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THEORETICAL CONSIDERATIONS OF MOLECULAR SIEVE EFFECTS

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SUMMARY

The separation of molecules in neutral gel and controlled-pore glass columns is studied on the basis of a statistical model. The theoretical results are compared with experimental results obtained from Sephadex gel columns. The good agreement between theoretical and experimental results over the whole working range enables complete calibration of columns with just one reference molecule and a knowledge of the void volume, V_0. By means of the derived equation, the molecular weight can be calculated from the elution volume with greater accuracy. The equation also permits the calculation of the elution volume of aggregates of molecules which can assume different sterical conformations with high frictional ratios. An accurate functional correlation between elution volume and molecular weight applicable over the whole working range not only makes analytical steric chromatography less time-consuming but also renders it more amenable to automation. Providing active transport can be neglected, the derived equation may also be valid for filtration across membranes and synapses.

INTRODUCTION

Although the use of gel filtration to estimate the dimensions of macromolecules was suggested by Lathe and Ruthven[1] in 1956, three years elapsed before the work of Porath and Flodin[2] on crossed-linked dextrans and the introduction of Sephadex opened the way to further developments. Since then, gel chromatography, together with sucrose density gradient centrifugation and analytical ultracentrifugation, has become an established technique for the characterization of macromolecular systems.

As in other areas of applied science, progress on the theoretical side has not been as rapid as that on the practical. Many attempts have been made to correlate elution volume with molecular size and shape and there exist a number of equations based on empirically-fitted curves, various geometrical models or statistical assumptions[3–9]. Most of the derived equations are, however, impracticable for normal laboratory purposes and the implied structural features of the gels are arbitrary. One

empirically-based equation[10,11] has nevertheless found wide acceptance; it expresses a logarithmic relationship between the elution volume, V_e, and the molecular weight, M

$$V_e = c_1 - c_2 \log (M) \quad (1)$$

where c_1 and c_2 are empirical constants. Eqn. 1 is used for simplicity, although it cannot be applied accurately over the whole working range of dextran gels (see Fig. 6), an obvious drawback in establishing a valid calibration curve.

This paper presents an equation which describes the functional correlation over the whole range of filtration, and which enables the influence of axial ratios on the elution volume to be calculated. The equation is very similar to an expression derived by Hjertén[12]: neither formula makes any assumptions as to the structure of the gel or the accessible space within the gel, but whereas Hjertén's derivation rests on thermodynamic considerations, the present treatment is based on a statistical model.

MATERIALS AND METHODS

Preparation of gel columns

Columns (100 × 2 cm I.D.) were filled with Sephadex G-25, G-50, G-75, G-100 and G-200 according to the procedure described by the manufacturer and equilibrated with acetate buffer (10 mM CH_3COONa, 60 mM KCl, 10 mM $MgCl_2$; pH 5.5).

Calibration proteins

The calibration of Sephadex gel columns was performed with the following molecules: α-alanine, phenol red, ribonuclease T1, pancreatic ribonuclease, myoglobin, lysozyme, cytochrome *c*, chymotrypsinogen, crotoxin, phosphofructokinase, hexokinase, haemoglobin, ovalbumin, glucose-6-phosphate dehydrogenase, aldolase, pyruvate kinase, transferrin (all from Boehringer, Mannheim, G.F.R.) and dextran blue (Pharmacia, Uppsala, Sweden).

The extinction of the elution solvent was measured at 252 and 280 nm with a 8300 Uvicord II photometer, and the elution volume was determined by collecting 1 ml fractions.

The measurements were repeated at least five times for each reference molecule in order to calculate the respective standard errors.

THEORETICAL

Preliminaries

Electron micrographs of swollen, frozen-etched Sephadex reveal a network of crossed-linked dextrans (Fig. 1). Gel filtration can be described as a diffusional partitioning of solute molecules between the external liquid phase (V_0) around the porous, bead-like particles, and the internal solvent phase (V_i), within the particles themselves. Molecules larger than the largest pores of the swollen Sephadex particles can move only in the volume V_0. Smaller molecules, however, penetrate the particles to an extent dependent on their size and shape. A schematical representation of how

Fig. 1. Electron micrograph of frozen-etched swollen Sephadex G-75.

partitioning may occur is illustrated in Fig. 2: the smaller molecule R_1 penetrates the "gel matrix" by pushing the fibres aside, whereas the larger molecule R_2 is, at first, excluded and has to move horizontally until it, in turn, is able to penetrate the matrix.

