G-200 is 18–22 g./g. of dry gel. Both V_e and V_e/V_0 for individual proteins also varied from one column to another.

Considerable heterogeneity of the thyroglobulin, which was freeze-dried material, was demonstrated in experiments with the highly swollen gel of lot no. To 46. As shown in Fig. 3 (lower diagram), part of

Table 1. *Molecular weights, diffusion coefficients, quantities and methods of estimation of proteins used in gel-filtration experiments*

Protein	10^{-3} × Mol.wt.	References	10^7 × Diffusion coefficients ($D^0_{20,w}$, $D_{20,w}$ or D_{20})	References	Amounts used (mg.) in gel filtration	Method of estimation
Glucagon	3·5	Bromer *et al.* (1957)	—	—	0·5–1	*E* at 220 or 230 mμ
Cytochrome *c* (from horse heart)	12·4	Margoliash (1962)	13·0	Edsall (1953)	0·5–2·5	*E* at 412 mμ
Myoglobin (from sperm whale)	17·8	Edmunson & Hirs (1962)	11·3*	Edsall (1953)	1–1·5	*E* at 407 mμ
Chymotrypsinogen	25	Wilcox, Krant, Wade & Neurath (1957); Hartley (1964)	9·5	Wilcox *et al.* (1957)	0·5–2	*E* at 220 or 230 mμ
Ovomucoid	26–30	Lineweaver & Murray (1947); Fredericq & Deutsch (1949); Rhodes, Bennett & Feeney (1960); Deutsch & Morton (1961)	6·0, 7·8	Deutsch & Morton (1961); Rhodes *et al.* (1960); Fredericq & Deutsch (1949)	1–3	*E* at 220 or 230 mμ
Ovalbumin	44–46	Warner (1954)	7·8	Warner (1954)	1–3	*E* at 230 mμ
Fetuin (from foetal calf serum)	43–50	Spiro (1960); Green & Kay (1963)	5·7	Spiro (1960)	1–2	*E* at 220 mμ
Malate dehydrogenase (from pig heart)	55–70	Thorne & Kaplan (1963)	5·2, 8·1	Thorne & Kaplan (1963); Siegel & Englard (1961)	0·03–0·1	Oxidation of NADH in the presence of excess of oxaloacetate (Ochoa, 1955)
Serum albumin (bovine)	65–70	Phelps & Putnam (1960); Hughes & Dintzis (1964)	5·9	Phelps & Putnam (1960)	2–8	*E* at 230 or 280 mμ
Transferrin (bovine)	65–70	Charlwood (1963)	5·3, 5·8†	Thompson (1956); Laurell & Ingelman (1947)	2–4	*E* at 230 mμ
Phosphatase (from *E. coli*)	75–80	Garen & Levinthal (1960)	7·3	Calc. for mol.wt. 78,000, by using Svedberg's equation and data from Garen & Levinthal (1960)	0·05–0·15	Hydrolysis of *p*-nitrophenyl phosphate at pH 8·0 (Garen & Levinthal, 1960)
Lactoperoxidase (from cow's milk)	80–85‡	Theorell & Pedersen (1944); Polis & Shmukler (1953)	5·9	Theorell & Pedersen (1944)	~ 1	*E* at 230 mμ
D-Glyceraldehyde 3-phosphate dehydrogenase (from rabbit muscle)	115–145	Taylor & Lowry (1956); Fox & Dandliker (1956); Elias, Garbe & Lamprecht (1960); Harris, Meriwether & Park (1963); Deal & Holleman (1964)	5·0, 5·5	Fox & Dandliker (1956); Taylor & Lowry (1956)	0·5–1·5	*E* at 220 mμ
Alcohol dehydrogenase (from yeast)	125–155	Hersh (1962); Armstrong, Coates & Morton (1963); Iwatsubo & Curdel (1963)	4·7	Hayes & Velick (1954)	0·3–3	Reduction of NAD^+ (Racker, 1955) or *E* at 230 mμ
Serum albumin dimer (present in bovine serum albumin)	130–140	2 × mol.wt. of serum albumin	—	—	~ 0·5	*E* at 220 mμ
Lactate dehydrogenase (from rabbit muscle)	130–140	Pesce, Stolzenbach, Freedberg & Kaplan (1963); Fromm (1963)	5·2	Fromm (1963)	0·02–0·1	Oxidation of NADH in the presence of excess of pyruvate (Kornberg, 1955)
Aldolase (from rabbit muscle)	140–150	Taylor & Lowry (1956); Hass & Lewis (1963); Stellwagen & Schachman (1962)	4·6	Taylor & Lowry (1956)	0·5–1	Reduction of NAD^+ in the presence of arsenate and excess of D-glyceraldehyde 3-phosphate dehydrogenase (Taylor, 1955); *E* at 220 mμ
Caeruloplasmin (human)	150–165	Kasper & Deutsch (1963)	3·8, 4·5	Kasper & Deutsch (1963); Sanders, Miller & Richard (1959)	1–3	*E* at 230 mμ
γ-Globulins (bovine)	150–170	Phelps & Putnam (1960)	4·1	Phelps & Putnam (1960)	1–3	*E* at 230 mμ
γ-Globulins (human)	150–170	Phelps & Putnam (1960)	3·8	Phelps & Putnam (1960)	1–6	*E* at 230 or 280 mμ
Fumarase (from pig heart)	185–225	Johnson & Massey (1957); Kanarek, Marler, Bradshaw, Fellows & Hill (1964)	4·05	Cecil & Ogston (1952)	0·05–0·15	Disappearance of L-malate followed at 260 mμ (Massey, 1955)

