4. These findings have been discussed in relation to the intermediary metabolism of lambs, sheep and rats.

The authors gratefully acknowledge the assistance of Mr N. S. Stenhouse of the C.S.I.R.O. Division of Mathematical Statistics, University of Adelaide, who carried out the statistical treatment of the data.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Bartlett, P. D., Grimmett, P., Beers, L. & Shelata, S. (1956). *J. biol. Chem.* **218**, 419.
 Burton, K. (1956). *Biochem. J.* **62**, 315.
 Caiger, P., Morton, R. K., Jarrett, I. G. & Filsell, O. H. (1962). *Biochem. J.* **85**, 351.
 Filsell, O. H., Jarrett, I. G., Atkinson, M. R., Caiger, P. & Morton, R. K. (1963). *Biochem. J.* **89**, 92.
 Fishkin, A. F. & Lata, G. F. (1958). *Endocrinology*, **63**, 162.
 Franz, J. M. & Lata, G. F. (1957). *Endocrinology*, **60**, 602.
 Gallagher, C. H. (1959). *Aust. J. agric. Res.* **10**, 854.
 Gallagher, C. H. & Buttery, S. H. (1959). *Biochem. J.* **72**, 575.
 Handschumacher, R. E., Mueller, G. C. & Strong, F. M. (1951). *J. biol. Chem.* **189**, 335.
 Holdsworth, E. S., Neville, E., Nader, C., Jarrett, I. G. & Filsell, O. H. (1963). *Biochim. biophys. Acta* (in the Press).
 Jarrett, I. G. & Filsell, O. H. (1958). *Aust. J. exp. Biol. med. Sci.* **36**, 433.
 Jarrett, I. G. & Filsell, O. H. (1960). *Aust. J. exp. Biol. med. Sci.* **38**, 347.
 Jarrett, I. G., Jones, G. B. & Potter, B. J. (1964). *Biochem. J.* **90**, 189.
 Jarrett, I. G. & Potter, B. J. (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 207.
 Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
 Klein, H. & Lipmann, F. (1953). *J. biol. Chem.* **203**, 101.
 Krebs, H. A. & Kornberg, H. L. (1957). *Ergebn. Physiol.* **49**, 212.
 McCandless, E. L. & Dye, J. A. (1950). *Amer. J. Physiol.* **162**, 434.
 McCarthy, R. D., Shaw, J. C. & Lakshmanan, S. (1958). *Proc. Soc. exp. Biol., N.Y.*, **99**, 560.
 McLean, P. (1958). *Biochim. biophys. Acta*, **30**, 303.
 Novelli, G. D. (1953). *Physiol. Rev.* **33**, 525.
 Olson, R. E. & Kaplan, N. O. (1948). *J. biol. Chem.* **175**, 515.
 Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
 Thompson, M. & Mayer, J. (1962). *Amer. J. Physiol.* **202**, 1005.

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

Estimation of the Molecular Weights of Proteins by Sephadex Gel-Filtration

By P. ANDREWS

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

(Received 30 August 1963)

When a mixture of molecules is passed through a column of porous gel granules, the molecules may appear in the effluent in order of decreasing size. Fractionation is believed to occur when diffusion of the molecules into the gel pores is restricted but not prevented because of their size, and because they pass through the column at rates that are related inversely to the fluid volume accessible to them within the column. Lathe & Ruthven (1956) suggested that the dimensions of protein and polysaccharide molecules might be estimated by this technique, now widely known as gel-filtration. However, evaluation of their suggestion is difficult because of uncertainty about the size and shape of macromolecules in solution (Schachman, 1960). Although molecular weight is unlikely to be a good approximation for size in comparing such dissimilar molecules as proteins and polysaccharides, the correlation between molecular weights and gel-filtration behaviour of dextrans (Granath & Flodin,

1961) indicates that, for a homogeneous series of macromolecules, size and molecular weight are closely related. Andrews (1962) obtained evidence from experiments with agar-gel columns that this was also true for a number of proteins, and showed that gel-filtration can be used as a comparative method to give useful estimates of the molecular weights of proteins.

Agar gels can be used to separate proteins over very wide molecular-weight ranges (Polson, 1961; Andrews, 1962; Steere & Ackers, 1962), whereas over smaller ranges of molecular weight much better separations can be obtained with gel-filtration media composed of cross-linked dextrans (Sephadex, manufactured by Pharmacia, Uppsala, Sweden) (Flodin, 1962) or polyacrylamide (Hjertén & Mosbach, 1962). Therefore a more critical appraisal of the relationship between the molecular weights of proteins and their gel-filtration behaviour is possible with these media than with agar gels.

The present paper describes experiments in this connexion with two of the Sephadex-gel-filtration media (G-75 and G-100). A preliminary report has already been published (Andrews & Folley, 1963).

MATERIALS AND METHODS

Proteins. Information on the source and purity of the non-enzymic proteins and ribonuclease used in this work is included in Table 1. Of the other enzymes, crystalline yeast alcohol dehydrogenase, crystalline yeast hexokinase and analytical-reagent-grade horseradish peroxidase were obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany, purified *Escherichia coli* phosphatase from Worthington Biochemical Corp., Freehold, N.J., U.S.A., partially purified glycerol dehydrogenase from L. Light and Co. Ltd., Colnbrook, Bucks., crude glucose 6-phosphate dehydrogenase from British Drug Houses Ltd., Poole, Dorset, and crude intestinal alkaline phosphatase from Armour Laboratories, Eastbourne, Sussex. The rennin was crystalline, adenosine deaminase from calf intestine was highly purified (Brady & O'Connell, 1962) and alkaline phosphatase from cow's milk was partially purified (R. L. J. Lyster, unpublished work).

Samples were very kindly given by Professor T. G. Brady, Professor A. Neuberger, Dr R. Aschaffenburg, Dr O. K. Behrens, Dr G. C. Cheeseman, Dr W. A. Jones, Dr J. C. Kendrew, Dr R. L. J. Lyster, Dr E. Margoliash and Dr F. Sanger.

Preparation of gel columns. Each column was packed in a vertical glass tube (2.4 cm. internal diam.) across which was fused a sintered-glass disk (porosity 1) to support the gel. A layer of sand (0.5 cm. depth) prevented blockage of the disk by gel particles.

Sephadex-gel-filtration media G-75 [lot no. To 6360; particle size (U.S. standard) 100–250 mesh] and G-100 (lot no. To 1291; particle size 140–400 mesh) (Pharmacia, Uppsala, Sweden) were suspended in water and allowed to swell for 2–3 days, and then the smallest particles were removed by decantation. Suspensions of the gels were de-aerated under reduced pressure before use.