The derivation of the function is based on the following assumptions: (1) the fibres in the porous regions of the gel are distributed randomly; (2) the size of the penetrable area, $A(r)$, is determined by the square of the longest axis of the largest molecule that can penetrate; (3) the probability, $w(r)$, of finding an area $A(r)$ at height h is normally distributed; and (4) the volume $(V_e - V_0)$ is proportional to the total area $A_t(r')$ which can be penetrated by a molecule with a radius r', *i.e.*, the distribution of $A(r)$ is the same along the whole length of the column.

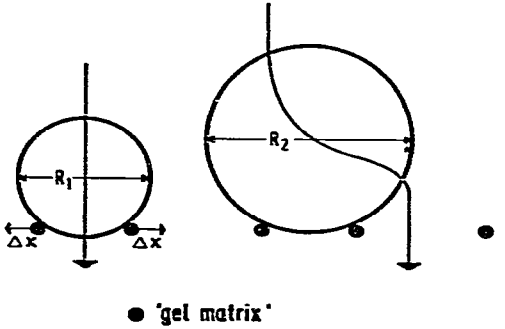

Fig. 2. Schematic representation of a gel filtration process.

An arbitrary Gaussian probability distribution of the penetrable areas $A(r)$ is shown in Fig. 3; the distribution of accessible areas for a molecule with a radius r' is indicated by hatching.

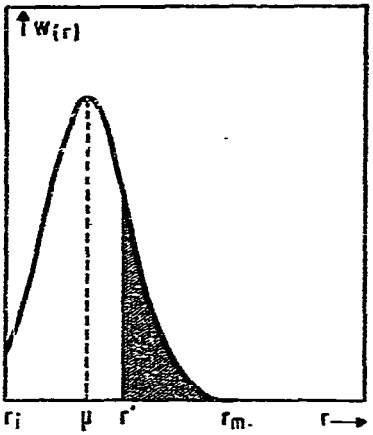

Fig. 3. Arbitrary Gaussian distribution of penetrable areas.

RESULTS

The total penetrable area, $A_t(r')$, within a small vertical distance, Δx, for a molecule with a radius r' is the sum over all areas $A(r)$ with $r' \leqslant r \leqslant r_m$, where r_m is the radius of the largest molecule that can penetrate the gel particles. Thus:

$$A_t(r') = \sum_{r' \leqslant r \leqslant r_m} w(r)\, A(r) \tag{2}$$

Assuming a continuous distribution:

$$A_t(r') = \int_{r' \leqslant r \leqslant r_m} W(r)\, \mathrm{d}A \tag{3}$$

Inserting the Gaussian distribution function, eqn. 3 becomes:

$$A_t(r') = \int_{A(r')}^{A(r_m)} \exp\left[-(r - \langle r \rangle)^2/2\sigma^2\right] \mathrm{d}A \tag{4}$$

Substituting $r^2 = A/\pi$ and $(V_e - V_0) \propto A_t$, we obtain

$$V_e = k_1 \exp\left(-\frac{r^2 - 2\mu r}{2\sigma^2}\right) - k_2 \operatorname{erf}\left(\frac{r - \mu}{\sqrt{2}\sigma}\right) + k_3 \tag{5}$$

where

$$\mu \equiv \langle r \rangle \tag{6}$$

$$k_1 \equiv L\sqrt{2\pi}\sigma \exp\left(-\frac{\mu^2}{2\sigma^2}\right) \tag{7a}$$

$$k_2 = 2L\sqrt{\pi}\mu \tag{7b}$$

$$k_3 = L\sqrt{\pi}\left[2\mu \operatorname{erf}\left(\frac{r_m - \mu}{2\sigma}\right) - \sqrt{2}\sigma \exp\left(-\frac{(r_m - \mu)^2}{2\sigma^2}\right)\right] + V_0 \tag{7c}$$

$$\operatorname{erf}(x) = {}_0\int^x \exp(-t^2)\,dt, \tag{8}$$

and L is a constant.