Catalase (from ox liver)	230–250	Samejima & Yang (1963)	4·1	Sumner & Gralén (1938)	0·05–0·2	Disappearance of H_2O_2 followed at 240 mμ (Chance & Maehly, 1955)
R-Phycoerythrin	250–270‡	Eriksson-Quensel (1938)	4·0	Tiselius & Gross (1934)	0·8–1·5	*E* at 564 mμ
α-Conarachin	285–305	Dechary, Talluto, Evans, Carney & Altschul (1961); Evans, Carney & Neucere (1963)	—	—	0·6–2	*E* at 220 or 280 mμ
Fibrinogen (bovine)	330–340	Shulman (1953*a*); Scheraga & Laskowski (1957)	2·0	Shulman (1953*a*)	1–3	*E* at 280 mμ
Apoferritin	460–490	Harrison (1959, 1963)	3·6	Rothen (1944)	0·5–1·5	*E* at 280 mμ
Urease	470–510‡	Sumner, Gralén & Eriksson-Quensel (1938); Creeth & Nichol (1960); Gorin, Robbins & Reithel (1964)	3·3	Creeth & Nichol (1960)	2–5 (crude)	See the text
β-Galactosidase (from *E. coli*)	510–530	Sund & Weber (1963)	3·1	Sund & Weber (1963)	0·02–0·5	Hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (Wallenfels, 1962)
Thyroglobulin (bovine)	660–680	Edelhoch (1960)	2·5	Edelhoch (1960)	1–3	*E* at 280 mμ
Ferritin (from horse spleen)	700–800§	Harrison (1959)	—	—	0·5–2	*E* at 280 mμ
α-Crystallin (bovine)	790–840	Bloemendal, Bont, Jongkind & Wisse (1962); Björk (1963)	2·2	Björk (1963)	1–3	*E* at 230 or 280 mμ

* Determined on protein from horse heart.
† Values are for human and porcine transferrins respectively.
‡ Allowance is made for an error (about 10% too high) in values derived from sedimentation measurements made in early models of the analytical ultracentrifuge (cf. Miller & Golder, 1952; Taylor, 1952; Shulman, 1953*b*).
§ Heterogeneous as regards iron content.

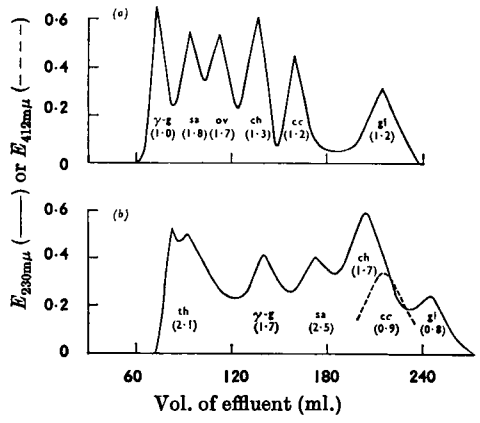

Fig. 3. Elution diagrams for separation of proteins on Sephadex columns [2·5 cm. × 50 cm.; equilibrated with 0·05 M-tris–HCl buffer, pH 7·5, containing KCl (0·1 M)]: (*a*) Sephadex G-100; (*b*) Sephadex G-200. Abbreviations: cc, cytochrome *c*; ch, chymotrypsinogen; γ-g, human γ-globulins; gl, glucagon; ov, ovalbumin; sa, bovine serum albumin; th, thyroglobulin. The values in parentheses refer to quantities (mg.) used.

it was eluted from the column at the void volume, and the remainder gave a peak 10 ml. later. In view of the tendency of thyroglobulin to aggregate on freeze-drying and storage (Shulman & Armenia, 1963), the second of these peaks is attributed to thyroglobulin monomer, and the one at the void volume to aggregated material.

Working range and exclusion limit of G-200. The relationship between V_e and log (mol. wt.) shown in Fig. 1 is linear only in the molecular-weight range 40 000–200 000, but with the more swollen gel (Fig. 2) linearity extends to about 500 000. The useful working range of Sephadex G-200 depends on the extent to which the gel has swollen, and evidently varies also from lot to lot. The lower molecular-weight limit for useful fractionation of polypeptides and proteins is probably ~5000, whereas the upper limit may be anywhere from 500 000 (Fig. 1) to 10^6 (Fig. 2), or possibly even higher, depending on the gel. The lower molecular-weight limit for complete exclusion from the pores of Sephadex G-200 will also depend on the gel. Fig. 1 indicates about 10^6 as the exclusion limit for globular proteins on one column of gel, whereas a reasonable extrapolation of the graph in Fig. 2 indicates about 3×10^6 as the limit for another column of more expanded gel. The narrow shape of the blue dextran peak eluted from the latter column strongly suggested that it was at the void volume, despite the expanded nature of the gel. An extrapolation of data given by Wieland *et al.* (1963) also indicates an exclusion limit of over 10^6 for

globular proteins on Sephadex G-200. As with other Sephadex gels, the exclusion limit for globular proteins is much greater in terms of molecular weight than that specified by the manufacturers for dextrans, not less, as indicated by Morris & Morris (1964).

Estimation of the sub-unit molecular weight of glutamate dehydrogenase. Various amounts of glutamate dehydrogenase (0·06–0·8mg.), admixed with human γ-globulins (3mg.) and bovine serum albumin (6mg.) to serve as standards, were subjected to gel filtration on a Sephadex G-200 column equilibrated with 0·05M-tris–hydrochloric acid, pH 7·0, containing potassium chloride (0·1M). The dehydrogenase in the column effluent fractions was estimated by its enzymic activity, and the other proteins by extinction at 235mμ. γ-Globulins and serum albumin were eluted in the same positions each time. Glutamate dehydrogenase was eluted ahead of γ-globulins, but its elution volume varied inversely with the amount used. The relationship between amount used and molecular weight (calculated from V_e, from Fig. 1) is shown in Fig. 4. Extrapolation of the graph to zero protein concentration gives a molecular weight of 252000 for the dissociated enzyme. Recovery of enzymic activity in the column effluents was 90–100% in experiments with 0·2mg. or more of enzyme, but fell to ~20% in the experiment with 0·06mg., and none was detected in an experiment with 0·02mg.

