THE DETERMINATION OF PROTEIN MOLECULAR WEIGHTS OF UP TO 225,000 BY GEL-FILTRATION ON A SINGLE COLUMN OF SEPHADEX G-200 AT 25° AND 40°

A. A. LEACH AND P. C. O'SHEA
*The Gelatine and Glue Research Association**, *London* (*Great Britain*)
(Received May 28th, 1964)

INTRODUCTION

The determination of the molecular weights of proteins by means of gel-filtration on cross-linked dextran gels (Sephadex) has been described by a number of authors. WHITAKER[1] obtained an excellent linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume, V, to the column void volume, V_0. Using Sephadex G-75 he covered the molecular weight range 13,000–40,200 and with Sephadex G-100 the range 13,000–76,000. WIELAND, DUESBERG AND DETERMANN[2] found a similar correlation on Sephadex G-200 for molecular weights of 13,000–150,000. Using Sephadex G-75 and G-100 ANDREWS[3] also obtained the relationship for molecular weights in the range 3,500–150,000.

The present paper extends the work of WHITAKER[1] up to molecular weights of 225,000 on columns of Sephadex G-200; evidence is provided showing that considerable extension beyond this is possible. Alternative conditions in which the column temperature and buffer ionic strength are increased to minimise possible aggregation effects are also described.

EXPERIMENTAL

Materials

Proteins of known molecular weight. Thyroglobulin; porcine, lyophilized, water soluble, I = 0.9 %. Catalase; *ex* ox-liver, supplied as a suspension. α-Globulins; equine, fraction IV. γ-Globulins; bovine, fraction II. Alcohol dehydrogenase; *ex* yeast, supplied as a suspension. Ovalbumin; *ex* chicken-egg, lyophilized, salt-free. Lysozyme; *ex* egg-white, two times crystallized, lyophilized. Cytochrome c; *ex* horse-heart, Fe = 0.43 %. All these proteins were obtained from L. Light & Co., Ltd. Serum albumin; bovine, crystallized, from Armour Laboratories. Pepsin; crystallized, 1:60,000, from Sigma Chemical Co.

Dextran-gel. Sephadex G-200, Lot No. To. 3016 supplied by Pharmacia, Sweden.

Citrate buffer. A 0.2 M solution was prepared by dissolving 21.0 g "Analar", citric acid, $C_6H_8O_7 \cdot H_2O$, in 200 ml N NaOH and diluting to 500 ml with distilled water. The pH of this buffer when diluted with an equal volume of water is 5.0 ± 0.1.

* 2a Dalmeny Ave., London N.7.

Small differences from this value can be corrected with N HCl or N NaOH. 0.25 g thymol/l was added as a preservative. The buffer was used undiluted for preparing the ninhydrin reagent, and diluted with an equal volume of water for column development. When used with the column at 40°, NaCl (5 % w/v) was added to the buffer. When used at 40°, the buffers were boiled to remove air and stored in the reservoir under liquid paraffin; this prevents the formation of bubbles within the column.

Methyl cellosolve. Technical methyl cellosolve was shaken with acidified aqueous ferrous sulphate and distilled. The first 5 % and the last 10 % of the distillate were rejected.

Ninhydrin reagent. This was prepared as described by MOORE AND STEIN[4] by dissolving 0.08 g "Analar" stannous chloride, $SnCl_2 \cdot 2\,H_2O$ in 50 ml of the undiluted citrate buffer. This solution was mixed thoroughly with 50 ml methyl cellosolve containing 2 g "Analar" ninhydrin. The solution could be used for three weeks if it was stored under nitrogen and in the dark.

Diluent. The solution was obtained by mixing equal volumes of water and absolute alcohol. Alternatively, re-distilled n-propanol could be used instead of the alcohol.