Each column was prepared by pouring a thin slurry of gel particles in buffer solution into a vertical tube, already partly filled with buffer, and at the same time allowing excess of liquid to percolate through the growing gel bed. The addition of gel was continued until a bed height of 51–52 cm. was obtained, and then a solvent reservoir was connected to the top of the column and the flow of buffer maintained at a rate of approx. 30 ml./hr. for 2 days. By this time the bed had settled to a constant height, which was then adjusted to 50 cm. (bed volume, 226 ml.) by the addition or removal of gel, already equilibrated with buffer, as necessary. Some columns gave variable results when newly packed, but showed consistent behaviour after 2–4 days' washing with buffer.

Even packing of each column was checked by watching the passage through it of a band of coloured protein (e.g. cytochrome c). Irregularities developed at the top of most columns after repeated use. These were readily detected by skewness of coloured bands, or poor definition of peak maxima in elution diagrams. Faults were corrected by replacing the top 0.5–1 cm. of such columns with fresh gel, then stirring the top 2–3 cm. and allowing the gel

particles to settle again with buffer solution flowing through the column. Columns were eluted continuously with buffer solution when not in use.

Procedure for column runs. Except where otherwise stated, experiments were performed with columns equilibrated with 0.05 M-tris hydrochloride buffer, pH 7.5, containing KCl (0.1 M), and a flow rate through the columns of approx. 30 ml./hr. (6.6 ml./cm.²/hr.) was maintained by the application of positive or negative hydrostatic pressure to the tops of the columns by adjustment of the position of the solvent reservoir. All the experiments were done at room temperature (20 ± 3°).

Proteins were dissolved in the equilibration buffer (2 ml.) and the solution was applied to the top of a column by layering under the buffer already present. The density of the protein solution was increased by the addition of sucrose (approx. 5 mg.) when the weight of protein was less than 5 mg. Collection of the column effluent in fractions, with a fraction collector (Aimer Products Ltd., London) fitted with a 3 ml. siphon, was begun when half the protein solution was judged to have entered the column. The variation in fraction volume was ± 2% (determined by weight).

Examination of column effluents. Proteins were estimated spectrophotometrically by using the whole of each effluent fraction in a cuvette with a 1 cm. light-path. The wavelength was selected according to the quantity of protein used (Table 2); cytochrome c-551 and haemoglobin were estimated at 408 m μ and 410 m μ respectively. The reference cuvette contained a sample of column effluent collected before the emergence of protein. In experiments involving extinction measurements at 215 m μ , the maintenance of a steady flow of buffer through a column during application of the sample, and at other times, was necessary to avoid irregularities in background absorption. Indian ink was estimated at 900 m μ , and sucrose determined by the anthrone method (Trevelyan & Harrison, 1952).

Enzymic activities were estimated by spectrophotometric methods in 1 cm. cuvettes by using samples (0.05–1.0 ml.) from appropriate effluent fractions, in total volumes of 3 ml., at 25°. Assay mixtures for the alkaline phosphatases from milk and intestine contained 1.0 M-mole of tris hydrochloride buffer, pH 9.5, 4 μ moles of *p*-nitrophenyl phosphate and 10 μ moles of MgCl₂; the linear change in extinction at 410 m μ with time was measured. For hexokinase, assay mixtures contained 250 μ moles of tris hydrochloride buffer, pH 7.5, 10 μ moles of ATP, 10 μ moles of MgCl₂, 50 μ moles of D-glucose, 0.7 μ mole of NADP and sufficient glucose 6-phosphate dehydrogenase to effect an extinction change at 340 m μ of approx. 1.0/min. when, in a separate experiment, 10 μ moles of glucose 6-phosphate (Boehringer und Soehne) were added and hexokinase was omitted. In the hexokinase assays the measured extinction changes at 340 m μ were linear with time for at least 4 min. and did not exceed 0.15/min. Other enzymes were assayed as indicated in Table 4.

The effluent volume corresponding to maximum concentration of a solute (elution volume, V_e) (Gelotte, 1960; Flodin, 1962) was estimated to the nearest 1 ml. from an elution diagram, by extrapolating both sides of the solute peak to an apex.

Thin-layer gel-filtration with Sephadex G-100. Thin gel layers were prepared on glass plates (20 cm. × 10 cm.) with

apparatus manufactured by C. Desaga G.m.b.H., Heidelberg, Germany, and used as described by Determann (1962) and Johansson & Rymo (1962). Because of the swelling of Sephadex G-100 particles that occurs on the addition of buffer solution, dry material of very fine particle size (400 mesh; generously supplied by Pharmacia, Uppsala, Sweden) proved more satisfactory for the preparation of uniform layers than did coarser grades. The gel particles were swollen in 0.05 M-tris hydrochloride buffer, pH 7.5, containing KCl (0.1 M) for 3 days before use. A slurry of suitable consistency for making thin layers was obtained by allowing a suspension of the particles in buffer to settle for half an hour, then removing as much excess of buffer as possible through a narrow tube with gentle suction. Layers 0.5 mm. thick were generally more uniform than thinner layers. The flow of buffer through the layers was maintained for several hours before the application of samples, and the flow rate was regulated by altering the angle of tilt of the glass plates (usually 10–20° from horizontal). Good separations were obtained when the flow rate was sufficient to move cytochrome *c* (regularly used as reference substance) through 6–8 cm. during either 8 hr. or 16 hr. Proteins (30–50 µg. of each) were applied to the plates in tris hydrochloride buffer (1–3 µl.), and subsequently detected as brown spots by placing the wet plates in a closed vessel containing iodine vapour. The distances between the point of application and centre of each spot were measured.

RESULTS

Elution volumes of proteins of known molecular weight. The proteins listed in Table 1 were used in various combinations (three, four or five proteins at a time), together with sucrose and indian ink in some experiments, to determine their elution volumes from Sephadex G-75 and G-100 columns. Indian ink often left dark stains on the columns, so its routine use in Sephadex gel-filtration is not recommended. The elution volumes of indian ink and thyroglobulin were identical on both gels. The admixture of colourless and coloured proteins in the weight ratio of about 3:1 facilitated accurate estimation of the separation between some proteins differing only a little in molecular weight, e.g. α -lactalbumin and myoglobin, for they could then be estimated independently in the same column effluent by measuring the extinction at appropriate wavelengths.

The effect of protein concentration on elution volume of all the proteins listed in Table 1 was investigated, in experiments with either Sephadex G-75 or G-100 columns. None of the following proteins showed any variation over the concen-

Table 1. *Proteins used in gel-filtration experiments*

The mol.wt. values were obtained by the following methods: AAS, calculation (to the nearest 100) from the amino acid sequence; AAA, calculation (to the nearest 100) from amino acid analyses; U, ultracentrifugal methods.