Molecular-weight estimation with Sephadex G-100. The finding that γ-globulins are eluted from Sephadex G-200 in the position expected for typical carbohydrate-free globular proteins of molecular weight about 205000 (see Fig. 1 and Discussion section) necessitates a revision of the higher molecular-weight end of the V_e–log(mol.wt.) graph for Sephadex G-100 given by Andrews (1964), since its shape was based in part on the assumption that γ-globulins behaved typically according to their molecular weight (~160000) during gel filtration. The revised curve is given in Fig. 5, with positions for lactate dehydrogenase and blue dextran included. The disposition of points relative to the curve is similar to that on the Sephadex G-200 graph (Fig. 1).

Molecular weights above 100000 previously estimated for several enzymes by Sephadex G-100 gel filtration (Andrews, 1964) now appear to be too low. Revised estimates, derived from the previously used elution volumes by use of Fig. 5, are given in Table 2. The values for yeast glucose 6-phosphate dehydrogenase and milk alkaline phosphatase were checked in experiments with Sephadex G-200. The molecular weight and gel-filtration behaviour of

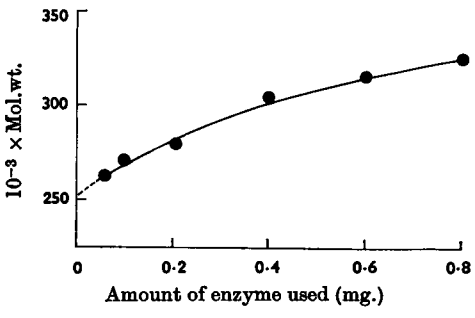

Fig. 4. Effect of concentration on the molecular weight of glutamate dehydrogenase, estimated by gel filtration on a Sephadex G-200 column equilibrated with 0·05M-tris–HCl, pH 7·5, containing KCl (0·1M). Samples were applied to the column in 2ml. of this solution.

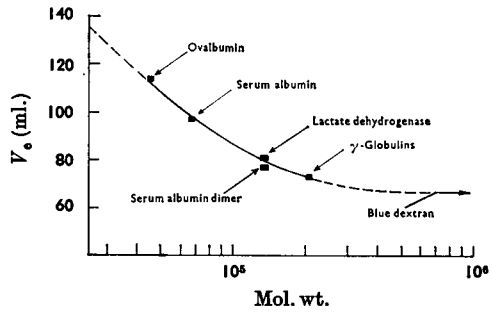

Fig. 5. Revised part of V_e–log(mol.wt.) graph for proteins on a Sephadex G-100 column (2·4cm. × 50cm.) at pH 7·5. Results are plotted as in Fig. 1 except that glycoproteins are not separately indicated. The point for γ-globulins corresponds to mol.wt. 200000–210000. Further details are given in the text.

Table 2. *Molecular weights estimated by Sephadex gel filtration*

Elution volumes were determined in experiments with a Sephadex G-100 column (Andrews, 1964) and converted into molecular weights with the revised G-100 calibration graph (Fig. 5). The results for glucose 6-phosphate dehydrogenase and milk alkaline phosphatase were checked in experiment with a Sephadex G-200 column. Further details are given in the text.

Enzyme	Mol.wt. (±10%)
Alkaline phosphatase (from bovine intestine)	130000
Alkaline phosphatase (from cow's milk)	180000
Glucose 6-phosphate dehydrogenase (from yeast)	128000
Glycerol dehydrogenase (from *Aerobacter aerogenes*)	140000

yeast alcohol dehydrogenase, which was also used in the G-100 experiments, are discussed below.

Comparison between Sephadex G-100 and G-200 for protein fractionation and molecular-weight estimation. The slope of the V_e–log(mol.wt.) graph for Sephadex G-200 (Fig. 1) is greater than that of the corresponding graph for G-100 (Fig. 5) for molecular weights above 60000, so in principle G-200 is the better gel for protein fractionation and molecular-weight estimation at molecular weights above this. However, a comparison of elution diagrams obtained under closely similar conditions with Sephadex G-100 and G-200 columns (Fig. 3) shows that in general the peaks obtained with G-100 are narrower than those obtained with G-200. Consequently, G-100 is probably the better gel for molecular weights up to $\sim$130000.

DISCUSSION

Carbohydrate-free proteins. Figs. 1 and 2 show graphically that, allowing for uncertainties in many of the molecular weights, good correlation exists between molecular weight and gel-filtration behaviour on Sephadex G-200 columns for the carbohydrate-free proteins listed in Table 1. The elution volumes of sucrose and blue dextrans are helpful in delineating the molecular-weight range over which a column will effect separation. As in earlier experiments (Andrews, 1964), glucagon is assumed to exist as monomer and to behave according to its molecular weight, but as a deviation from such behaviour might be undetected through the lack of other small proteins for comparison, the exact shape of the curve at the lower-molecular-weight end is uncertain. The concentration-dependent aggregation of chymotrypsinogen is allowed for in plotting its position (Andrews, 1964). Agreement between the results with different gels is demonstrated by Figs. 1 and 5, which show similar dispositions of points in the overlapping parts of Sephadex G-200 and G-100 curves.