Procedure

Packing of the column. The Sephadex G-200 was suspended in enough buffer to ensure that after swelling the suspension was still sufficiently fluid to allow air-bubbles to escape readily. The gel was allowed to swell for at least 1 day, preferably 3 days, before pouring into the column, otherwise the swelling continued in the column giving very slow flow rates. The swelling was carried out in the buffer used at the column temperature to be employed. Two jacketed columns of 0.9 cm and 2.0 cm diameter and both 110 cm high with sintered glass plates at the bottom were used. The 2 cm diameter column, which gives the higher resolution, was used for most of the present work. The less accurate 0.9 cm diameter column was examined to establish that it could be used in cases where only very small amounts of material are available. The columns were maintained at 25° ± 1° or 40° ± 1°, including during the preparation of the columns. A cork was placed in the column outlet and the suspension of Sephadex poured down the column surface to minimise the formation of bubbles. When a 10 cm layer of the gel particles had settled out, the cork was removed, whereupon more rapid column packing was achieved. The column was kept filled with the suspension; after prolonged settling and passage of buffer (500 ml with the larger column) through the column, a stable level of gel-particles was achieved. The gel was packed to a height of 105–110 cm in both columns.

Preparation of protein solutions. 0.5–10 mg of each protein was dissolved in 1–2 ml of buffer and if necessary filtered. In solutions containing more than one protein the total did not exceed 10 mg/ml. The catalase and alcohol dehydrogenase were supplied as suspensions. The solid was centrifuged off from these, washed with water, re-centrifuged, shaken with the buffer for 30 min, re-centrifuged and 2 ml of the clear solution placed on the column. In the case of catalase the concentration obtainable was very low.

Chromatography. 1–2 ml of the protein solution was run very slowly down the column surface onto the gel. This was to minimise disturbance of the gel; the usual procedure of covering the surface with filter paper was abandoned, as the point at which buffer or protein solution had just sunk into the gel could not be judged accu-

rately. After the solution had sunk into the gel, two 1 ml portions of the buffer were used to wash in any solution adhering to the column, the first being allowed to sink into the gel before the second portion was used. The column was connected to the buffer reservoir and the column developed. A drop-counting fraction-collector was employed and fractions of approximately 1 ml collected. Each fraction had to be weighed as the drop size varied throughout the development, particularly when a protein was emerging. WHITAKER[1] reports an error of only 0.5–1.0 % in the elution volume from this cause; in the present work variations in drop size of as much as 15–20 % have been recorded. A siphon fraction-collector is to be preferred. The number of drops on the drop recorder was used to judge the exact point of addition of the sample to the column. Proteins were added as mixtures when compatible, or singly if not. One protein could be placed on a column following another, provided there was sufficient elution volume between them, and that the molecular weight of the second was not sufficiently great to enable it to catch up the first during elution. As soon as all the material placed on the column had been eluted the column was ready for re-use. The flow rate never exceeded 0.1 ml/min per cm^2 cross-section of column (about 16 ml/h on the large column), slower than any system used by WHITAKER[1], who showed that flow rates of 0.18–0.42 $ml \cdot min^{-1} \cdot cm^{-2}$ were satisfactory and that higher flow rates of 0.62 $ml \cdot min^{-1} \cdot cm^{-2}$ gave low elution volumes.

Colorimetry. 2 ml of the ninhydrin reagent was added to each fraction, the tube capped and heated in a bath of vigorously boiling water for 20 min. The tubes were cooled and the contents diluted with 5 ml of alcohol–water (1:1) and the optical density measured at 570 mμ on a Hilger Uvispek using 0.5 or 2.0 cm cells. An alternative procedure is to measure the U.V. absorption directly on the fractions to give the position of the protein peaks (see ANDREWS[3]). This requires the use of a preservative which does not absorb in the U.V., or the more rapid development of the column in the absence of preservative. In the absence of a preservative the prolonged use of a single column packing may give rise to fungal or bacterial growth. The use of ninhydrin enables one to use a simple colorimeter, and after development select only the tubes in the region of an elution peak for measurement.

Column void volume. The column void volume was checked on the first run on each freshly packed column, and then on alternate runs using thyroglobulin. Thyroglobulin has a molecular weight of 650,000 (see Table I), which is above the exclusion limit for Sephadex G-200; it passes through the column unhindered and its elution volume is therefore equal to the column void volume. Elution volumes were interpolated to the nearest 0.1 ml by triangulation.