Protein	Description and supplier	Mol.wt.	Method	Reference
Glucagon	Purified; Eli Lilly and Co.	3500	AAS	Bromer <i>et al.</i> (1957)
Cytochrome <i>c</i> -551 (from <i>Pseudomonas fluorescens</i>)	Purified (Ambler, 1963 <i>a</i>)	9000*	AAS	Ambler (1963 <i>b</i>)
Cytochrome <i>c</i> (from horse heart)	Type II; Sigma Chemical Co.	12400*	AAS	Margoliash (1962)
Ribonuclease (from ox pancreas)	4 × crystallized; Worthington Biochemical Corp.	13700	AAS	Hirs, Moore & Stein (1956)
α -Lactalbumin	Crystalline	15500	AAA	Gordon & Ziegler (1955)
Myoglobin (from sperm whale)	Crystalline	17800*	AAS	Edmundson & Hirs (1962)
Trypsin inhibitor (from soya bean)	Crystalline; L. Light and Co. Ltd.	21500	U	Wu & Scheraga (1962)
Cytochrome <i>c</i> dimer	Prepared from horse-heart cytochrome <i>c</i>	24800*	(2 × 12400)	Margoliash & Lustgarten (1962)
Chymotrypsinogen	3 × crystallized; Worthington Biochemical Corp.	25000†	Various	Wilcox, Krant, Wade & Neurath (1957)
β -Lactoglobulin A	Crystalline	36500	AAA	Gordon, Basch & Kalan (1961); Piez, Davie, Folk & Gladner (1961)
Ovalbumin	5 × crystallized	45000†	Various	Warner (1954)
Haemoglobin (bovine)	2 × crystallized; Sigma Chemical Co.	~64500‡		
Serum albumin (bovine)	Fraction V; Sigma Chemical Co.	67000†	U	Phelps & Putnam (1960)
Serum albumin dimer	Present in the bovine serum albumin	134000	(2 × 67000)	Hartley, Peterson & Sober (1962); Sogami & Foster (1962)
γ -Globulins (human)	Fraction II; Sigma Chemical Co.	160000†	U	Phelps & Putnam (1960)
Thyroglobulin (bovine)	Purified; Nutritional Biochemicals Corp.	670000	U	Edelhoeck (1960)

* Including the haem group.

† Given in the reference cited as the best estimate from data by several authors.

‡ The mol.wt. of human haemoglobin is 64450 (including haem groups) by amino acid analysis (Hill, Konigsberg, Guidotti & Craig, 1962).

tration ranges that were studied (the smallest quantity used was 0.2–0.3 mg.): 50-fold concentration range: α -lactalbumin, ovalbumin, serum albumin, γ -globulins; 25-fold concentration range: cytochrome *c*, ribonuclease, thyroglobulin; tenfold concentration range: myoglobin, soya-bean trypsin inhibitor, serum albumin dimer; fourfold concentration range: glucagon, *Pseudomonas* cytochrome *c*-551, cytochrome *c* dimer. Table 2, which is designed to assist in the choice of wavelength for

Table 2. Spectrophotometric estimation of proteins in Sephadex-column effluents

The quantities indicated, after passage through Sephadex G-75 and G-100 columns (2.4 cm. $\times$ 50 cm.), gave maximum extinction readings of 0.2–0.4 (1 cm. cuvette) at the wavelengths indicated for the effluent.

Wavelength for estimation (m μ)	Weight (mg.)		
	215	230	280
Protein			
Glucagon	0.4	0.8	—
Cytochrome <i>c</i>	0.5	1.3 (412 m μ)	—
α -Lactalbumin	0.3	0.7	4
Myoglobin	0.4	1.5 (407 m μ)	—
Trypsin inhibitor (from soya bean)	0.3	1.5	—
Chymotrypsinogen	0.3	0.8	4
Ovalbumin	0.4	1.0	8
Serum albumin	0.4	0.8	8
γ -Globulins	0.2	0.5	3
Thyroglobulin	0.2	0.5	3

spectrophotometric estimation of proteins in column effluents, was compiled from the results of these experiments.

For chymotrypsinogen, the elution volume decreased slightly with increasing concentration over a tenfold range (139 ml. for 0.3 mg. of protein on a Sephadex G-100 column, 136 ml. for 3 mg.). This behaviour indicates a concentration-dependent aggregation of the molecules (Schwert, 1951; Massey, Harrington & Hartley, 1955) which should be negligible in the experiment with 0.3 mg. of protein (see the Discussion section). Elution volumes of β -lactoglobulin and haemoglobin showed considerable variation with protein concentration, and this was investigated further (see below).

Elution volumes for a group of proteins sometimes differed by 1 ml. or 2 ml. in separate experiments with the same column, possibly as a result of temperature changes, but separation between the proteins was usually unaffected. Sets of elution volumes from columns that were identical in size and packed with gel from the same batch differed from each other by as much as 4 ml. for each protein, owing presumably to differences in density of the column packing.

Relationship between molecular weight and elution volume. Fig. 1 shows plots of elution volume, V_e , from Sephadex G-75 and G-100 columns against log(molecular weight) for the proteins listed in Table 1 (except β -lactoglobulin and haemoglobin),

Fig. 1. Plots of elution volumes, V_e , against log(molecular weight) for proteins on Sephadex G-75 (●) and G-100 (○) columns [2.4 cm. $\times$ 50 cm.; equilibrated with 0.05 M-tris hydrochloride buffer, pH 7.5, containing KCl (0.1 M)].

plus sucrose. Each set of results was obtained with one column, but results with other columns fitted almost identical curves. For Sephadex G-75 the experimental points lie close to a straight line over the molecular-weight range 3000–35000, and for Sephadex G-100 over the range 5000–60000. However, the graphs indicate that the useful working range of each gel extends well above these values, to about 70000 for Sephadex G-75 and 150000 for Sephadex G-100.

Effect of pH on the V_e -molecular weight relationship. Experiments with γ -globulins, bovine serum albumin, ovalbumin, soya-bean trypsin inhibitor and α -lactalbumin, with both Sephadex G-75 and G-100 columns, were carried out over a wide range of pH and in a variety of buffers, as follows: approx. 0.1N-hydrochloric acid, pH 1.3; 5 mM-succinic acid-potassium hydroxide, containing potassium chloride (0.1M), pH 4.1, 5.0 and 5.6; 0.05M-tris hydrochloride containing potassium chloride (0.1M), pH 8.5; 0.1M-boric acid-sodium hydroxide, containing potassium chloride, pH 9.9 and 10.7. Plots of V_e against $\log(\text{molecular weight})$ at each pH were similar to those at pH 7.5 (Fig. 1), except that the slope of the linear parts of the graphs increased to a small extent with decrease in pH.