Where molecular weights are uncertain, the most likely values indicated by the literature data often correspond to values suggested by the gel-filtration results. Thus pig-heart malate dehydrogenase fits the general pattern if its molecular weight proves to be about 60000, a value which is well within the published range and close to those (62000, 65000) for the ox-heart enzyme reported by Siegel & Englard (1961) and Grimm & Doherty (1961) respectively. The molecular weight for yeast alcohol dehydrogenase generally supported by the literature is about 150000, again in agreement with its gel-filtration behaviour on Sephadex G-200, and also in agreement with its behaviour on Sephadex G-100 when the revised graph (Fig. 5) for that gel is used for interpretation of results. The earlier conclusion (Andrews, 1964) that the gel-filtration behaviour of this enzyme on Sephadex G-100 corresponded to a lower molecular weight (125000–130000) was probably incorrect. A recent estimate (194000) of the molecular weight of fumarase (Kanarek *et al.* 1964) is much closer to the value (approx. 180000) indicated for it by gel filtration than is the earlier value of 220000. Even at the low concentrations used in gel filtration, rabbit-muscle lactate dehydrogenase behaved approximately according to its molecular weight, and showed no indication of dissociation into sub-units, such as reported by Millar (1962) for the ox-heart enzyme (however, cf. Pesce, McKay, Stolzenbach, Cahn & Kaplan, 1964). On the other hand, agreement between molecular weight and gel-filtration behaviour may prove to be less satisfactory for glyceraldehyde 3-phosphate dehydrogenase when its precise molecular weight is known (cf. Harris, 1964).

Of the carbohydrate-free proteins listed in Table 1, catalase and ferritin show the most significant deviations from the general V_e–log(mol.wt.) relationship. The behaviour of ferritin, however, is readily explained by reference to that of apoferritin, which does conform to the relationship. Ferritin and apoferritin were eluted from a Sephadex G-200 column with very nearly the same volume of eluent, and well away from the void volume of the column, despite the difference between their molecular weights. Apoferritin, which is the protein component of ferritin, has the form of a hollow, approximately spherical shell with an outer diameter of approx. 122 Å, and this dimension is unchanged when, in ferritin, the central cavity is occupied by a micelle of hydrated ferric oxide, the iron forming on average about 20% of the molecular weight (Harrison, 1959, 1963). The elution diagrams for ferritin indicated a homogeneous protein, although ferritin molecules generally show a wide range of molecular weights as a result of variations in their iron content. Catalase behaved on gel filtration, compared with the other proteins, as a globular protein of molecular weight 195000, although its currently accepted molecular weight is about 240000. As experiments with different amounts of enzyme gave no indication of a concentration-dependent dissociation of the molecule, the result may be due to its retardation during gel filtration by weak adsorption to the gel, or possibly in solution it has a more compact structure than that of most globular proteins. Samejima & Yang (1963) point out that its low intrinsic viscosity (3·9ml./g.) implies a compact conformation of the molecule, but as the structure so indicated is that of many globular proteins, which have about the same intrinsic viscosity (Tanford, 1958), the problem is unresolved.

Glycoproteins. The curves in Figs. 1 and 2 are

taken as a 'standard' V_e–log(mol. wt.) relationship, representing the gel-filtration behaviour of 'typical' carbohydrate-free globular proteins. Figs. 1 and 2 show that glycoproteins do not on the whole conform to this standard relationship. The presence of carbohydrate in glycoprotein molecules has already been suggested as the cause of the anomalous gel-filtration behaviour of ovomucoid by Whitaker (1963) and of horse-radish peroxidase by Andrews (1964). Gel filtration indicates that some glycoproteins have more expanded structures than those of typical globular proteins, which may well be due to a greater hydration in solution of carbohydrate chains as compared with polypeptide chains. For the purpose of molecular-weight estimation, a correlation between the carbohydrate content of glycoproteins and the extent to which they deviate from the standard behaviour of globular proteins would be useful. However, although the information in Table 3 indicates that the glycoproteins of higher carbohydrate content generally have the more expanded structures, a simple relationship is not apparent. The behaviour of ovalbumin and transferrin indicates that a carbohydrate content of only 3–4% may have little effect on molecular size, as compared with typical globular proteins, although the structure of transferrin may be influenced by its metal-binding properties. An expanded structure seems to be inconsistent with such properties, since caeruloplasmin, despite its carbohydrate content, behaves in gel filtration as a typical globular protein. Charlwood's (1963) revision of the molecular weight of transferrin is supported by its gel-filtration behaviour, which is much more consistent with a molecular weight of approx. 70000 than with one of approx. 90000. If small carbohydrate contents have negligible effects on the density of glycoprotein structures, the deviations of γ-globulins and fibrinogen from typical behaviour presumably are due to their shapes (see below). One consequence of the structural differences between caeruloplasmin and γ-globulins is that, although they have about the same molecular weight, they are separable by gel filtration (see Killander, 1964).