In accordance with Oncley[13], the molecular weight, M, of globular particles can be expressed as

$$M = \alpha r^3, \quad \text{with} \quad \alpha = \frac{4\pi}{3}\frac{N}{(\bar{v} + \delta\bar{v}_s)} \tag{9}$$

where N is Avogadro's number, $\bar{v}$ is the partial specific volume, δ is the number of grams of solvent per gram of dry macromolecular material and $\bar{v}_s$ is the specific volume of pure solvent.

Eqn. 5 can now be written

$$V_e = k_1 \exp\left(-\frac{M^{2/3} - 2\tilde{\mu}M^{1/3}}{2\tilde{\sigma}^2}\right) - k_2 \operatorname{erf}\left(\frac{M^{1/3} - \tilde{\mu}}{\sqrt{2}}\right) + k_3 \tag{10}$$

where

$$\tilde{\mu} = \alpha^{1/3}\mu \tag{11a}$$

and

$$\tilde{\sigma} = \alpha^{1/3}\sigma \tag{11b}$$

For flexible and fibrous molecules, $M = \beta r^2$ can be used instead of $M = \alpha r^3$ (ref. 14).

The value of V_0 has to be measured directly. A knowledge of the exclusion limits to the gel enable r_m to be derived from eqn. 9. The parameters μ and σ can be treated as calibration constants. It is also possible, however, to estimate σ, subject to a small error probability, from:

$$\sigma \approx (r_m - \mu)/3 \tag{12}$$

Alternatively, μ and σ could be estimated from micrographs of Sephadex particles by making allowances for the extensive hydrophilic envelope surrounding each polysaccharide chain.

As can be seen from Fig. 4, the theoretical curve calculated from eqn. 10 agrees extremely well with the experimental results obtained from Sephadex G-75 gel filtration of globular proteins over the complete range of M from zero to the exclusion limits of the gel—in other words, even for small, totally non-excluded molecules.

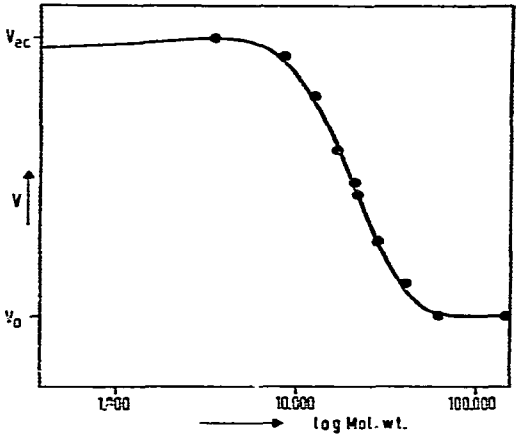

Fig. 4. Relationship between elution volume V_e and logarithm of molecular weight for Sephadex G-75 according to eqn. 10. The empirical data are indicated by closed circles.

An estimate of the sample mean μ of the distribution function for five different types of Sephadex is given in Table I. Calculations with various μ show that the value of μ has a significant influence on the theoretical results only in the range of lower M values. In the working range of the gel, therefore, $\mu = 0$ can be used for all gels. Thus, eqn. 10 reduces to

$$V_e = k_1 \exp\left(-\frac{M^{2/3}}{c}\right) + k_2 \tag{13}$$

where

$$k_1 = L\sqrt{2\pi}\,\sigma \text{ (L is a constant)} \tag{14a}$$

$$c = 2a^{2/3}\sigma^2 \tag{14b}$$

$$k_2 \approx V_0 \tag{14c}$$

TABLE I

SAMPLE MEAN OF THE DISTRIBUTION FUNCTION $w(r)$ FOR FIVE DIFFERENT TYPES OF SEPHADEX

Material	*Molecular range of μ*
G-25	0– 100
G-50	100–1000
G-75	1000–3000
G-100	100–1000
G-200	0

