MOLECULAR WEIGHT DISTRIBUTION ANALYSIS BY GEL CHROMATOGRAPHY ON SEPHADEX

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INTRODUCTION

The original form of gel chromatography, gel filtration[1,2] on hydrophilic gels, has been used for a vast number of separations of macromolecular compounds, particularly of biological origin.

Using standardized conditions, gel filtration has also been successfully applied to the determination of molecular weights of a wide variety of proteins[3–5]. Hydrophilic gels have also been used for the preparative fractionation of water-soluble polymer homologues[6–10].

In this paper the application of gel filtration to molecular weight distribution analysis is described. In the field of organophilic polymers a similar method, called gel permeation chromatography, is well established today[11–15]. Our work was started primarily to facilitate certain studies on membrane permeability, glomerulus filtration, lymph penetration etc. in healthy humans and also pattern changes caused by certain diseases. Such studies can be carried out with a colloidal plasma volume expander as test substance, provided that it is possible to determine the molecular weight distribution of the penetrated or excreted material. A method of distribution analysis was desired for dealing with very dilute samples of body fluids and small amounts of substance. Preparative fractionation was thus out of the question.

WALLENIUS had earlier developed an advanced technique for turbidimetric titration of different molecular sizes of dextran in order to study their renal clearance after infusion in dog[16] and in humans[17]. Unfortunately, this method is too expensive for general use.

Thus, in view of the wide use of dextran in therapy and in the studies mentioned above, and encouraged by our experiences from the preparative fractionation of dextrans on Sephadex[6,7] the authors began two years ago to examine the possibility of calibrating Sephadex for molecular weight distribution analysis of dextran, preferentially within the $\bar{M}_w$ range 150,000 to 10,000.

Since this method has proved to be rapid and reproducible and well suited for advanced automation and because of its potential application to water-soluble polymers in general, the calibration procedure and some applications are reported below.

EXPERIMENTAL

Column and bed material

The column used had the dimensions 1.4 × 75 cm (PS 58/30 Quickfit tube) and

was provided with a cooling jacket and an applicator cup. Before packing, the volume of the column was calibrated over its entire length using water. The bed height could then be read from a mm-scale fitted on the mantle. On the basis of our experience from the fractionation of dextrans[7] a mixture of two Sephadex types, G-200 (Wr = 20.1 g/g) and G-100 (Wr = 9.8 g/g) was used. A dry weight ratio of 1 to 2 between G-200 and G-100 was chosen, so that both occupied approximately equal volumes in the swollen state. The mixture was allowed to swell in excess water several days before packing. This was carried out by pouring the homogeneous suspension through an extension tube into the water filled column. The water circulating in the jacket was thermostated at 20° ± 0.1°. To ensure complete equilibration, eluant was percolated through the bed some days before determining the void volume (V_0). The absence of any carbohydrate material in the eluate was checked before the column was considered ready for use.

It soon became evident that distilled water was not a satisfactory eluant. Occasionally the bed height changed suddenly even after a long period of operation. 0.3 % NaCl solution, with chlorobutanol as preservative, was therefore used throughout this work. The flow rate was kept at 6–7 ml/h (3.8–4.5 ml/cm²/h) by means of a constant hydrostatic pressure.

VOID VOLUME (V_0) AND PARTITION COEFFICIENT (K_{av})

Blue dextran of high molecular weight was used for the void volume determination. 0.5 ml of 0.5 % solution was transferred using a syringe provided with a Millipore filter adaptor, into the applicator cup just as the eluant soaked into the bed. The eluate was collected from the moment the last of the coloured solution vanished into the bed. For determination of V_0, the coloured eluate was collected in fractions of 0.3 ± 0.01 g. The extinction of the fractions was read at 650 mμ. The elution volume at the inflection point of the leading edge of the elution pattern was taken to represent the void volume V_0. The determination of V_0 was carried out once a week. The accuracy of the determination of V_0 was ± 0.1 ml (± 0.4 %).

In order to make the calibration independent of the individual experimental conditions and thus more generally applicable, the concept of the volume fraction available for a solute in the gel phase was introduced according to LAURENT AND KILLANDER[18]:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume of the species in question, V_t is the total volume and V_0 the void volume of the gel bed used. All these parameters can be determined accurately and the resulting K_{av} expresses the penetrability of a certain molecular size into a gel of defined porosity.

CORRELATION OF $\bar{M}_w$ AND K_{av} FOR DEXTRAN ON SEPHADEX

Several authors[5,18,19] have found that the elution behaviour of macromolecules primarily depends on the Stokes radius (or diffusion coefficient) of the species. From

this relationship other parameters, like molecular weight, can be derived assuming the necessary data are known. As the gel filtration (GF) method in this case was intended for routine analysis of molecular weight distributions of one particular polymer, a direct calibration of K_{av} *vs.* molecular weight was desirable.

Fractions of dextran, obtained by extraction fractionation[20] or preparative gel filtration, were used for the calibration. They were not sharp enough to be considered as monomolecular samples. Consequently the heterogeneity of the fractions had to be taken into account. The tentative procedure was as follows.