Effect of shape on gel-filtration behaviour. Current views do not suggest that the structures of proteins follow any simple rules; nevertheless Smith (1963) concludes from a consideration of the literature that some factor, probably shape, causes measured values of the frictional coefficients of globular proteins, and possibly other groups of proteins as well, to vary only within quite narrow limits. In addition, the partial specific volumes of globular proteins, and of fibrous proteins, fall into groups with approximately Gaussian distributions around mean values of about 0·73 ml./g. The implication that in solution the majority of globular proteins closely resemble one another in both shape and density accords well with their gel-filtration behaviour. Close conformity to the standard V_e–log(mol. wt.) relationship may well indicate for a protein an approximately spherical shape in solution, for two proteins which are probably spherical fit the relationship well. These are *E. coli* phosphatase, which has a frictional ratio of only 1·05 and in preparations for electron microscopy is seen as spheres of about 60 Å diameter (Garen & Levinthal, 1960), and apoferritin, which on X-ray-diffraction evidence consists of spherical molecules (see above). Minor differences in shape and density may then be the cause of the small deviations of globular proteins from the standard V_e–log(mol. wt.) relationship. Myoglobin, for example, has the form of a thick slab of dimensions 43 Å × 35 Å × 23 Å (Kendrew *et al.* 1958). Typical, perhaps, of such deviation, which limit the accuracy of molecular-weight estimation by the gel-filtration method, are those exhibited by proteins in the molecular-weight range 9000–25000 during gel filtration on Sephadex G-75 and G-100 (Andrews, 1964). The likelihood that bovine serum albumin has a molecular weight of 65000 (Hughes & Dintzis, 1964) whereas its gel-filtration behaviour indicates a value of 71000 (Fig. 1) suggests the molecule is somewhat elongated, as indeed may be the case (see Phelps & Putnam, 1960). However, bovine serum albumin monomer and dimer show proportionally similar deviations from standard behaviour, which suggests that their shapes are rather alike and that end-to-end linking of the monomers is unlikely to be the mode of dimerization. Spiro (1960) has calculated from hydrodynamic data that fetuin has a high axial ratio, and this also seems to be reflected in its gel-filtration behaviour, for although fetuin and ovomucoid both contain about the same proportion of carbohydrate, fetuin shows considerably the more deviation from standard behaviour (Table 3). As might be expected, the deviation of a protein molecule from standard behaviour seems to be greater the higher its axial ratio. Literature data place γ-globulins, fetuin and fibrinogen in that increasing order of axial ratio (Edelman & Gally, 1964; Spiro, 1960; Scheraga & Laskowski, 1957), and if the deviations from standard behaviour exhibited by γ-globulins and fibrinogen are not due appreciably to their carbohydrate content (see above), then gel-filtration results (Table 3) place the three proteins in the same increasing order of axial ratio.

Gel-filtration behaviour and diffusion coefficients. A relationship between the gel-filtration behaviour of proteins and their equivalent hydrodynamic radii (r) has been deduced by Laurent & Killander (1964) and Ackers (1964) in their theoretical treatments of the gel-filtration process. Since by the Stokes–Einstein law for the free diffusion of spherical particles, r and the diffusion coefficient D are related

Table 3. *Carbohydrate content and gel-filtration behaviour of glycoproteins*

The gel-filtration estimations of molecular weight are obtained from Figs. 1 and 2. Further details of literature values of the molecular weights are given in Table 1 and the Discussion section.

Glycoprotein	Total carbohydrate content (%)	Reference	Literature mol.wt. (A)	Apparent mol.wt. by gel filtration (B)	Deviation from 'standard' behaviour (%) $[(B-A/A)\times 100]$
γ-Globulins (human)	2·2	Rosevear & Smith (1961)	160000	205000	30
	3·1	Winzler (1960)			
Fibrinogen (bovine)	2·3	Winzler (1960)	330000	733000	120
Ovalbumin	3·5	Cunningham, Clouse & Ford (1963)	45000	41000	−10
Transferrin	3·9	Laurell (1960)	68000	74000	10
	5·5	Winzler (1960)			
Caeruloplasmin (human)	7·1	Winzler (1960)	160000	155000	0
	9·5	Laurell (1960)			
Thyroglobulin (bovine)	8·1	Spiro & Spiro (1963)	670000	1330000	100
Fetuin	19·2	Fisher, O'Brien & Puck (1962)	46000	115000	150
	22·5	Spiro (1960)			
Ovomucoid	21	Chatterjee & Montgomery (1962)	28000	55000	100

by $r=(1/D)\times$ constant when measurements are reduced to standard conditions, the first relationship is readily tested by plotting the elution volumes (Fig. 1) of the proteins listed in Table 1 against $1/D$, with $D^0_{20,w}$ or $D_{20,w}$ values in most cases (Fig. 6). Allowing for considerable uncertainty in many of the diffusion coefficients, and the possibility that free diffusion coefficients are not strictly applicable to restricted diffusion through porous gels, a useful correlation between V_e and D is indicated. The graph closely resembles ones obtained by Laurent & Killander (1964) and Ackers (1964) from their treatments of data from experiments with Sephadex gels. The glycoproteins which failed to fit the V_e–log(mol.wt.) relationship of typical globular proteins now conform moderately well, and catalase fits very well. The disparity for some proteins between measured diffusion coefficients and those indicated by the graph might be due to configurational or other small differences between the preparations used in measurements of D and in the gel-filtration experiments. The occurrence of configurational changes in protein structures could be at least a small source of error in the gel-filtration method of molecular-weight estimation, but in the likelihood that such changes cause inactivation of enzymes, molecular-weight estimations on enzymes which involve enzymic assay for their detection in column effluents seem least susceptible to these errors. Diffusion coefficients which lie well away from the curve in Fig. 6 are not necessarily erroneous. Although the value of D ($3\cdot8\times10^{-7}$ cm.^{2}/sec.) found for caeruloplasmin by Kasper & Deutsch (1963) is considerably lower than the value indicated by Fig. 6, when combined with a sedimentation coefficient

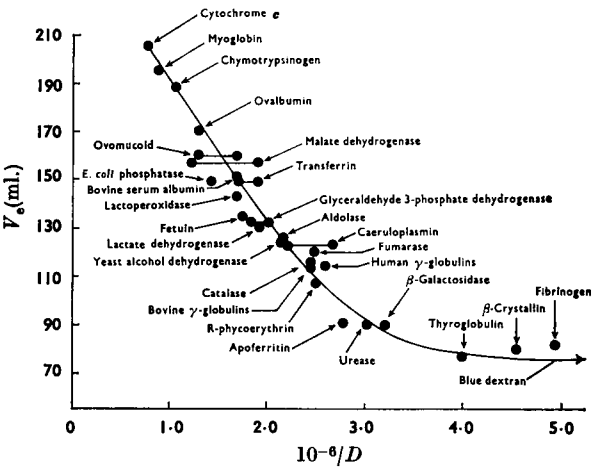

Fig. 6. Plot of elution volumes, V_e, against reciprocal of diffusion coefficient, D, for gel filtration of proteins on a Sephadex G-200 column (2·5 cm. × 50 cm.) at pH 7·5. Different values of $1/D$ for the same protein are joined by a horizontal line.

for the same preparation it gave a molecular weight (160000) which is confirmed by other determinations. On the other hand, none of the diffusion coefficients or molecular weights reported for pig-heart malate dehydrogenase accord with its gel-filtration behaviour, which indicates for it values of both properties similar to those recorded for the ox-heart enzyme (Siegel & Englard, 1961; Grimm & Doherty, 1961). According to Fig. 6, diffusion coefficients reported for transferrin are approximately

correct; so, as pointed out by Charlwood (1963), errors in determinations of its molecular weight seem to be mainly in the sedimentation measurements.