and the standard deviation σ can be estimated by:

$$\sigma \approx r_m/3 \tag{14d}$$

For different values of $c \propto \sigma^2$, the parameters k_1 and k_2 of eqn. 13 were determined for Sephadex G-75, G-100 and G-200 using the method of least squares. With the resultant values of k_1 and k_2, the theoretical elution volume V_e was calculated from eqn. 13. The averaged deviations between the theoretical and the empirical elution volumes, $f_w = [\Sigma(V_{\text{theoretical}} - V_{\text{empirical}})^2]^{1/2}/n$, were then plotted against the respective values of c (Fig. 5) in order to ascertain the magnitude of c which yields the smallest f_w value. The minima, thus determined (see Fig. 5), do not differ greatly from the corresponding values estimated from eqn. 14b: $c_{th}^{75} = 429.6$ ($M = 85{,}000$), $c_{th}^{100} = 882.0$ ($M = 250{,}000$) and $c_{th}^{200} = 2071.5$ ($M = 900{,}000$) (exclusion limits according to ref. 15).

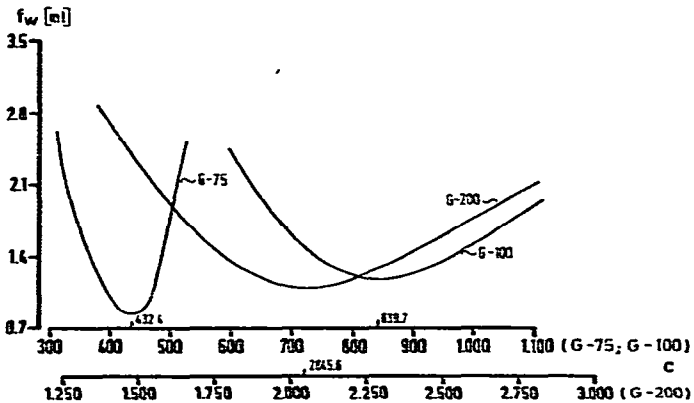

Fig. 5. Empirical approximation of the c calues for different types of Sephadex.

Empirical (V_{em}) and theoretical ($V_{ep}^{(13)}$; V_{st}; V_{1g}) values for the elution volumes of Sephadex G-75 and G-200 are compiled in Table II. In the case of $V_{ep}^{(13)}$, the optimal values for k_1 and k_2 of eqn. 13 were determined by the method of least squares; for V_{st}, k_1 was calculated from eqn. 13 using a single standard molecule ($M = 13{,}500$) and k_2 was evaluated from eqn. 14c. The values of V_{1g} were computed from the generally applied logarithmic relationship between V_e and M (eqn. 1), the parameters c_1 and c_2 being optimized by the method of least squares.

As can be seen from Table II, there is a close similarity between V_{em} and both $V_{ep}^{(13)}$ and V_{st}, but discrepancies exist between V_{em} and V_{1g}. Moreover, the good agreement between $V_{ep}^{(13)}$ and V_{st} demonstrates that a column could be calibrated accurately with only one, or maximally two (should a control be deemed necessary), reference molecules, providing, of course, the void volume had already been established. The conventional calibration procedure could thus be considerably shortened and the efficacy of the column correspondingly enhanced.

The calibration curves computed from a logarithmic and an exponential correlation between V_e and M, according to eqns. 1 and 13, respectively, are

TABLE II

ELUTION VOLUMES OF SEPHADEX G-75 AND G-200

For details see text.