TABLE I

PHYSICAL CHEMICAL DATA FOR THE DEXTRAN FRACTIONS USED IN THE CALIBRATION OF THE GF DISTRIBUTION ANALYSIS

Fraction No.	$\bar{M}_w$	$\bar{M}_n$	K_{av}	$D_{20,w} \times 10^7$[a] *(cm²/sec)*	*Stokes radius (r) (Å)*
1	147,000	91,000	0.04	2.6	82
2	130,000	83,000	0.043	2.77	78
3	96,000	66,000	0.064	3.2	67.5
4	76,000	53,000	0.085	3.58	60.5
5	58,000	46,000	0.110	4.10	53.0
6	48,300	38,500	0.148	4.46	48.5
7	41,700	33,500	0.173	4.77	
8	36,000	26,500	0.215	5.10	42.5
9	32,400	26,500	0.227	5.38	
10	27,800	21,800	0.266	5.8	37.7
11	22,400	18,500	0.316	6.4	
12	19,300	16,000	0.380	6.9	31.8
13	13,200	9,500	0.476		(26.5)
14	10,000	7,100	0.556	9.4	23.3
15	7,500	5,100	0.620		
16	6,100	4,160	0.671		
17	5,400	4,070	0.719		

[a] Extrapolated from values measured for a series of dextran fractions[21]. Stokes radius follows from the equation:

$$r = \frac{RT}{6\pi\eta D N}$$

where R = gas constant, T = absolute temperature, η = viscosity of the solvent, D = diffusion coefficient and N = Avogadro's number.

Seventeen well defined dextran fractions* within the $\bar{M}_w$ range 150,000 to 5,000 were chromatographed on the Sephadex gel mixture selected for this work. The data for these fractions are given in Table I. The weight average molecular weights, $\bar{M}_w$, were determined by the light scattering method with a Sofica photogoniodiffusometer using essentially the same technique as described previously[21]. The number average molecular weights, $\bar{M}_n$, were determined by the end group analysis using the Somogyi copper phosphate method[22]. Using these data, the theoretical molecular weight distributions of the fractions were then computed by applying the Lansing-Kraemer logarithmic number distribution function according to WILLIAMS AND SAUNDERS[23].

* The parent material was partially hydrolyzed and purified samples of dextran, synthesized by *Leuconostoc mesenteroides*, strain B 512.

The measured $\bar{M}_w$ value of each fraction was then noted on the differential plot of the corresponding Lansing-Kraemer distribution and the two fractional areas under the curve were measured planimetrically (Fig. 1). (Alternatively, the Lansing-Kraemer distribution may be presented as an integral curve and the position of the experimental $\bar{M}_w$ value read off on the Y-axis.)

The procedure adopted for these fractions and for all subsequent samples was as follows. Two milligrams of dextran dissolved in 2 ml water were applied as described for the void volume determination and eluted with 0.3 % NaCl solution. After a certain volume ($\lesssim V_0$), fractions of about 1.5 ml were taken and weighed to $\pm$ 0.01 g each. The dextran concentrations were determined with anthrone[24]. (Later these analyses were automated using the Technicon Auto Analyzer. The above conditions were so chosen that the dextran in the fractions could be determined without further dilution.)

Finally the elution curve, *i.e.* the dextran concentrations (γ/ml) *vs.* K_{av}, was drawn as well as the corresponding cumulative diagram (Fig. 1). The Y-axis position of the experimental $\bar{M}_w$ value according to the determination above was inserted in the cumulative elution diagram and serves as one point on the calibration curve for $\bar{M}_w$ *vs.* K_{av} for dextran on the gel in question.