R_{SA} values. The comparison of gel-filtration results for proteins was facilitated by expressing the rate at which a protein migrates through a gel-filtration column as a fraction of the rate at which serum albumin migrates through the same column. Thus:

$$R_{SA} = \frac{\text{migration rate of protein}}{\text{migration rate of serum albumin}} \\ = \frac{V_e \text{ for serum albumin}}{V_e \text{ for protein}}$$

Bovine serum albumin was chosen as standard because it was included in almost every column run. R_{SA} values calculated from the elution volumes of Fig. 1 are given in Table 3, together with values derived from experiments with 9% (w/w) agar-gel columns (Andrews, 1962). Variation in the values derived from different columns of the same gel was $\pm 2\%$.

Calibration of Sephadex G-100 columns for molecular-weight estimation. Sephadex G-100 columns for molecular-weight estimation were calibrated with glucagon, cytochrome c, α -lactalbumin, myoglobin, soya-bean trypsin inhibitor, chymotrypsinogen, ovalbumin, serum albumin, serum albumin dimer, γ -globulins and thyroglobulin. Elution volumes for these proteins were estimated as described above, and used to prepare graphs of the type shown in Fig. 1. Table 2 indicates amounts of the different proteins found suitable for these experiments.

Molecular-weight estimations. Elution volumes of a number of enzymes were determined on calibrated Sephadex G-100 columns, and their molecular weights (Table 4) calculated from appropriate calibration curves. When the catalytic activity of enzymes was used to determine their positions in column effluents, γ -globulins, serum albumin, ovalbumin and cytochrome c were included in each run to check column performance, and estimated by extinction measurements. The amounts of protein and wavelengths for their estimation were chosen to avoid appreciable contribution to the extinction by protein in the enzyme preparation. Thus, when the latter was less than 0.1 mg., the amounts of protein added were sufficient for their estimation at 230 m μ (412 m μ for cytochrome c), otherwise larger amounts of protein and estimation at 280 m μ and 412 m μ were used (Table 2). Rennin and adenosine deaminase (approx. 1 mg.) were each put through columns alone and estimated by the extinction at 220 m μ to determine approximately their positions in the effluents, then they were run in admixture with comparable amounts of γ -globulins, serum albumin and cytochrome c for molecular-weight estimation. In both cases, the enzyme proteins gave distinct peaks in the elution diagrams.

Table 3. R_{SA} values for elution of proteins and other compounds from Sephadex G-75, Sephadex G-100 and 9% (w/w) agar-gel columns at pH 7.5

The R_{SA} values are migration rates through a gel relative to that of serum albumin under the same conditions, and are calculated (see the text) from the elution volumes given in Fig. 2 for Sephadex gels and from data given by Andrews (1962) for the agar gel.

Substance	Sephadex G-75 gel	Sephadex G-100 gel	Granulated agar gel (9% agar)
Sucrose	0.355	0.445	0.64
Glucagon	0.38	0.45	—
Cytochrome c-551 (from <i>Pseudomonas</i>)	0.495	0.56	—
Cytochrome c	0.54	0.60	0.70
Ribonuclease	0.545	0.605	—
α -Lactalbumin	0.57	0.62	0.77
Myoglobin	0.59	0.635	—
Trypsin inhibitor (from soya bean)	0.66	0.69	—
Chymotrypsinogen	0.67*	0.70*	—
Cytochrome c dimer	0.685	0.72	—
Ovalbumin	0.85	0.85	—
Serum albumin	1.00	1.00	1.00
Serum albumin dimer	—	1.26	—
γ -Globulins	1.09	1.31	1.14
Thyroglobulin	1.09	1.45	1.38
Indian ink	1.09	1.45	—
Gastric mucin (from hog)	—	—	1.53

* Determined in experiments where aggregation is believed to be negligible (see the text).

Table 4. *Molecular weights estimated by gel-filtration on Sephadex columns*

Experimental details are given in the text. Since the columns were run at room temperature, the recovery of enzymic activity is expressed relative to that of enzyme kept at room temperature for the same length of time as that in the corresponding column run.				Molecular weight		
Enzyme	Wt. used (mg.)	Assay method	Recovery of enzymic activity (%)	Gel-filtration estimate	Literature value(s)	
Rennin (from calf intestine)	0.8	Protein estimated at 220 m μ	—	31 000	32 000 (see the text); 34 400 (Friedman, 1960)	
Adenosine deaminase (from calf intestine)	{ 1.2 0.014 }	Protein estimated at 230 m μ Disappearance of adenosine followed at 265 m μ (Kaplan, 1955)	— 94	35 000 33 500	See the text	
Peroxidase (from horseradish)	0.040	Oxidation of guaiacol (Maehly, 1954)	101	49 000	40 000 (Maehly, 1955)	
Hexokinase (from yeast)	0.25	Synthesis of glucose 6-phosphate in assay with glucose 6-phosphate dehydrogenase (see the text)	103	51 000	47 000 (Barnard & Ramel, 1962); 50 000 (Ågren, Engström & Eklund, 1963)	
Phosphatase (from <i>Escherichia coli</i>)	0.030	Hydrolysis of <i>p</i> -nitrophenyl phosphate at pH 8.0 (Garen & Levinthal, 1960)	100	74 000	75 000–80 000 (Garen & Levinthal, 1960)	
Alkaline phosphatase (from bovine intestine)	Not known	Hydrolysis of <i>p</i> -nitrophenyl phosphate at pH 9.5 (see the text)	—	115 000	100 000 (Engström, 1961)	
Alkaline phosphatase (from cow's milk)	0.010	Hydrolysis of <i>p</i> -nitrophenyl phosphate at pH 9.5 (see the text)	101	148 000	—	
Glucose 6-phosphate dehydrogenase (from yeast)	Not known	Reduction of NADP (Kornberg & Horecker, 1955)	103	110 000	—	
Glycerol dehydrogenase (from <i>Aerobacter aerogenes</i>)	Not known	Reduction of NAD (Burton, 1955)	104	120 000	—	
Alcohol dehydrogenase (from yeast)	0.6	Reduction of NAD (Racker, 1955)	—	126 000	129 000 (Armstrong, Coates & Morton, 1963); 150 000 (Hayes & Velick, 1954)

Fig. 2. Effect of concentration and pH on the molecular weight of bovine haemoglobin, estimated by Sephadex gel-filtration. $\circ$, pH 6.8 [0.04 M-potassium phosphate buffer containing KCl (0.1 M)]. $\bullet$, pH 7.5 [0.05 M-tris hydrochloride buffer containing KCl (0.1 M)]. $\triangle$, pH 4.9 [0.05 M-sodium acetate buffer containing KCl (0.1 M)].