RESULTS

Molecular weight of reference proteins

The protein molecular weights used in Fig. 1 and given in Table I are taken from the voluminous literature on the subject. The value selected for any protein, often from a wide range, is that which appears to represent the true value following an examination of the range of values quoted and their methods of determination. Selected literature values are also given in Table I.

The two blood fractions employed are not homogeneous. MCFARLANE[5] has demonstrated that the sedimentation diagrams for the plasma of normal cow, horse

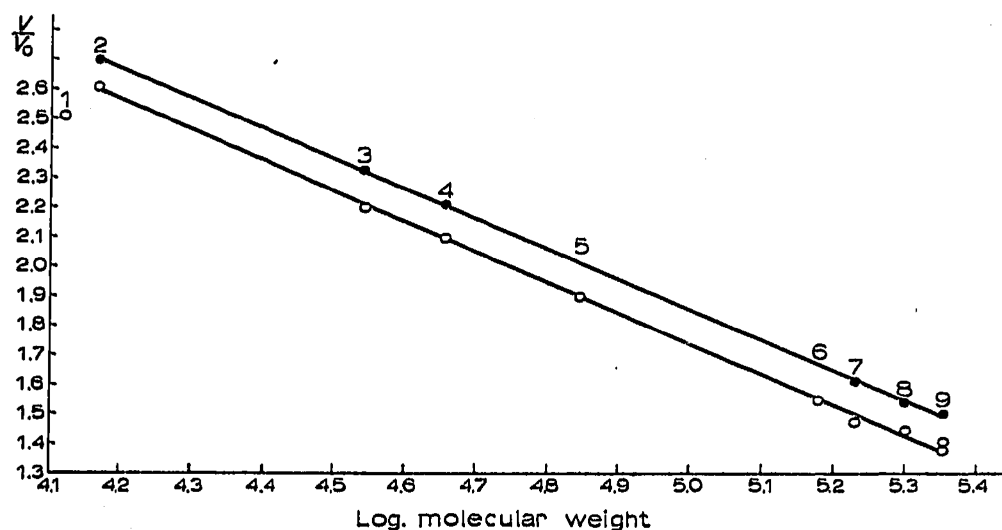

Fig. 1. Relationship between the ratio of the protein elution volume (V) to column void volume (V_0) and the protein molecular weight. ○ 25°, pH 5.0, 0.1 M citrate buffer; ● 40°, pH 5.0, 0.1 M citrate buffer plus 5% NaCl w/v. 1 = cytochrome c; 2 = lysozyme; 3 = pepsin; 4 = ovalbumin; 5 = serum albumin; 6 = alcohol dehydrogenase; 7 = γ-globulin; 8 = α-globulin; 9 = catalase.

and man are qualitatively similar. ONCLEY, SCATCHARD AND BROWN[6] have shown that fraction II of human plasma is predominantly γ-globulin and fraction IV is predominantly α-globulin. It is therefore assumed that fraction II of bovine plasma and fraction IV of equine plasma respectively are of similar composition; the results in Table I support this view.

Void volume

The elution volume of thyroglobulin (mol.wt. = 650,000), which is equal to the void volume for the 0.1 M citrate buffer 25° system, was in the range of 106.3 to 107.0 ml (8 determinations, S.D. ± 0.25 ml) for a column 108.5 cm high and 2.0 cm diameter, measured on alternate runs during a period of 4 weeks. Thus it is necessary to check the void volume of a single column only occasionally.

Effect of column diameter

V/V_0 for α-globulin and lysozyme were 1.440 and 2.603 on the 2 cm diameter column and 1.432 and 2.590 on the 0.9 cm diameter column with the lower temperature system.

Molecular weight and elution volumes

As expected from the earlier work mentioned above, eight of the nine proteins, covering a molecular weight range of 14,600–225,000, gave a linear plot of V/V_0 against log mol. weight. Using the form of equation found by WHITAKER[1], *i.e.*:

$$\text{log mol. wt.} = a\left(\frac{V}{V_0} - 1\right) + b$$

the equation for the lower temperature system was:

$$\text{log mol. wt.} = -0.959\left(\frac{V}{V_0} - 1\right) + 5.700 \qquad (1)$$

TABLE I

MOLECULAR WEIGHTS (mol. wt.) AND THE RATIOS OF ELUTION VOLUME (V) TO COLUMN VOID VOLUME (V_0) OF REFERENCE PROTEINS

System A: Elution at 25° with pH 5.0, 0.1 M citrate buffer.
System B: Elution at 40° with pH 5.0, 0.1 M citrate buffer plus 5% NaCl (w/v).