The cumulative elution profiles of the dextran fractions used in calibration are

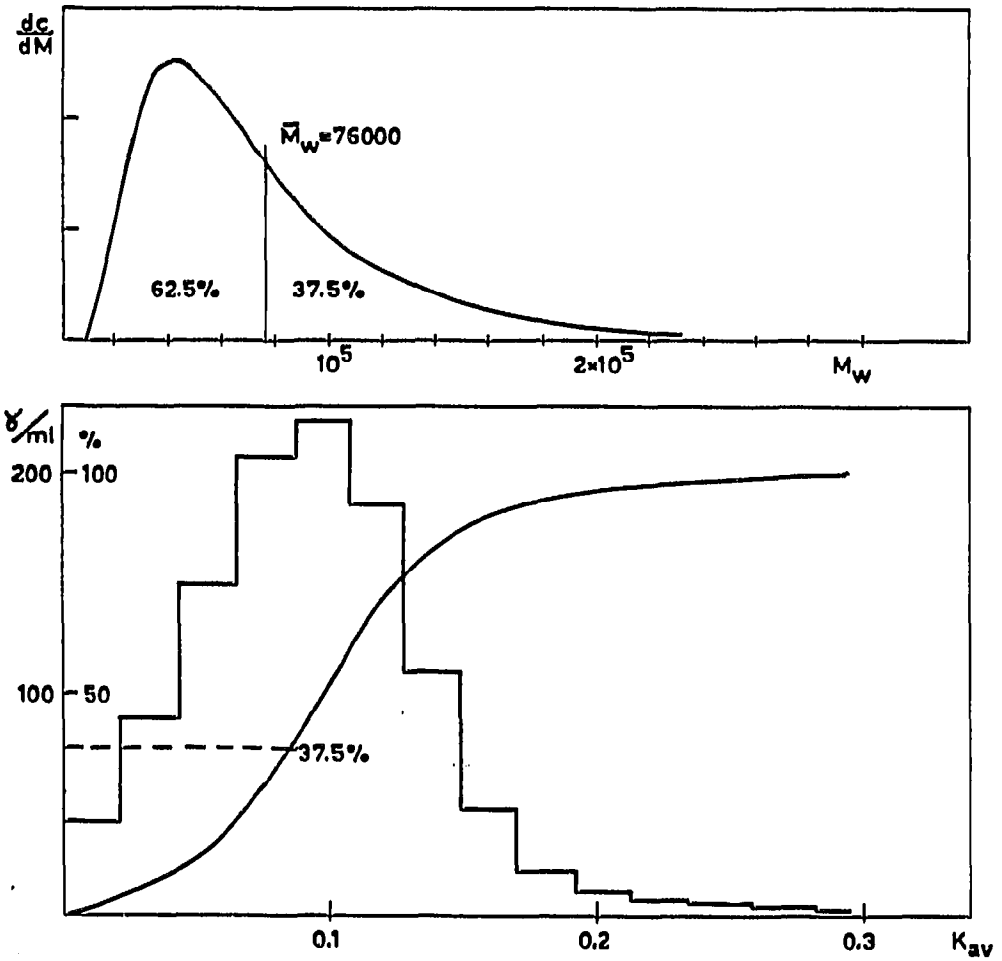

Fig. 1. Lansing-Kraemer distribution and elution diagram of dextran fraction No. 4 (Table I).

shown in Fig. 2. No significative displacement of the curve was observed when the sample charged was 20 mg/2 ml, 2 mg/2 ml or 0.5 mg/2 ml, respectively.

The correlation between log $\overline{M}_w$ and K_{av} for dextran within the range of $\overline{M}_w \sim$ 150,000 to 5,000 is plotted in Fig. 3. The correlation is linear only within a very limited range, thus diverging from the observations of several authors when chromatographing globular proteins on Sephadex[25–27]. This discrepancy very likely depends on a non-Gaussian pore distribution of the mixed gel used. For the same reason the results obtained cannot be treated according to the theories proposed by PORATH[27] and LAURENT AND KILLANDER[18]. The correlation between corresponding Stokes radii and K_{av} was deduced using the diffusion coefficients in Table I.

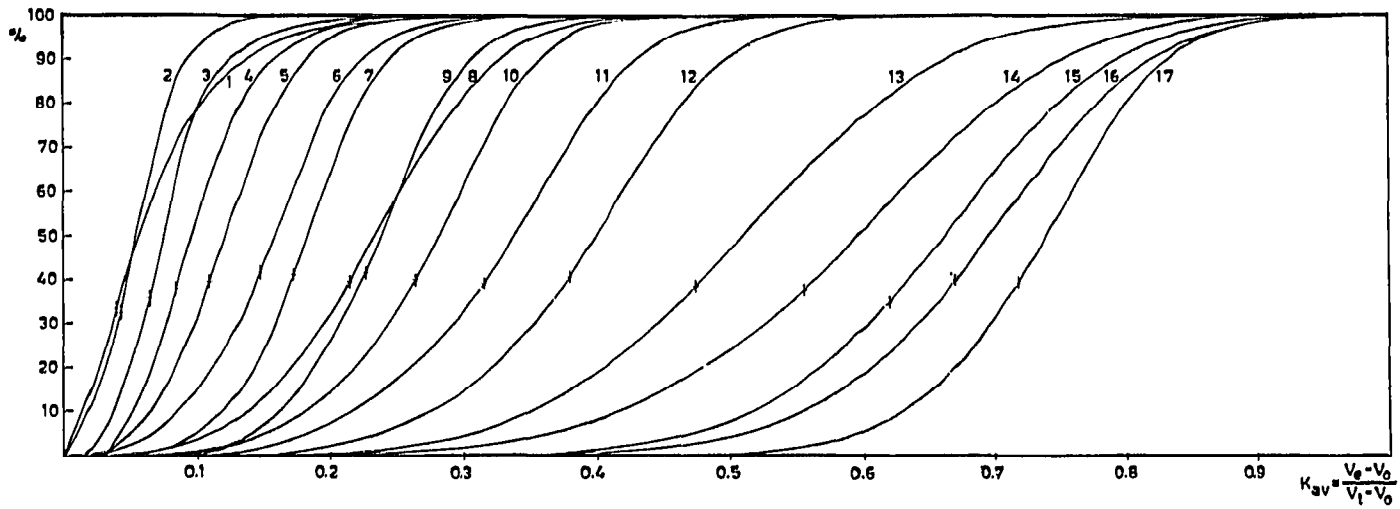

Fig. 2. Cumulative elution curves of the dextran fractions in Table I.