Gel filtration applied to molecular-weight estimation. Molecular-weight estimation by gel filtration involves in principle a comparison between the gel-filtration behaviour of the compounds under investigation with that of related compounds which conform to an appropriate relationship between molecular weight and gel-filtration behaviour. At present the method seems to be particularly useful for carbohydrate-free globular proteins, which probably include the majority of enzymes, although further possibilities are indicated above. As few globular proteins conform exactly to one relationship between molecular weight and gel-filtration behaviour, the choice of proteins as molecular-weight standards can considerably influence the results of molecular-weight estimations, or other conclusions about gel-filtration behaviour. Some uniformity in the basic data for such experiments is clearly desirable. Hence it is suggested that the V_e–log (mol. wt.) curves given in Figs. 1, 2 and 5 and elsewhere (Andrews, 1964) are taken as the standard relationship of this sort for globular proteins in the molecular-weight range 10000–800000. The full V_e–log (mol. wt.) curves for Sephadex gels are of the general shape expected if, as is probable, the pore sizes in each gel follow a normal distribution. Although the middle part of each curve is linear, these parts seem to be particularly short in some curves for Sephadex G-200 gels, and vary in length according to previous treatment of the gel. The useful parts of the curves extend well beyond the linear stretches.

Equivalent curves, or parts of such curves, should be obtainable for any gel-filtration column which works in an appropriate molecular-weight range by determining the elution volume from it of proteins selected from those already studied and taking into account, in plotting the results, any deviations they show from the standard relationship. Thus the choice of reference proteins is not necessarily limited to those which conform to the standard V_e–log (mol. wt.) relationship, for γ-globulins and ferritin are useful for the purpose provided that their atypical behaviour is recognized. On the other hand proteins such as haemoglobin and β-lactoglobulin which show marked dissociation on gel filtration (Andrews, 1964) are unsuitable, as also are those such as lysozyme which appear to interact with gel-filtration media (Whitaker, 1963), since their behaviour may well depend very much on the experimental conditions. However, both haemoglobin and lysozyme have been used as reference substances in gel filtration (Morris, 1964; Sluyser & Li, 1963). In molecular-weight estimations, the inclusion whenever possible of several standard proteins in column runs with unknowns is recommended, as this eliminates uncertainties about variations in elution volumes from run to run. Purified enzymes are particularly useful for this purpose, since they can be located by specific assay methods and generally they need only to be used in microgram amounts, thus making negligible contributions to the protein content of samples. Some guidance in the choice of standard proteins is given by Andrews (1964) and Downey & Andrews (1965). A value of 10% for the uncertainty in molecular weights of carbohydrate-free globular proteins estimated by the gel-filtration method (Andrews, 1964) still seems appropriate, as practically all the proteins of this type listed in Table 1 appear to conform within this limit to the standard V_e-log (mol. wt.) relationship.

Winzor & Scheraga (1963, 1964) showed that the negative concentration-dependence of the migration rate of proteins which is frequently observed in the ultracentrifuge can be detected in gel-filtration experiments when large sample volumes are applied to a column to maintain a constant protein concentration in the protein zone, and frontal analysis is applied to the migration of the zone. Calculations based on these authors' results with ovalbumin indicate that on columns of Sephadex G-200 or G-100 of the size used in the present and earlier experiments (Andrews, 1964) the advancing edge of a zone containing 5mg. of ovalbumin would be eluted 3–4ml. later than that of a zone containing 1mg. of ovalbumin. The corresponding difference in apparent molecular weight is $\sim$4000. Clearly such an effect would decrease the value of the gel-filtration method of molecular-weight estimation. However, although it could easily be detected, the effect is not observed with ovalbumin or many other proteins, even over wide concentration ranges (Andrews, 1964 and above; Whitaker, 1963; Ackers, 1964), when small sample volumes are used, and protein migration through the columns is measured by the elution volumes at which solutes are at maximum concentration.

Concentration-dependent dissociation of glutamate dehydrogenase. The possibilities both of investigating association–dissociation behaviour (Andrews, 1964; Winzor & Scheraga, 1963, 1964) and of estimating the molecular weights of enzymes at extremely low concentrations by gel filtration suggests a means of studying the concentration-dependent dissociation of glutamate dehydrogenase. At a concentration of several milligrams/ml. this enzyme has molecular weight $\sim 10^6$, but dilution of the solution induces dissociation into sub-units which form a system in rapid association–dissociation equilibrium. Dissociation is apparently incomplete until solutions are too dilute for molecular-

weight estimations by conventional methods. According to Frieden (1963) the question of sub-unit molecular weight is still unresolved, although the best estimates put it in the range 250000–350000. On the basis of gel-filtration experiments with Sephadex G-200, Rogers & Thompson (1963) set an upper limit of 340000 to the molecular-weight range of the enzyme in the concentration range 0·4–200 μg./ml. More recently Bitensky, Yielding & Tomkins (1964) conclude from thiol titrations that the dilution dissociation of the enzyme is complete at a concentration of 30 μg./ml. Fig. 4 shows that enzyme applied to the column at this concentration, and diluted approximately tenfold during passage through the column, was still not completely dissociated. Although complete dissociation was not observed, a sub-unit molecular weight $\sim$250000 is clearly indicated. The loss of enzymic activity at the highest dilutions, noted also by Frieden (1963), does not necessarily imply that the sub-units are inactive, but only that they are more unstable when separated from one another than when associated.