Protein	*V/V_0 in system A*	*V/V_0 in system B*	*Mol. wt. used in Fig. 1*	*Mol. wt. calculated from eqn. (1)*	*Mol. wt. calculated from eqn. (2)*	*Literature values of mol. wt.*	*References*
Thyroglobulin	1	1	650,000			650,000	7
						650,000	8
Catalase	1.377–1.401[a]	1.503	225,000	207,000[a],	225,000	225,000	9
	1.374–1.400	1.488–1.515		228,000		225,000	10
						248,000	11
α-Globulin	1.439[b]	1.547	200,000	191,000	208,000	200,000[c]	6
	1.440	1.525					
γ-Globulin	1.469	1.617	171,000	177,000	177,000	170,000	12
	1.476	1.600				171,000	13
Alcohol dehydrogenase	1.538		151,000	152,000		150,000	14
	1.544					151,000	15
Serum albumin	1.890		70,000	70,000		69,000	16
	1.891					70,300	17
						70,400	18
						71,300	19
Ovalbumin	2.081	2.211	45,000	45,200	45,500	44,400	20
	2.101	2.211				45,000	21
						45,160	22
						45,200	23
Pepsin	2.198	2.318	35,500	35,600	35,500	35,000	24
	2.200	2.322				35,500	25
						35,500	26
Lysozyme	2.616	2.697	14,700	14,600	15,100	14,700	27
	2.590	2.700				14,700	28
						14,800	29
						14,900	28
Cytochrome c	2.508[a,d]		13,200	17,900[a,d]		13,000	30
	2.511					13,400	31

[a] See Results.
[b] Small peak at 1.458.
[c] Human material.
[d] See Discussion.

and for the higher temperature and higher ionic strength system with six of the proteins the equation was:

$$\log \text{mol. wt.} = -0.981 \left(\frac{V}{V_0} - 1\right) + 5.845 \qquad (2)$$

With catalase the peak was very small and flat due to its low solubility in the buffer. To fractions in the region where elution was expected, 10 ml of 1% H_2O_2 was added; the tubes which gave the most rapid evolution of oxygen were in the same position as those detected colorimetrically thus confirming the position of this peak.

For the case of cytochrome c which did not fall on the straight line see Discussion.

Reproducibility

The greatest difference in duplicates of $V/V_0 - 1$ was about 2% for the low temperature system and 4% at the higher temperature. The peaks were broader at the higher temperature.

DISCUSSION

The present work demonstrates that protein molecular weights of at least 225,000 can be determined with Sephadex G-200 with the conditions described. At 25° on a column where the void volume was 106.7 ml, catalase (mol.wt. = 225,000) was eluted at 147.0 ml, indicating that the column can be used in determinations of molecular weights in excess of 225,000.

Cytochrome c behaved as though its molecular weight was 17,900 against literature values of about 13,200. Using acetate buffers WHITAKER[1] found this protein to behave normally. It is of interest to note that this situation was reversed with lysozyme, behaving normally with the present system. It is probably preferable to use the Sephadex G-75 system of WHITAKER[1] for proteins of such low molecular weights, for the slope of V/V_0 *versus* log. mol. wt. is greater. WHITAKER[1] showed that increasing the temperature from 3.3° to 25° resulted in small decreases in V/V_0 and that increases in the ionic strength of the buffer gave slight increases in V/V_0. In the present work the resultant effect of the increase in temperature from 25° to 40° and the addition of 5% NaCl (w/v) to the buffer was an increase in V/V_0. The higher temperature and ionic strength system is designed for the examination of gelatins and their degradation products where aggregation effects must be eliminated.

It should be noted that even if the thyroglobulin was not completely excluded by the Sephadex G-200 the accuracy of the method would not be impaired provided all the measurements for other proteins were made relative to its elution volume for any given column.