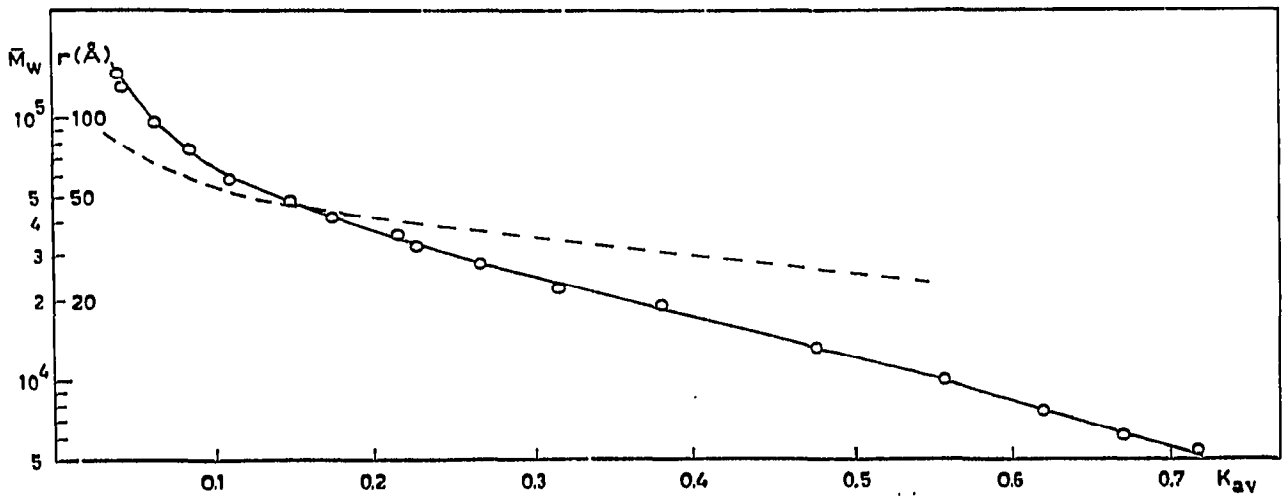

Fig. 3. The correlation curve log $\overline{M}_w$ *vs.* K_{av} for dextran on Sephadex G-200 + G-100. The dotted curve represents Stokes radius *vs.* K_{av}.

DETERMINATION OF MOLECULAR WEIGHT DISTRIBUTIONS

Having calibrated the gel for a specific polymer, the determination of molecular weight distributions of unknown samples of the same polymer is simple and rapid. The experimental conditions are the same as described for the calibration runs. The elutions are usually carried out during the night and the fractions analyzed the following day. The calibration curve is then used to convert the K_{av} values of the cumulative elution curve into molecular weights. Although this can readily be performed manually, a computer program has been worked out, affording both a tabulated and graphical presentation of the cumulative and differential molecular weight distributions and also the molecular weight averages of interest*.

RESOLUTION

Several authors have studied the gel chromatographic process by applying the general plate height equation. The contribution of longitudinal diffusion to the broadening of the zone was found to be negligible even in rather long columns and with slow flow rates[28,29]. The fact that monomolecular species and very narrow fractions of polymers are eluted as Gaussian curves is primarily due to the non-perfect resolution power of the chromatographic system[30]. In order to check the influence of the broadening phenomenon in our system when dealing with evaluation of polydisperse distributions the following experiments were carried out.

A series of six fractions obtained by preparative gel filtration of a dextran on Sephadex G-200[10] were run separately, charging 2 mg of each fraction, on calibrated columns. The computed $\bar{M}_w$ values were within the range of 63,000 to 15,000. The elution diagrams, with the areas scaled in proportion to the size of each fraction, are presented in Fig. 4a. The distribution of the parent sample was then reconstructed by summing the individual distributions in Fig. 4a. In another experiment, appropriate amounts of each constituent fraction were dissolved to give a total sample of 2 mg/2 ml which was subsequently charged on the gel. Both the calculated and the experimental elution curve are drawn in Fig. 4b. The agreement is good indicating that the broadening in the elution profiles of the subfractions is compensated in the elution of the heterogeneous sample.

The resolving power of the chromatographic system used here was further tested by rechromatography of one of the eluted fractions from a sample of considerable heterogeneity. The experimentally determined values for the parent polymer were $\bar{M}_w = 35{,}000$ and $\bar{M}_n = 22{,}000$ ($\bar{M}_w/\bar{M}_n = 1.6$). The sample applied on the column contained 20 mg/2 ml and it emerged within an eluant volume of 65 ml. A fraction near the peak was selected for rechromatography (1.25 mg/2.04 ml) representing a cut-out between 33,000 and 37,000 on the calculated distribution. The rechromatographed distribution had a nearly symmetrical shape (Fig. 5) and gave $\bar{M}_w = 36{,}500$, $\bar{M}_n = 34{,}500$ ($\bar{M}_w/\bar{M}_n = 1.057$).

The overlap of the adjacent molecular sizes, shown in this experiment, apparently is due to the limited resolution of the column. For a general analysis of the elution diagrams the calibration should be based on both the molecular sizes and the resolution[31].

* The program for dextran was coded by Dr. K. VOGEL in Fortran IV for CD 3600 Computer.