M	V_{em} (ml)	$V_{cp}^{(13)}$ (ml)	V_{st} (ml)	$V_{cp}^{(16)}$ (ml)	V_{tg} (ml)
Sephadex G-75: $V_0 = 67.2$ ml, $V_t = 254.5$ ml					
11,000	225.2	224.4	225.1	222.7	209.4
13,500	199.6	199.0	199.7	199.8	192.9
16,900	171.9	172.4	173.2	173.9	174.9
20,000	149.4	153.7	154.9	154.6	161.3
24,000	133.5	133.1	134.0	132.7	145.0
30,000	118.8	115.0	116.1	113.2	128.6
60,000	74.2	74.4	76.0	73.6	72.8
75,000	67.2	67.9	69.7	69.3	54.8
	$f_w =$	±0.7 ml	±0.9 ml	±1.1 ml	±3.6 ml
Sephadex G-200: $V_0 = 67.0$ ml, $V_t = 259.6$ ml					
25,000	190.0	188.5	190.1	185.1	178.1
37,500	173.2	170.6	173.9	170.9	164.3
52,500	153.0	154.0	158.9	156.3	152.8
58,000	150.0	149.6	154.3	151.5	149.4
67,000	137.0	142.5	147.5	144.3	144.5
123,000	112.5	113.6	118.6	112.4	123.8
158,000	101.0	103.1	107.4	100.4	115.2
237,000	98.6	89.0	91.3	85.7	101.4
1,000,000	67.0	70.8	65.9	75.1	52.3
	$f_w =$	±1.4 ml	±1.8 ml	±2.0 ml	±3.5 ml

illustrated in Fig. 6; the closed circles represent empirical values. In order to emphasize the differences between the curves, a linear scale is used for M instead of the customary logarithmic.

If calibration is conducted with just one or two reference molecules, it is advisable to select the molecules from the M ranges given in Table III. The table also contains a list of the most appropriate empirical c values (see also Fig. 5) for Sephadex G-75, G-100 and G-200. In the given deviation interval i, changes in

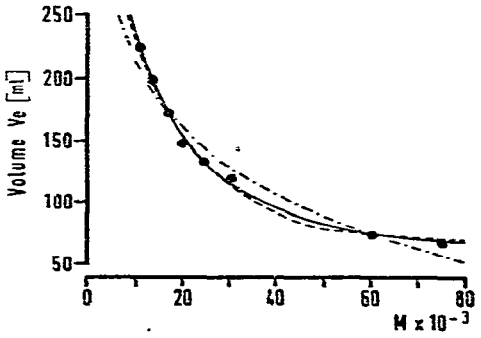

Fig. 6. Comparison of empirical and theoretical data for Sephadex G-75: —·—·—, according to the generally applied eqn. 1; ———, according to eqn. 13; — — —, according to eqn. 16a.

TABLE III

BEST VALUES FOR c AND M RANGE FOR CALIBRATION WITH STANDARD PROTEINS

In the given interval, i, changes in f_w are less than 0.2 ml.

Material	$c \pm i$ *(eqn. 13)*	$c_0 \pm i$ *(eqn. 16)*	*M range*
G-75	432 ± 15	15,700 ± 1300	4000– 50,000
G-100	840 ± 30	29,500 ± 5000	5000–100,000
G-200	2050 ± 60	90,500 ± 20,000	5000–200,000

f_w are less than 0.2 ml (indicated by the dashed lines in Fig. 7) and the parameter k_1 derived from eqn. 13 is more or less constant. The larger the mean size of the dextran pores, the smaller is the range over which k_1 can be varied without significantly affecting the f_w value; on the other hand, the more accurately can k_1 be derived.

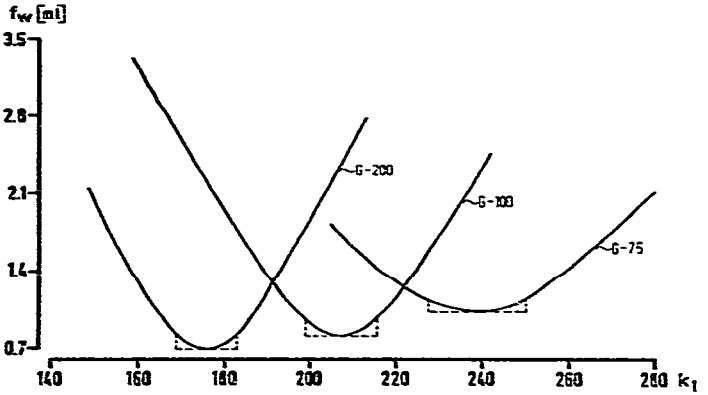

Fig. 7. Variation of the parameter k_1 of eqn. 13 within a given interval, according to Table III, for different types of Sephadex.