REFERENCES

Ackers, G. K. (1964). *Biochemistry*, **3**, 723.

Andrews, P. (1962). *Nature, Lond.*, **196**, 36.

Andrews, P. (1964). *Biochem. J.* **91**, 222.

Andrews, P., Bray, R. C., Edwards, P. & Shooter, K. V. (1964). *Biochem. J.* **93**, 627.

Armstrong, J. McD., Coates, J. H. & Morton, R. K. (1963). *Biochem. J.* **86**, 136.

Bitensky, M. W., Yielding, K. L. & Tomkins, G. M. (1964). *Fed. Proc.* **23**, 263.

Björk, I. (1963). *Exp. Eye Res.* **2**, 339.

Bloemendal, H., Bont, W. S., Jongkind, J. F. & Wisse, J. H. (1962). *Exp. Eye Res.* **1**, 300.

Bromer, W. W., Staub, A., Diller, E. R., Bird, W. L., Sinn, L. G. & Behrens, O. K. (1957). *J. Amer. chem. Soc.* **79**, 2794.

Cecil, R. & Ogston, A. G. (1952). *Biochem. J.* **51**, 494.

Chance, B. & Maehly, A. C. (1955). In *Methods in Enzymology*, vol. 2, p. 764. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Charlwood, P. A. (1963). *Biochem. J.* **88**, 394.

Chatterjee, A. K. & Montgomery, R. (1962). *Arch. Biochem. Biophys.* **99**, 426.

Creeth, J. M. & Nichol, L. W. (1960). *Biochem. J.* **77**, 230.

Cunningham, L. W., Clouse, R. W. & Ford, J. D. (1963). *Biochim. biophys. Acta*, **78**, 379.

Deal, W. C. & Holleman, W. H. (1964). *Fed. Proc.* **23**, 264.

Dechary, J. M., Talluto, K. F., Evans, W. J., Carney, W. B. & Altschul, A. M. (1961). *Nature, Lond.*, **190**, 1125.

Deutsch, H. F. & Morton, J. I. (1961). *Arch. Biochem. Biophys.* **93**, 654.

Downey, W. K. & Andrews, P. (1965). *Biochem. J.* **94**, 642.

Edelhoch, H. (1960). *J. biol. Chem.* **235**, 1326.

Edelman, G. M. & Gally, J. A. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 846.

Edmunson, A. B. & Hirs, C. H. W. (1962). *J. molec. Biol.* **5**, 663.

Edsall, J. T. (1953). In *The Proteins*, vol. 1, part B, p. 634. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Elias, H.-G., Garbe, A. & Lamprecht, W. (1960). *Hoppe-Seyl. Z.* **319**, 22.

Eriksson-Quensel, I.-B. (1938). *Biochem. J.* **32**, 585.

Evans, W. J., Carney, W. B. & Neucere, H. J. (1963). *Nature, Lond.*, **198**, 1303.

Fisher, H. W., O'Brien, D. & Puck, T. D. (1962). *Arch. Biochem. Biophys.* **99**, 241.

Flodin, P. (1961). *J. Chromat.* **5**, 103.

Fox, J. B. & Dandliker, W. B. (1956). *J. biol. Chem.* **218**, 53.

Fredericq, E. & Deutsch, H. F. (1949). *J. biol. Chem.* **181**, 499.

Frieden, C. (1963). *J. biol. Chem.* **238**, 3286.

Fromm, H. J. (1963). *J. biol. Chem.* **238**, 2938.

Garen, A. & Levinthal, C. (1960). *Biochim. biophys. Acta*, **38**, 470.

Gorin, G., Robbins, J. E. & Reithel, F. J. (1964). *Fed. Proc.* **23**, 264.

Green, W. A. & Kay, C. M. (1963). *Arch. Biochem. Biophys.* **102**, 359.

Grimm, F. C. & Doherty, D. G. (1961). *J. biol. Chem.* **236**, 1980.

Harris, I. (1964). *Nature, Lond.*, **203**, 30.

Harris, I., Meriwether, B. P. & Park, J. H. (1963). *Nature, Lond.*, **198**, 154.

Harrison, P. M. (1959). *J. molec. Biol.* **1**, 69.

Harrison, P. M. (1963). *J. molec. Biol.* **6**, 404.

Hartley, B. S. (1964). *Nature, Lond.*, **201**, 1284.

Hass, L. F. & Lewis, M. S. (1963). *Biochemistry*, **2**, 1368.

Hayes, J. E. & Velick, S. F. (1954). *J. biol. Chem.* **207**, 225.

Hersh, R. T. (1962). *Biochim. biophys. Acta*, **58**, 353.

Hughes, W. L. & Dintzis, H. M. (1964). *J. biol. Chem.* **239**, 845.

Iwatsubo, M. & Curdel, A. (1963). *C.R. Acad. Sci., Paris*, **256**, 5224.

Johnson, P. & Massey, V. (1957). *Biochim. biophys. Acta*, **23**, 544.

Kanarek, L., Marler, E., Bradshaw, R. A., Fellows, R. E. & Hill, R. L. (1964). *J. biol. Chem.* **239**, 4207.

Kasper, C. B. & Deutsch, H. F. (1963). *J. biol. Chem.* **238**, 2325.

Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G., Wyckoff, H. & Phillips, D. C. (1958). *Nature, Lond.*, **181**, 662.

Killander, J. (1964). *Biochim. biophys. Acta*, **93**, 1.

Kornberg, A. (1955). In *Methods in Enzymology*, vol. 1, p. 441. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Laki, K. (1951). *Arch. Biochem.* **32**, 317.