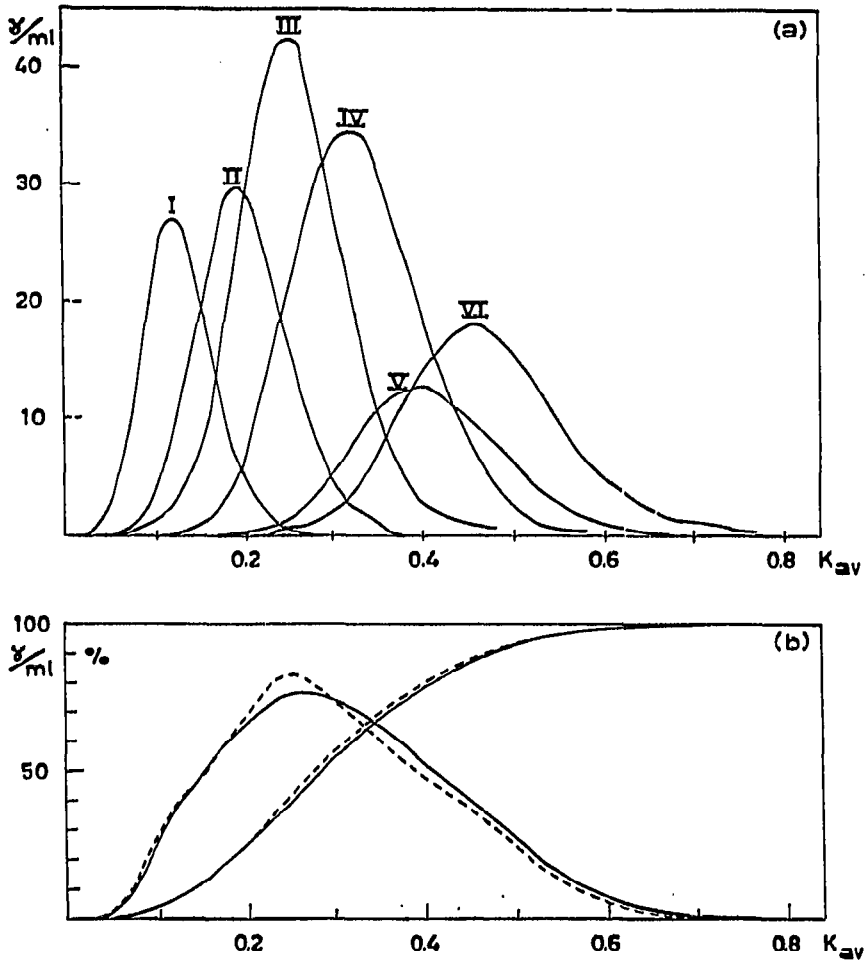

Fig. 4. (a) Elution curves for the constituent fractions of a dextran ($\overline{M}_w \sim 32{,}000$, $\overline{M}_n \sim 24{,}000$) (b) Continuous line: Elution curve after remixing the fractions in Fig. 4a ($\overline{M}_w = 29{,}700$, $\overline{M}_n = 23{,}400$). Dotted line: The calculated summed curve of the elution profiles of the fractions in Fig. 4a ($\overline{M}_w = 30{,}200$, $\overline{M}_n = 24{,}000$).

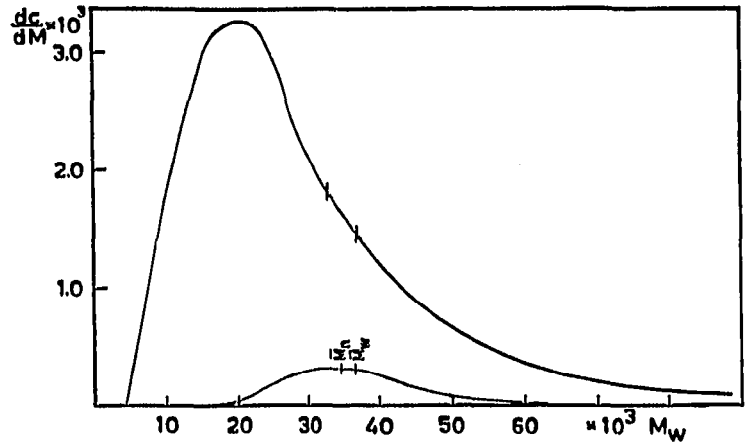

Fig. 5. Molecular weight distribution obtained by rechromatography of a cut-out from an elution pattern of dextran.

During the preparation of this manuscript, a study of the gel permeation chromatographic (GPC) process, partly by experiments similar to those described above, was published by ADAMS, FARHAT AND JOHNSON[14]. Their comparison between calculated molecular weights derived from various fractionation methods and the experimentally determined values showed that fractional precipitation analysis resulted in too narrow molecular weight distributions and consequently in too low $\bar{M}_w/\bar{M}_n$ ratios for the sample, no matter whether the original distributions were narrow or broad. GPC analysis again had a tendency to result in broader distributions than was expected from the experimental molecular weights. An improved resolution was obtained by decreasing the flow rate, however.

Of the very large number of runs in which the molecular weights calculated from the gel filtration analysis above have been compared with measured values, the heterogeneity of the GF distributions is regularly somewhat less than indicated by the experimental values of molecular weights. The difference is due primarily to the higher number average molecular weights (see Table II). This discrepancy, illustrated also by a slight shift of the calibration curve when $\bar{M}_n$ values are correlated with the corresponding K_{av} values, very likely depends on a non-perfect presentation of the true distributions by the Lansing-Kraemer equation.