Fig. 3. Effect of concentration on the molecular weight of β -lactoglobulin A, estimated by Sephadex gel-filtration. The protein was dissolved in 0.05 M-tris hydrochloride buffer, pH 7.5, containing KCl (0.1 M).

In duplicate experiments for all the enzymes listed in Table 4, the maximum difference between molecular-weight estimates for the same enzyme was 5 %.

Bovine haemoglobin and β -lactoglobulin A. Elution volumes for bovine haemoglobin and β -lactoglobulin A, determined at pH 7.5 in experiments with 1–2 mg. of either protein, were lower than expected from their molecular weights (Table 1), indicating dissociation of the molecules into sub-units under the conditions of the experiments. The effect of varying the haemoglobin concentration on its estimated molecular weight at pH 4.9, 6.8 and 7.5 is shown in Fig. 2, and of varying β -lactoglobulin concentration on its molecular weight at pH 7.5 in Fig. 3.

Thin-layer gel-filtration with Sephadex G-100. Correlation between molecular weight and migration rate in thin layers of Sephadex G-100 was investigated with nine of the proteins listed in Table 1. They were usually run in groups of three, and measurements from separate experiments were

Fig. 4. Plot of migration distance against log(molecular weight) for thin-layer gel-filtration of proteins on Sephadex G-100. Experimental details are given in the text.

correlated by expression on the common basis of 6 cm. migration by cytochrome *c*. In a plot of migration distance against log(molecular weight) (Fig. 4) the points lie on a curve, as may be predicted from the results of column experiments. Evidently the molecular weights of proteins can be estimated from their behaviour in thin-layer gel-filtration, although better resolution may be attainable with gel columns.

DISCUSSION

The proteins used to test for correlation between molecular weight and gel-filtration behaviour were chosen because their molecular weights are accurately known. Column runs were performed with mixtures of proteins to cross-check the behaviour of each one against that of several others. Although several proteolytic enzymes of known molecular weight are available in purified form they were not included in the experiments because they are clearly unsuitable for admixture with other proteins. The effect of protein concentration on the elution volumes of β -lactoglobulin and haemoglobin showed that they could not be used as molecular-weight standards, because of dissociation of the molecules into sub-units; their behaviour is discussed below. The decrease in elution volume of chymotrypsinogen with increasing protein concentration suggests reversible concentration-dependent aggregation even in dilute solution (Schwert, 1951; Massey *et al.* 1955). Sedimentation and diffusion

data indicate that this protein exists almost entirely in the monomeric form at a concentration of 1 mg./ml., so it is likely that in a gel-filtration experiment in which the initial protein concentration was 0.15 mg./ml. the measured elution volume was that of the monomer. Glucagon behaves as monomer in glycine buffer, pH 9.5, as judged by approach-to-equilibrium studies in the ultracentrifuge (Professor E. L. Smith, personal communication), and since there is no evidence to suggest otherwise it is assumed to exist as monomer during gel-filtration at pH 7.5.

The relationship between the molecular weights of proteins and their elution volumes from Sephadex columns (Fig. 1) is similar to that for dextrans (Granath & Flodin, 1961). In turn, the behaviour of dextrans is in accordance with Porath's (1962) theoretical treatment of the gel-filtration process, which predicts a linear relationship between the square roots of their molecular weights and the cube roots of their distribution coefficients. Distribution coefficient, K_d , is defined as the volume fraction of solvent imbibed by the gel which is accessible to a solute, and is related to elution volume, V_e , by:

$$V_e = V_o + K_d V_i$$

where V_o , the void volume, is the elution volume of a solute completely excluded from the internal cavities of the gel, and V_i is the volume of solvent imbibed by the gel. Further support for Porath's (1962) treatment is provided by the existence of a similar relationship between molecular weight and K_d of proteins that are not too large to enter the internal cavities of Sephadex G-75 and G-100 gels (Fig. 5) and Sephadex G-200 gel (Wieland, Duesberg & Determann, 1963). In the calculation of K_d values for Fig. 5, V_e for thyroglobulin was taken as

equal to V_o ($K_d = 0$) and V_e for sucrose as equal to $V_o + V_i$ ($K_d = 1$).

The slopes of each curve in Fig. 1 are directly related to resolving power, so the linear parts define the molecular weights between which each gel gives optimum separation of proteins. In addition, the complete curves indicate that the useful working range of each gel extends well beyond these limits. From the relative slopes of the two curves, the resolving power of Sephadex G-75 in its optimum range is only slightly better than that of Sephadex G-100 in the same range. The 50 cm. columns used in the experiments described above did not, however, give the best resolution attainable with Sephadex gels. Other experiments have shown that, in accordance with the recommendations of Flodin (1961, 1962), improved resolution can be obtained by using longer columns, narrower sieve-fractions of gel and smaller gel particles. By such means the presence of serum albumin dimer in preparations of serum albumin can be detected with Sephadex G-75 (King & Norman, 1962) which, as Fig. 1 shows, needs good resolution. The R_{sA} values (Table 3) are useful for correlating gel-filtration results from different columns because, unlike elution volumes, they are not a function of column size but depend only on the nature of the gel and the solutes themselves. It is suggested that sets of such values, not necessarily calculated with serum albumin as standard, may be useful for characterizing gel-filtration media.

The curves in Fig. 1 indicate that the molecular weights of proteins that are just excluded from the Sephadex gels used in the experiments described above are about 110 000 and 300 000 for Sephadex G-75 and G-100 respectively. Although these values may differ a little for other batches of Sephadex G-75 and G-100 gels, they are considerably greater than the lower molecular-weight limits given in the manufacturers' specifications for dextrans that are completely excluded from these gels (40 000–50 000 and 100 000 for Sephadex G-75 and G-100 respectively). Evidently dextrans in solution have more expanded structures than do proteins of the same molecular weight, and application of the manufacturers' values without qualification to the behaviour of proteins on these gels is misleading.

Approximate values for the maximum and minimum pore diameters in each gel have been calculated from the gel-filtration results by assuming that the pores are circular in cross-section and that the appropriate diameters are equal to the diameters of spherical protein molecules that are just excluded at those points. Other assumptions are that the protein molecules have partial specific volumes of 0.73 ml./g. and are hydrated in solution

Fig. 5. Relationship between (molecular weight)^{1/2} and (distribution coefficient)^{1/3} for gel-filtration of proteins (Table 1) on Sephadex G-75 (●) and G-100 (○) columns. For explanation of the terms and reference to the theoretical basis of the relationship, see the Discussion section.

to the extent of 0.3 g. of water/g. of protein. For Sephadex G-75 the minimum and maximum molecular weights at which the V_e -log(molecular weight) curve has any slope are 2000 and 110 000, and the corresponding pore diameters are about 18 Å and 70 Å, whereas for Sephadex G-100 the limits of 2500 and 300 000 indicate minimum and maximum pore diameters of 20 Å and 100 Å respectively. The significance of these values clearly depends on the shape of cavities inside the gel particles. If they are conical (Porath, 1962), with the greatest diameter at the particle surface, then maximum pore size applies to the surface opening of the widest cavity and minimum pore size to the diameter at the inner end of the narrowest cavity. However, the suggestion is that the calculated values are reasonable estimates of the spacings between dextran chains and cross-links that determine the properties of each gel.