Largier, J. F. & Polson, A. (1964). *Biochim. biophys. Acta*, **79**, 626.

Laurell, C. B. (1960). In *The Plasma Proteins*, vol. 1, p. 349. Ed. by Putnam, F. W. New York: Academic Press Inc.

Laurell, C. B. & Ingelman, B. (1947). *Acta chem. scand.* **1**, 770.

Laurent, T. C. & Killander, J. (1964). *J. Chromat.* **14**, 317.

Lineweaver, H. & Murray, C. W. (1947). *J. biol. Chem.* **171**, 565.

Margoliash, E. (1962). *J. biol. Chem.* **237**, 2161.

Massey, V. (1955). In *Methods in Enzymology*, vol. 1, p. 729. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Millar, D. B. S. (1962). *J. biol. Chem.* **237**, 2135.
Miller, G. L. & Golder, R. H. (1952). *Arch. Biochem. Biophys.* **36**, 249.
Morris, C. J. O. R. (1964). *J. Chromat.* **16**, 167.
Morris, C. J. O. R. & Morris, P. (1964). *Separation Methods in Biochemistry*, p. 387. London: Pitman and Sons Ltd.
Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Olson, J. A. & Anfinsen, C. B. (1952). *J. biol. Chem.* **197**, 67.
Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D. & Kaplan, N. O. (1964). *J. biol. Chem.* **239**, 1753.
Pesce, A., Stolzenbach, F., Freedberg, I. & Kaplan, N. O. (1963). *Fed. Proc.* **22**, 241.
Phelps, R. A. & Putnam, F. W. (1960). In *The Plasma Proteins*, vol. 1, p. 143. Ed. by Putnam, F. W. New York: Academic Press Inc.
Polis, B. D. & Shmukler, H. W. (1953). *J. biol. Chem.* **201**, 475.
Racker, E. (1955). In *Methods in Enzymology*, vol. 1, p. 500. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Rhodes, M. B., Bennett, N. & Feeney, R. E. (1960). *J. biol. Chem.* **235**, 1686.
Rogers, K. S. & Thompson, T. E. (1963). *Fed. Proc.* **22**, 290.
Rosevear, J. W. & Smith, E. L. (1961). *J. biol. Chem.* **236**, 425.
Rothen, A. (1944). *J. biol. Chem.* **152**, 679.
Samejima, T. & Yang, J. T. (1963). *J. biol. Chem.* **238**, 3256.
Sanders, B. E., Miller, O. P. & Richard, M. N. (1959). *Arch. Biochem. Biophys.* **84**, 60.
Scheraga, H. A. & Laskowski, M. (1957). *Advanc. Protein Chem.* **12**, 1.
Shulman, S. (1953*a*). *J. Amer. chem. Soc.* **75**, 5846.
Shulman, S. (1953*b*). *Arch. Biochem. Biophys.* **44**, 230.
Shulman, S. & Armenia, J. P. (1963). *J. biol. Chem.* **238**, 2723.
Siegel, L. & Englard, S. (1961). *Biochim. biophys. Acta*, **54**, 67.
Sluyser, L. & Li, C. H. (1963). *Nature, Lond.*, **200**, 1007.
Smith, M. H. (1963). *Biochem. J.* **89**, 45P.
Spiro, R. G. (1960). *J. biol. Chem.* **235**, 2860.
Spiro, R. G. & Spiro, M. J. (1963). *Fed. Proc.* **22**, 538.
Stellwagen, E. & Schachman, H. K. (1962). *Biochemistry*, **1**, 1056.
Sumner, J. B. & Gralén, N. (1938). *J. biol. Chem.* **125**, 33.
Sumner, J. B., Gralén, N. & Eriksson-Quensel, I-B. (1938). *J. biol. Chem.* **125**, 37.
Sund, H. & Weber, K. (1963). *Biochem. Z.* **337**, 24.
Tanford, C. (1958). In *Symposium on Protein Structure*, p. 35. Ed. by Neuberger, A. London: Methuen and Co. Ltd.
Taylor, J. F. (1952). *Arch. Biochem. Biophys.* **36**, 357.
Taylor, J. F. (1955). In *Methods in Enzymology*, vol. 1, p. 311. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Taylor, J. F. & Lowry, C. (1956). *Biochim. biophys. Acta*, **20**, 109.
Theorell, H. & Pedersen, K. O. (1944). In *The Svedberg*, p. 523. Ed. by Tiselius, A. & Pedersen, K. O. Uppsala, Sweden: Almqvist and Wiksells Boktryckeri A.B.
Thompson, T. E. (1956). Cited by Gostling, L. J., in *Advanc. Protein Chem.* **11**, 429.
Thorne, C. J. R. & Kaplan, N. O. (1963). *J. biol. Chem.* **238**, 1861.
Tiselius, A. & Gross, D. (1934). *Kolloid Z.* **66**, 11.
Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
Wallenfels, K. (1962). In *Methods in Enzymology*, vol. 5, p. 212. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Warner, R. C. (1954). In *The Proteins*, vol. 2, part A, p. 435. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Whitaker, J. R. (1963). *Analyt. Chem.* **35**, 1950.
Wieland, T., Duesberg, P. & Determann, H. (1963). *Biochem. Z.* **337**, 303.
Wilcox, P. E., Krant, J., Wade, R. D. & Neurath, H. (1957). *Biochim. biophys. Acta*, **24**, 72.
Winzler, R. J. (1960). In *The Plasma Proteins*, vol. 1, p. 309. Ed. by Putnam, F. W. New York: Academic Press Inc.
Winzor, D. J. & Scheraga, H. A. (1963). *Biochemistry*, **2**, 1263.
Winzor, D. J. & Scheraga, H. A. (1964). *J. phys. Chem.* **68**, 338.