TABLE II

MOLECULAR WEIGHTS OF DEXTRANS MEASURED AND CALCULATED FROM GF ANALYSIS RESPECTIVELY

Measured values			*Calculated values**		
$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$
110,000	80,000	1.38	108,000	82,000	1.32
72,000	40,500	1.78	70,400	44,900	1.57
38,400	25,900	1.48	37,700	27,800	1.36
20,800	14,500	1.43	19,800	15,600	1.27
11,200	6,700	1.67	11,100	7,400	1.50

* Calculated from the GF distribution diagrams according to the equations

$$\bar{M}_w = \Sigma w_i M_i \text{ and } \bar{M}_n = \frac{1}{\Sigma \frac{w_i}{M_i}}$$

APPLICATIONS

The molecular weight distribution analysis described above has been for the past two years in regular use in our laboratories. It has been used in a great number of investigations pertaining to clinical dextran. Most of this work will be reported in the medical literature[32]. A short review of a few experiments will be given below, however, to illustrate the applicability of this new method.

Fig. 6 shows the shift in the molecular weight distribution of dextran in urine, collected at specified time intervals after the intravenous infusion of 500 ml dextran (Rheomacrodex®, 10%), in a normovolemic healthy person. The dextrans isolated from the urine samples were subjected to molecular weight determination by light scattering and end group analysis. Separately 2 mg of each dextran were applied on a

column and the molecular weight distribution determined (see Fig. 6 and Table III). These figures are complementary to the distribution analyses for dextran in serum performed by ARTURSON AND WALLENIUS[17] using the turbidimetric titration technique. In accordance with these previous studies, the human renal threshold for dextran molecules was found to be about 55,000 ($\overline{M}_w$).

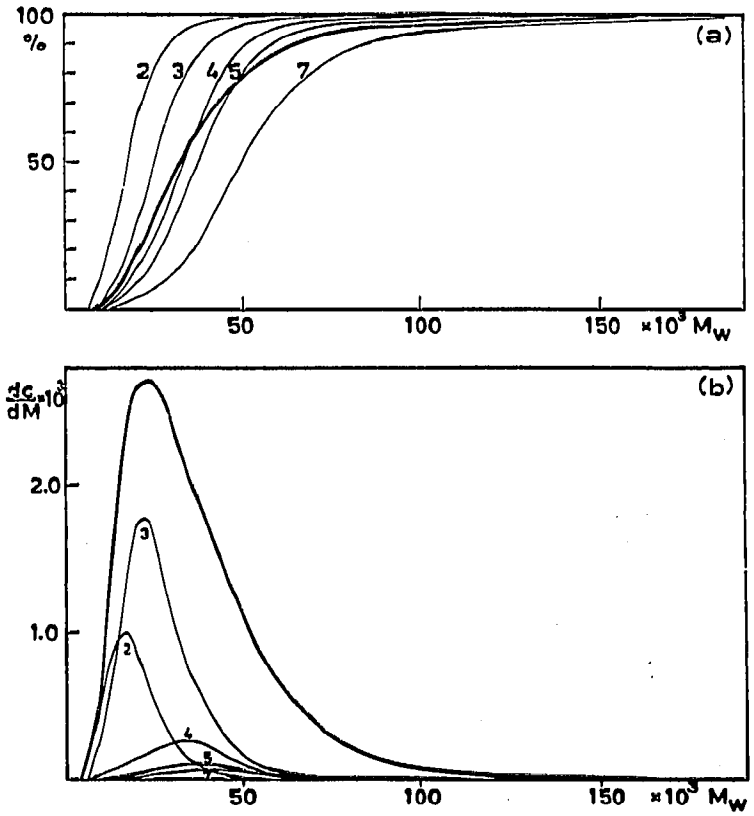

Fig. 6. (a) Integral curves for the molecular weight distributions of dextran in human urine collected at specified times after infusion of 500 ml Rheomacrodex (see Table III). The thick curve shows the distribution of the Rheomacrodex infused. (b) The corresponding frequency curves. The thick curve represents the amount and distribution of the dextran infused. The areas of the other dextran curves are proportional to the amounts excreted in the urine.

Hours after infusion	*Curve*	*Per cent of the amount infused*
0–1	2	18
1–4	3	38
4–8	4	8
8–12	5	3.4
—	6	3.7
24–48	7	3.0

The distribution analysis of dextran in serum is complicated by the high blank readings caused by proteins. Elimination of proteins by precipitation with zinc sulphate has proved suitable. The serum glucose appears as a separate peak at $K_{av} = 1$ and does not interfere with the evaluation of the molecular weight distribution of dextran in the sample. Occasionally the urine samples, mostly from small animals

TABLE III

MOLECULAR WEIGHT DATA ON EXCRETED DEXTRAN IN URINE COLLECTED AT INTERVALS AFTER INFUSION

Hours after infusion	GF analysis					
	Calculated values			Measured values		
	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$
0–1	19,300	16,300	1.18	20,000	16,400	1.22
1–4	26,700	23,300	1.15	27,000	20,500	1.32
4–8	35,100	31,300	1.12	—	26,200	—
8–12	39,000	33,400	1.17	42,000	31,300	1.34
12–24	—	—	—	49,000	31,800	1.54
24–28	55,900	45,600	1.22	56,000	—	—
Rheomacrodex® infused	37,200	27,000	1.38	38,000	24,000	1.58

such as rabbits and mice, cause difficulties and isolation of dextran prior to gel chromatography is recommended. A detailed study of the treatment of biological fluids for GF analysis is proceeding in our laboratories and will be published elsewhere.