Although Sephadex G-75 and G-100 function almost equally well for the separation of proteins with molecular weights up to approx. 50 000, Sephadex G-100 is the preferred gel for molecular-weight estimation because its useful range extends to approx. 150 000, well above that of Sephadex G-75. The wider range also facilitates calibration with readily available proteins of known molecular weight. The amount of any protein needed for a molecular-weight estimation clearly depends on the sensitivity of the method used to measure its concentration in column effluents or detect its presence in thin layers of gel. Therefore only catalytic amounts of enzymically active proteins are necessary (Table 4) and their molecular weights can be determined at concentrations similar to those employed for their assay. The method may well be applicable to the study of complex systems of enzymes, as purification of individual enzymes is not essential, but, in such cases, the possibility of interaction between solute molecules should be particularly noted. In experiments with non-enzymic proteins, 100–200 µg. of purified material is sufficient for a molecular-weight estimation.

Molecular weights of enzymes estimated by the gel-filtration method are compared with literature values in Table 4. The best estimates of the molecular weight of rennin are probably 32 000, calculated from its amino acid composition (Dr G. C. Cheeseman, personal communication), and 34 400, derived from its sedimentation and diffusion properties (Friedman, 1960), whereas the gel-filtration estimate was 31 000. The molecular weight of adenosine deaminase has not been reported, but its sedimentation coefficient (approx. 3s; Professor T. G. Brady, personal communication) indicates a value of 30 000–40 000, and gel-filtration confirms this. Adenosine deaminase exists as a mixture of five or more isoenzymes (Brady & O'Connell, 1962),

so if sub-unit structure is responsible for this phenomenon (cf. lactate dehydrogenase; Appella & Markert, 1961; Wilson, Cahn & Kaplan, 1963) the molecular weight of an adenosine-deaminase sub-unit may be no more than 9000. The behaviour of horseradish peroxidase on Sephadex columns indicated a molecular weight (49 000) greater than the accepted value of 40 000 (the evidence for which is summarized by Maehly, 1955). If the peroxidase contains nearly 20 % of carbohydrate (Maehly, 1955) it may well have a more expanded structure than that of more typical proteins, and gel-filtration would then give a high molecular-weight estimate. Molecular weights reported for intestinal alkaline phosphatase are 60 000 (Schramm & Armbruster, 1954) and 100 000 (Engström, 1961), whereas gel-filtration of a crude preparation gave a value of 115 000. Again, the difference between the latter two values may be due to association of carbohydrate with the enzyme (Engström, 1961). Gel-filtration of yeast alcohol dehydrogenase indicated a molecular weight of 126 000, in good agreement with the value of 129 000 determined by Armstrong, Coates & Morton (1963) by using the approach-to-equilibrium method in the ultracentrifuge, but lower than the older value of 150 000 (Hayes & Velick, 1954).

Both bovine haemoglobin and β -lactoglobulin gave single peaks in elution diagrams, but except at the higher β -lactoglobulin concentrations the elution volumes corresponded to molecular weights that were less than established values (Figs. 2 and 3). Both proteins have sub-unit structures, and the gel-filtration behaviour is evidence for rapidly established association-dissociation equilibria between complete molecules and sub-units, such as observed with a number of proteins in ultracentrifugation experiments (Schachman, 1960). Osmotic-pressure, light-scattering and sedimentation data show that considerable concentration-dependent dissociation of haemoglobin molecules occurs in solutions of high ionic strength at neutral pH (Gutfreund, 1949; Rossi-Fanelli, Antonini & Caputo, 1961), and at moderate ionic strength at high and low pH (Field & O'Brien, 1955; Hasseroth & Vinograd, 1959; Vinograd & Hutchinson, 1960). Dissociation at moderate ionic strength and neutral pH, conditions similar to those used in the gel-filtration experiments, is normally so small that it has only been detected by the formation of hybrid haemoglobins (Huehns & Beaven, 1963). However, from the molecular-weight estimates (Fig. 2), considerable dissociation of haemoglobin occurred during gel-filtration, even at pH 6.8. Clearly the inference is that dissociation was enhanced while the haemoglobin was passing through the column. This may have occurred through disturbance of equilibrium by

diffusion of the smaller sub-unit aggregates into internal cavities of the gel which are not accessible to larger aggregates, followed by dissociation of the latter in an attempt to restore equilibrium.

At the highest protein concentrations used in the β -lactoglobulin A experiments, the elution volumes corresponded to molecular weights (35 000, 37 500) close to the accepted value (36 500), whereas a reasonable extrapolation of the other results to zero protein concentration gave about half this value (Fig. 3). As dissociation of β -lactoglobulin into half-molecules, which readily occurs in the pH range 2–4 (Timasheff & Townend, 1961), has also been detected by ultracentrifugation in dilute solution at a pH close to that used in the gel-filtration experiments (Georges, Guinand & Tonnelat, 1962), it does not seem to be increased during gel-filtration as markedly as with haemoglobin. Gel-filtration may well have application in the study of proteins that show association–dissociation phenomena, particularly in the determination of monomer or sub-unit molecular weights because molecular-weight measurements are possible at very low protein concentrations. Although protein concentration during gel-filtration is difficult to estimate in terms of equivalent concentration in free solution, the expression of results as in Figs. 2 and 3 should be helpful in such investigations.

Some consideration and evaluation of possible errors in molecular weights estimated by gel-filtration is appropriate. The acidic groups in Sephadex gels (Gelotte, 1960; Flodin, 1962) cause retention of strongly basic proteins on the gels (Cruff, 1961), and exclusion of phosvitin (Dr T. E. Barman, unpublished work) and other strongly acidic macromolecules (Hay, Wicken & Baddiley, 1963; Posner, 1963) from the inner cavities. Whether or not these ionic interactions can be eliminated by certain conditions of ionic strength and pH is uncertain, but moderate salt concentrations alone are sufficient to overcome the retention of other proteins on Sephadex gels from solutions of very low ionic strength (Glazer & Wellner, 1962; Miranda, Rochat & Lissitzky, 1962). The anomalous behaviour of pancreatic amylase on Sephadex columns indicates that interaction between enzyme and gel might also occur because of structural similarities between the gel and a normal substrate for the enzyme (Flodin, 1962). Clearly, any undetected interaction between solute and gel can lead to erroneous conclusions about the molecular weight of the solute, but on present evidence this problem seems unlikely to detract seriously from the general usefulness of the method. No difficulties should arise from irregularities in the distribution of pore sizes in the gels, because both the method of their preparation (Flodin, 1962) and the regularity of the curves in

Fig. 1 indicates that in each gel the distribution is smooth. In this respect, however, difficulties might arise in experiments with mixtures of gels.