ACKNOWLEDGEMENT

This paper is published by permission of the Director of Research and Council of The Gelatine and Glue Research Association.

SUMMARY

The present paper describes the use of a cross-linked dextran-gel, Sephadex G-200, for the determination of protein molecular weights of up to 225,000. Evidence is provided which indicates that measurements beyond this are possible. A second system is described, employing a column temperature of 40° and a buffer of high ionic strength which is for use with proteins, such as gelatin, where aggregation effects must be eliminated.

REFERENCES

1 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
2 T. WIELAND, P. DUESBERG AND H. DETERMANN, *Biochem. Z.*, 337 (1963) 303.
3 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.

4 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
5 A. S. MCFARLANE, *Biochem. J.*, 29 (1935) 660.
6 J. L. ONCLEY, G. SCATCHARD AND A. BROWN, *J. Phys. Colloid Chem.*, 51 (1947) 184.
7 M. HEIDELBERGER AND K. O. PEDERSEN, *J. Gen. Physiol.*, 19 (1935) 95.
8 Y. DERRIEN, R. MICHEL, K. O. PEDERSEN AND J. ROCHE, *Biochim. Biophys. Acta*, 3 (1949) 436.
9 J. B. SUMNER, A. L. DOUNCE AND V. L. FRAMPTON, *J. Biol. Chem.*, 136 (1940) 343.
10 K. AGNER, *Biochem. J.*, 32 (1938) 1702.
11 J. B. SUMNER AND N. GRALÉN, *J. Biol. Chem.*, 125 (1938) 33.
12 K. O. PEDERSEN, *Thesis*, Uppsala, 1945, p. 148.
13 H. GUTFREUND, *Trans. Faraday Soc.*, 50 (1954) 628.
14 J. E. HAYES AND S. F. VELICK, *J. Biol. Chem.*, 207 (1954) 225.
15 J. H. R. KÄGI AND B. L. VALLEE, *J. Biol. Chem.*, 235 (1960) 3188.
16 G. SCATCHARD, A. C. BATCHELDER AND A. BROWN, *J. Am. Chem. Soc.*, 68 (1946) 2320.
17 S. M. KLAINER AND G. KEGELES, *Arch. Biochem. Biophys.*, 63 (1956) 247.
18 W. B. DANDLIKER, *J. Am. Chem. Soc.*, 76 (1954) 6036.
19 S. M. KLAINER AND G. KEGELES, *J. Phys. Chem.*, 59 (1955) 952.
20 H. B. BULL, *J. Am. Chem. Soc.*, 67 (1945) 4.
21 A. V. GÜNTELBERG AND K. LINDERSTRØM-LANG, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 27 (1949) 1.
22 H. B. BULL, *J. Biol. Chem.*, 137 (1941) 143.
23 D. S. YASNOFF AND H. B. BULL, *J. Biol. Chem.*, 200 (1953) 619.
24 H. A. DIEU, *Bull. Soc. Chim. Belges*, 65 (1956) 603.
25 J. ST. L. PHILPOT AND I. B. ERIKSSON-QUENSEL, *Nature*, 132 (1933) 932.
26 F. A. BOVEY AND S. S. YANARI, in P. D. BOYER, H. LARDY AND K. MYRBÄCK (Editors), *The Enzymes*, Vol. 4, Academic Press, New York, 1960, p. 63.
27 C. FROMAGEOT AND M. PRIVAT DE GARILHE, *Biochim. Biophys. Acta*, 4 (1950) 509.
28 J. C. LEWIS, N. S. SNELL, D. J. HIRSCHMANN AND H. FRAENKEL-CONRAT, *J. Biol. Chem.*, 186 (1950) 23.
29 M. HALWER, G. C. NUTTING AND B. A. BRICE, *J. Am. Chem. Soc.*, 73 (1951) 2786.
30 H. THEORELL AND Å. ÅKESSON, *J. Am. Chem. Soc.*, 63 (1941) 1804.
31 S. M. ATLAS AND E. FARBER, *J. Biol. Chem.*, 219 (1956) 31.