An interesting application of the GF analysis was checking the permeability of a Visking membrane used in a dialysis study of different plasma volume expanders. The molecular weight distribution of the dextran in the dialysate is shown in Fig. 7. According to this, only 7.5 % of the permeating material had a molecular weight over 20,000.

Molecular weight distribution of inulin. Preparative fractionations of inulin have been performed previously on Sephadex[7]. These fractions were used for the calibration of the gel filtration method for the distribution analysis of inulin. Average molecular weights for the fractions were determined by light scattering and vapor pressure osmometry. Fig. 8a shows the elution diagram for the inulin preparation studied (Gurr Ltd., London). Two of the fractions are included for comparison. Fig. 8b

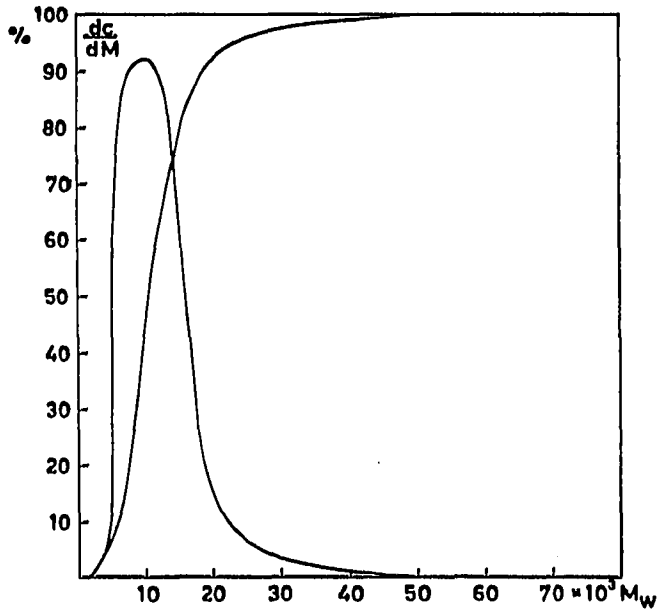

Fig. 7. Molecular weight distribution of dextran dialyzed through the Visking tube 28/32.

presents the resulting molecular weight distribution ($\bar{M}_w$ calc. = 7,900 and $\bar{M}_n$ = 7,000). The corresponding measured values were $\bar{M}_w$ = 7,000 and $\bar{M}_n$ = 6,300. The calibrated correlation, log $\bar{M}_w$ *vs.* K_{av} implies that the inulin molecule has a considerably more compact structure than dextran. This may be expected when considering the 1 → 2 fructosidic linkages of the inulin[33] chain.

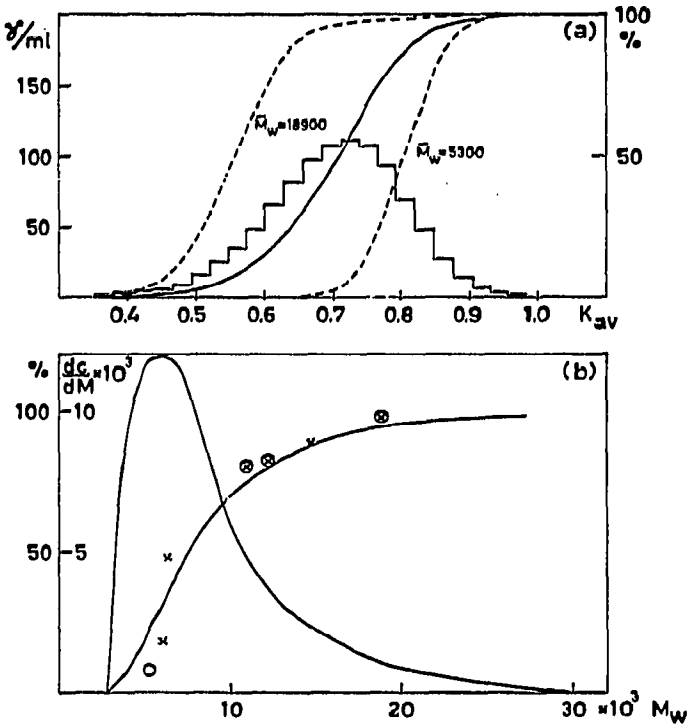

Fig. 8. (a) Elution diagram for inulin. The dotted lines show the cumulative curves for fractions $\bar{M}_w$ = 18,900 and $\bar{M}_w$ = 5,300, respectively. (b) Molecular weight distribution of inulin. Lines are constructed to show the integral and frequency curves, calculated from the elution diagram above. The dots and crosses represent fractions from a preparative gel filtration of the same batch inulin[7].