The most likely causes of error in protein molecular weights estimated by gel-filtration are the inaccurate estimation of elution volumes, differences in shape between one protein and another, and density differences between solvated protein molecules, resulting in different ratios between size and molecular weight. A way of assessing the magnitude of error is to assume that deviation of points from the curves in Fig. 1 and the differences between estimated and literature values in Table 4 are due to these causes. The greatest deviation in Fig. 1 occurs with *Pseudomonas* cytochrome *c*-551, for which the difference between known molecular weight and molecular weight calculated from elution volumes is approx. 10%. This uncertainty also covers the probable errors in the results of Table 4, except for horseradish peroxidase and intestinal alkaline phosphatase which have been discussed above. Therefore the possibility is that estimates with Sephadex G-100 of the molecular weights of globular proteins in the range 10 000–150 000 will be accurate to within 10%. Estimates for other types of protein, including glycoproteins, may be less accurate if globular proteins are used for calibration.

Molecular weights estimated by Sephadex gel-filtration are probably more reliable than those obtained by agar gel-filtration (Andrews, 1962) because of better resolution and less possibility of interaction between solute and gel. Adsorption effects may well have caused the significant differences between molecular weights estimated by the two methods for intestinal (115 000 and 92 000) and milk (148 000 and 105 000) alkaline phosphatases, for in each case the agar-gel estimate is the smaller. In this case, the Sephadex-gel estimates must also be accepted with reserve. Clearly, the value of the gel-filtration method will be enhanced if such difficulties are overcome by the development of other gel-filtration media.

Comparison between proteins of known and unknown molecular weight forms the basis of several methods that may be used for estimation of molecular weights or diffusion coefficients. Besides gel-filtration, they include density-gradient centrifugation (Martin & Ames, 1961), dialysis (Craig, 1960), electrodialysis (Pierce & Free, 1961), ultra-filtration (Rosenfeld, 1963), diffusion through agar gels (Allison & Humphrey, 1960; Ackers & Steere, 1962; Schantz & Lauffer, 1962) and starch-gel electrophoresis (Smithies, 1962). Although each method may have its special applications, gel-filtration may prove to be the most useful because of its simplicity in operation, wide application and equivalence with preparative procedures.

SUMMARY

1. Correlation between elution volumes, V_e , and molecular weight was investigated for gel-filtration of proteins of known molecular weight on Sephadex G-75 and G-100 columns at pH 7.5.

2. Graphs of V_e against log(molecular weight) were linear over the molecular-weight ranges 3000–35000 and 5000–60000 for Sephadex G-75 and G-100 respectively. Within these limits the gels give optimum separation of proteins according to molecular weight, but their useful working ranges extend to about 70000 and 150000 respectively.

3. The lower molecular-weight limits for complete exclusion of proteins from Sephadex G-75 and G-100 were estimated as 110000 and 300000 respectively.

4. The relationship between V_e and molecular weight was similar over the pH range 1.3–10.7.

5. The use of Sephadex G-100 columns for estimating the molecular weights of proteins was described; 10 μ g. of protein with enzymic activity, or 100 μ g. of non-enzymic protein, may be sufficient for an estimation. Purification of the protein is unnecessary if it can be estimated in column effluents by a specific method.

6. Molecular weights of enzymes estimated in this way were in agreement with literature values. In cases where molecular weights determined by other methods have not been published, Sephadex-gel-filtration estimates were: adenosine deaminase, 34000; yeast glucose 6-phosphate dehydrogenase, 110000; *A. aerogenes* glycerol dehydrogenase, 120000; milk alkaline phosphatase, 148000.

7. Bovine haemoglobin showed very marked concentration-dependent dissociation on Sephadex G-100 columns, and dissociation of β -lactoglobulin A into half-molecules also occurred.

8. Good correlation was observed between distances migrated and molecular weights of proteins in thin-layer gel-filtration with Sephadex G-100.

9. Potential sources of error in molecular-weight estimation by Sephadex gel-filtration were discussed. The maximum uncertainty in values estimated by the column procedure for globular proteins in the range 10000–150000 was put at $\pm 10\%$. For other types of protein the possibility is that values will be less reliable, if globular proteins are used for calibration of the columns.