DISCUSSION

In the present paper, eqn. 13 is derived which permits a more accurate calibration of molecular sieve columns than that afforded by the conventional logarithmic relationship between V_e and M (eqn. 1). An optimal calibration can be achieved by employing the method of least squares to determine the unknown parameters; alternatively, the latter can be ascertained by adopting the less time-consuming, but slightly less exact, procedure of measuring the V_0 and V_e values of at least one reference molecule (the M value of which should lie within the range given in Table III).

The derived equation might also provide a better understanding of the theoretical implications underlying the processes of filtration across membranes and synapses, always assuming, of course, that active transport can be neglected. The parameter c is a constant and well-defined characteristic of the filtration medium and

the solute, the precision of its determination being such, for example, that small productivity changes in 1965, designed to improve the quality of Sephadex G-25, G-50 and G-75, were readily detectable. The next step would obviously seem to be the compilation of a table of c values covering all the different filtration media and isochemical substances: the c values could be calculated from a consideration of the exclusion limits of the molecular sieve material and the partial specific volume, $\bar{v}$, of the molecules, although a more accurate procedure would be to evaluate c using the method of least squares.

For globular molecules with low frictional ratios, the elution volume and molecular weight can be determined from

$$V_e - V_0 = k \exp(-M^{2/3}/c) \tag{15a}$$

whilst more flexible and elliptical molecules, with $M \propto r^2$ (ref. 14), will obey, according to eqn. 5:

$$V_e - V_0 = K \exp(-M/c_0) \tag{16a}$$

As can be seen from Table II, the elution volumes ($V_{cp}^{(16)}$) calculated from eqn. 16a are very similar to those obtained empirically (see also Fig. 6). The best c values for Sephadex G-75, G-100 and G-200 are listed in Table III.

Eqns. 15a and 16a can also be used to estimate M values directly from logarithmic calibration curves simply by rewriting them in the form

$$-\log(V_e - V_0) = A_1 M^{2/3} + B_1 \tag{15b}$$

and

$$-\log(V_e - V_0) = A_2 M + B_2 \tag{16b}$$

It is interesting to note that eqns. 15b and 16b, derived from a statistical model, are analogous to eqns. 20 and 19, respectively, derived by Hjertén[12] on the basis of thermodynamic considerations. The coefficients A_1 and A_2 in the present equations are essentially dependent on the partial specific volume $\bar{v}$ of the solute and the standard deviation σ of the penetrable areas $A(r)$; σ itself is determined by the distribution of the interfaces between the solute particles and the gel, and the pressure brought to bear on the system. Similarly, the corresponding coefficients C_2 and C_1 in Hjertén's equations are mainly dependent on the partial specific volume $\bar{v}$ of the solute, the interfacial tension, γ, and the pressure, p.

Finally, the molecular weights of rod-like molecules or aggregates of molecules with high frictional ratios can be calculated from the respective elution volumes with greater accuracy than that provided by conventional methods, by a statistical consideration of the orientation of the axes of symmetry of the molecular structures to the gel "network". The theoretical procedure can be simplified by assuming that molecular structures which attempt to penetrate the gel obliquely will orientate themselves either parallel or perpendicular to the "network" depending on the angle of approach. The total penetrable area accessible to such molecular structures can be expressed by

$$A_t(a,b) = \alpha_1 A_t(a) + \alpha_2 A_t(b) \tag{17}$$

where a,b = lengths of the two longest axes of symmetry, and α_t = the probability of axis a or b entering the gel parallel to the "network" ($\Sigma\alpha_t = 1$). The parameter α_t is known from investigations on the rotation of rigid rod-shaped particles in a solution and its treatment is therefore subject to probability theory. The basic equation describing the distribution of particulate orientations was derived by Peterlin and Stuart[16].

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