Fig. 9 presents the elution diagram of a sample of the polysaccharide pullulan*. The native glucan was synthesized by *Pullularia pullulans* with sucrose as the carbon source[34]. The elution pattern is symmetrical and gives, when converted to molecular weights using the dextran calibration curve, a distribution with $\bar{M}_w$ = 14,000 and $\bar{M}_n$ = 10,300. The measured values obtained earlier in our laboratory were $\bar{M}_w$ = 18,000 (dn/dc = 0.152 as for dextran was used in the calculation) and $\bar{M}_n$ = 10,000. These results would suggest a similar molecular shape in solution for the polyglucoses dextran and pullulan.

The gel filtration method has recently been applied in our laboratory to obtain molecular weight distributions of the degraded gelatine and polyvinylpyrrolidone preparations marketed as plasma volume expanders. Details of these experiments will be reported elsewhere.

* This sample was kindly supplied by Dr. H. BOUVENG, Svenska Träforskningsinstitutet, Stockholm.

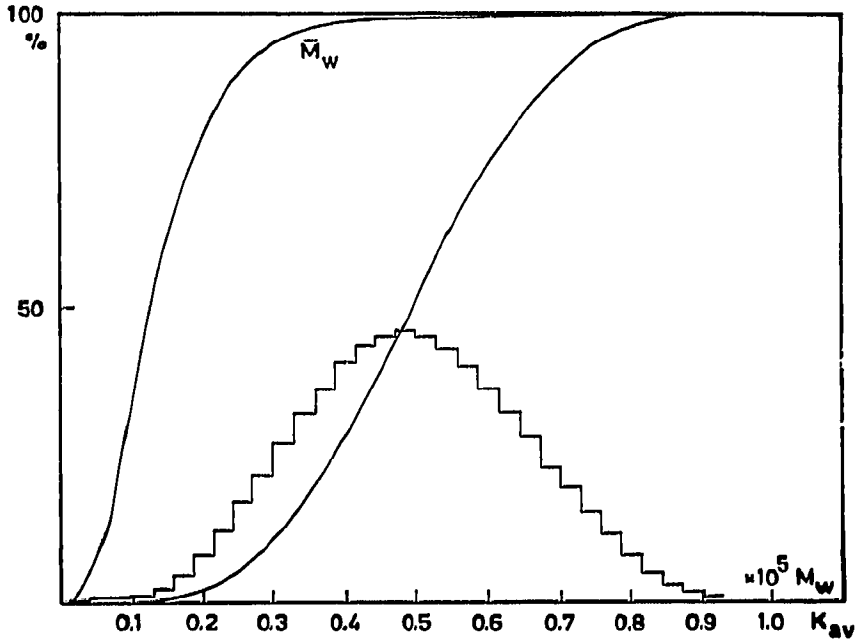

Fig. 9. Elution diagram and corresponding molecular weight distribution of pullulan.

DISCUSSION

The gel chromatography technique described above provides a new tool for the routine analysis of molecular weight distributions of water-soluble polymers within a fairly broad molecular weight range. The method is essentially equivalent to the gel permeation chromatographic procedure recently developed for polymers in organic solvents.

For routine analyses of a given polymer an initial calibration of the elution properties of a series of fractions from the same polymer is required. Once the polymer/gel system in question has been calibrated, only the void volume of the bed has subsequently to be determined, preferably however, at frequent intervals. An improved check of the total volume by adding KCl into the samples has recently proved feasible. The determination of V_e ($= V_t$) for KCl is carried out by flame photometry. A continuous method, for example precision refractometry or recording spectrophotometry, is recommended for the determination of concentration in the fractions, whereby the collecting of individual fractions is eliminated. The carbohydrates may be assayed by an Auto Analyzer which operates perfectly with the anthrone method and handles about forty samples per hour. The use of a peristaltic pump for attaining a constant flow rate reduces the need of weighing each fraction apart from a few sarnple tests. The necessary input data, *i.e.* the gel bed parameters, the polymer concentrations and amounts per fraction volume are then recorded together with the corresponding elution volumes and the derived K_{av} values respectively. A computer program using the equation for the calibration curve converts the input information to integral and differential distributions. Molecular weight averages and the printing of the diagrams are easily included in the program.

With polymers for which the calibration $\bar{M}_w$ *vs.* K_{av} is not available a distribution in terms of molecular dimensions may be derived from the gel chromatography

data. Provided the coefficients of sedimentation or diffusion are known, the Stokes radii can then be used to calculate molecular weights and frictional ratios[5,10].

The molecular weight distribution analysis using the present types of Sephadex as the gel material may be applied to molecular sizes below 100 Å (Stokes radius), corresponding to dextran $\bar{M}_w < 200{,}000$. On agarose gels, now available in the bead form (Sepharose®, Pharmacia Fine Chemicals), a considerably greater range of molecular sizes can be resolved. The procedure for the molecular weight distribution analysis on Sepharose is at present being studied and will be presented separately.

SUMMARY

A column chromatographic method for the molecular weight distribution analysis of water-soluble polymers has been developed with Sephadex as gel material. Very small amounts of polymer are needed and the procedure is well suited to an automated routine analysis. Some applications are illustrated.

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