REFERENCES

- Ackers, G. K. & Steere, R. L. (1962). *Biochim. biophys. Acta*, **59**, 137.
- Ågren, G., Engström, L. & Eklund, S. (1963). *Biochim. biophys. Acta*, **67**, 143.
- Allison, A. C. & Humphrey, J. H. (1960). *Immunology*, **3**, 95.
- Ambler, R. P. (1963a). *Biochem. J.* **89**, 341.
- Ambler, R. P. (1963b). *Biochem. J.* **89**, 349.
- Andrews, P. (1962). *Nature, Lond.*, **196**, 36.
- Andrews, P. & Folley, S. J. (1963). *Biochem. J.* **87**, 3p.
- Appella, E. & Markert, C. L. (1961). *Biochim. biophys. Res. Commun.* **6**, 171.
- Armstrong, J. McD., Coates, J. H. & Morton, R. K. (1963). *Biochem. J.* **86**, 136.
- Barnard, E. A. & Ramel, A. (1962). *Biochem. J.* **84**, 72p.
- Brady, T. G. & O'Connell, W. (1962). *Biochim. biophys. Acta*, **62**, 216.
- Bromer, W. W., Staub, A., Diller, E. R., Bird, W. L., Sinn, L. G. & Behrens, O. K. (1957). *J. Amer. chem. Soc.* **79**, 2794.
- Burton, R. M. (1955). In *Methods in Enzymology*, vol. 1, p. 397. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Craig, L. C. (1960). In *Analytical Methods of Protein Chemistry*, vol. 1, p. 104. Ed. by Alexander, P. & Block, R. J. London: Pergamon Press Ltd.
- Cruft, H. J. (1961). *Biochim. biophys. Acta*, **54**, 611.
- Determann, H. (1962). *Experientia*, **18**, 430.
- Edelhoc, H. (1960). *J. biol. Chem.* **235**, 1326.
- Edmundson, A. B. & Hirs, C. H. W. (1962). *J. molec. Biol.* **5**, 663.
- Engström, L. (1961). *Biochim. biophys. Acta*, **52**, 36.
- Field, E. O. & O'Brien, J. R. P. (1955). *Biochem. J.* **60**, 656.
- Flodin, P. (1961). *J. Chromat.* **5**, 103.
- Flodin, P. (1962). In *Dextran Gels and their Applications in Gel Filtration*. Uppsala: Pharmacia.
- Friedman, L. (1960). *Diss. Abstr.* **20**, 4510.
- Garen, A. & Levinthal, C. (1960). *Biochim. biophys. Acta*, **38**, 470.
- Gelotte, B. (1960). *J. Chromat.* **3**, 330.
- Georges, C., Guinand, S. & Tonnelat, J. (1962). *Biochim. biophys. Acta*, **59**, 737.
- Glazer, A. N. & Wellner, D. (1962). *Nature, Lond.*, **194**, 862.
- Gordon, W. G., Basch, J. J. & Kalan, E. B. (1961). *J. biol. Chem.* **236**, 2908.
- Gordon, W. G. & Ziegler, J. (1955). *Arch. Biochem. Biophys.* **57**, 80.
- Granath, K. A. & Flodin, P. (1961). *Makromol. Chem.* **48**, 160.
- Gutfreund, H. (1949). In *Haemoglobin*, p. 197. Ed. by Roughton, F. J. W. & Kendrew, J. C. London: Butterworth and Co. Ltd.
- Hartley, R. W., Peterson, E. A. & Sober, H. A. (1962). *Biochemistry*, **1**, 60.
- Hasseroth, U. & Vinograd, J. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 12.
- Hay, J. B., Wicken, A. J. & Baddiley, J. (1963). *Biochim. biophys. Acta*, **71**, 188.
- Hayes, J. E. & Velick, S. F. (1954). *J. biol. Chem.* **207**, 225.
- Hill, R. J., Konigsberg, W., Guidotti, G. & Craig, L. C. (1962). *J. biol. Chem.* **237**, 1549.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1956). *J. biol. Chem.* **219**, 623.
- Hjertén, S. & Mosbach, R. (1962). *Analyt. Biochem.* **3**, 109.
- Huehns, E. R. & Beaven, G. H. (1963). *Fed. Proc.* **22**, 596.
- Johansson, B. G. & Rymo, L. (1962). *Acta chem. scand.* **16**, 2067.

- Kaplan, N. O. (1955). In *Methods in Enzymology*, vol. 2, p. 475. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- King, T. P. & Norman, P. S. (1962). *Biochemistry*, **1**, 709.
- Kornberg, A. & Horecker, B. L. (1955). In *Methods in Enzymology*, vol. 1, p. 323. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Lathe, G. H. & Ruthven, C. R. J. (1956). *Biochem. J.* **62**, 665.
- Maehly, A. C. (1954). *Meth. biochem. Anal.* **1**, 357.
- Maehly, A. C. (1955). In *Methods in Enzymology*, vol. 2, p. 801. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Margoliash, E. (1962). *J. biol. Chem.* **237**, 2161.
- Margoliash, E. & Lustgarten, A. (1962). *J. biol. Chem.* **237**, 3397.
- Martin, R. G. & Ames, B. N. (1961). *J. biol. Chem.* **236**, 1372.
- Massey, V., Harrington, W. F. & Hartley, B. S. (1955). *Disc. Faraday Soc.* **20**, 24.
- Miranda, F., Rochat, H. & Lissitzky, S. (1962). *J. Chromat.* **7**, 142.
- 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.
- Pierce, J. G. & Free, C. A. (1961). *Biochim. biophys. Acta*, **48**, 436.
- Piez, K. A., Davie, E. W., Folk, J. E. & Gladner, J. A. (1961). *J. biol. Chem.* **236**, 2912.
- Polsno, A. (1961). *Biochim. biophys. Acta*, **50**, 565.
- Porath, J. (1962). *Proc. int. Symp. Pharmaceutical Chemistry, Florence*. London: Butterworth & Co. Ltd. (in the Press).
- Posner, A. M. (1963). *Nature, Lond.*, **198**, 1161.
- Racker, E. (1955). In *Methods in Enzymology*, vol. 1, p. 500. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Rosenfeld, M. (1963). *Biochim. biophys. Acta*, **75**, 241.
- Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1961). *J. biol. Chem.* **236**, 391.
- Schachman, H. K. (1960). *Brookhaven Symp. Biol.* **13**, 49.
- Schantz, E. J. & Lauffer, M. A. (1962). *Biochemistry*, **1**, 658.
- Schramm, G. & Armbruster, O. (1954). *Z. Naturf.* **9B**, 114.
- Schwert, G. W. (1951). *J. biol. Chem.* **190**, 799.
- Smithies, O. (1962). *Arch. Biochem. Biophys.* suppl. **1**, 125.
- Sogami, M. & Foster, J. F. (1962). *J. biol. Chem.* **237**, 2514.
- Steere, R. L. & Ackers, G. K. (1962). *Nature, Lond.*, **196**, 475.
- Timasheff, S. N. & Townend, R. (1961). *J. Amer. chem. Soc.* **83**, 470.
- Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
- Vinograd, J. & Hutchinson, W. D. (1960). *Nature, Lond.*, **187**, 216.
- Warner, R. C. (1954). In *The Proteins*, vol. 2, part A, p. 435. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
- Wieland, Th., 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.
- Wilson, A. C., Cahn, R. D. & Kaplan, N. O. (1963). *Nature, Lond.*, **197**, 331.
- Wu, Y. V. & Scheraga, H. A. (1962). *Biochemistry*, **1**, 698.

Biochem. J. (1964) **91**, 233

The Hydrolysis of Triphosphoinositide by Extracts of Ox Brain

By W. THOMPSON* AND R. M. C. DAWSON

Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(Received 7 October 1963)

Sloane-Stanley (1953) reported that brain lipid fractions rich in inositol were rapidly attacked by brain homogenates with the liberation of free and combined phosphate as well as free and combined inositol. Continuing these studies Rodnight (1956) obtained evidence which suggested that the initial product of the breakdown was inositol monophosphate and that its enzymic release was activated by Ca^{2+} ions. However, the interpretation of these results is complicated in view of the recent findings that brain contains at least three

inositol-containing phospholipids, namely mono-, di- and tri-phosphoinositide (Dittmer & Dawson, 1961; Dawson & Dittmer, 1961; Brockerhoff & Ballou, 1961a).

A number of isotopic investigations *in vivo* with labelled phosphate have suggested that brain phosphoinositides are metabolically very active compared with most of the other phospholipids (see, e.g., Ansell & Spanner, 1960). The availability of a method for preparing triphosphoinositide in a relatively pure form (Dittmer & Dawson, 1961) has led us to investigate the enzymic reactions involved in its catabolism and to charac-

* Present address: Charles H. Best Institute, University of Toronto, Toronto, Ontario, Canada.