THE CORRELATION BETWEEN MOLECULAR WEIGHT AND ELUTION BEHAVIOUR IN THE GEL CHROMATOGRAPHY OF PROTEINS

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INTRODUCTION

During their experiments with swollen granules of starch ten years ago, LATHE AND RUTHVEN[1] found a relationship between the molecular weights and the elution volumes of polysaccharides and polypeptides. After the introduction of cross linked dextran gels (Sephadex) by PORATH AND FLODIN[2], interest was focussed mainly on the isolation and purification of macromolecules by the so-called gel filtration (for further information *cf.* ref. 3). Because of the expensive and complicated equipment required for conventional methods of molecular weight determination of macromolecules attempts were soon made to correlate elution behaviour and molecular weight quantitatively (*cf.* Table I).

In order to compare the elution volumes (V_e) of different columns it is expedient to divide them by a column constant, *e.g.* the total volume (V_t) or the void volume (V_0). The K_d value[3], initially introduced, does not seem to be very appropriate, since it affords two additional measurements. This is a drawback to the very fruitful theory of PORATH[7], based on ball-like molecules (radius r) and conical pores. As the K_d value is proportional to the volume (r^3) and the molecular weight is expressed by r^2, there is a linear correlation between the cube root of the K_d value and the square root of the molecular weight. The proportionality of molecular weight and r^2 is only true for the statistical treatment of flexible macromolecules with equal segments and an effective radius r. It is striking, that PORATH's relation was found valid not only for dextran fractions[4,7] and oligostyrenes[34] but also for globular proteins[8,9,17].

Using a similar model but regarding the globular structure of proteins ($M \sim r^3$) and avoiding the K_d value, SQUIRE[38] deduced a relationship between the reduced elution volume (V_e/V_0) and the molecular weight. Data from different authors do not agree very well with his equations.

LAURENT AND KILLANDER[36] designed a simple physical model for the gel network. They assumed that the dextran chains are straight rigid rods (radius r_r), which are infinitely long and distributed at random in the gel, and used OGSTON's[45] equation for the available volume for spherical particles (radius r_s) in such a system. The standard curves derived from that function are not linear.

In view of a comparison between the K_d value and the equilibrium distribution constant, ACKERS[37] claimed that the mechanism of gel chromatography on Sephadex G-200 or agarose was different to that on other gels. This restricted diffusion mechanism for G-200 gel is based on a model in which the fluid regions within the gel

TABLE I

SYSTEMATIC INVESTIGATIONS ABOUT THE CORRELATION BETWEEN MOLECULAR WEIGHT AND ELUTION VOLUME

Gel	*Type of substance*	*Solvent*	*Relation*	*Authors and references*
Sephadex	polysaccharides	buffer	$V_e/V_t \sim \log M$	GRANATH AND FLODIN[4]
Rubber	paraffins	toluene	$V_e/V_0 \sim \log M$	BREWER[5]
Agar	proteins	buffer	$V_e = f(\log M)$	ANDREWS[6]
Sephadex	polysaccharides	buffer	$K_d^{1}/_{3} \sim M^{1}/_{2}$	PORATH[7]
Sephadex	proteins	buffer	$K_d^{1}/_{3} \sim M^{1}/_{2}$	WIELAND *et al.*[8] and AURICCHIO AND BRUNI[9]
Sephadex	proteins	buffer	$V_e/V_0 \sim \log M$	WHITAKER[10] and a further 6 papers[11–16]
Sephadex	proteins	buffer	$V_e \sim \log M$	ANDREWS[17] and a further 15 papers[18–32]
Sephadex	oligothymidylic acid	buffer	$\ln K_d \sim M$	HOHN AND POLLMANN[33]
Polymethyl-methacrylate	oligostyrenes	chloroform	$K_d^{1}/_{3} \sim M^{1}/_{2}$ *	DETERMANN *et al.*[34]
Agarose	proteins	buffer	$V_e \sim \log M$	LARGIER AND POLSON[35]
Sephadex	proteins	buffer	$K_{av} = f(r_s, r_t)$	LAURENT AND KILLANDER[36]
Sephadex	proteins	buffer	$K_d = f(a/r)$	ACKERS[37]
Sephadex	proteins	buffer	$(V_e/V_0)^{1}/_{3} \sim M^{1}/_{3}$	SQUIRE[38]
Styragel	cellulose nitrate / polystyrene / polymethacrylate	tetrahydrofuran	$V_e = f(M^{1}/_{2}[\eta])$	MEYERHOFF[39]
Styragel	polyethylenes, polyethers	trichlorobenzene etc.	$V_e \sim$ log chain length	MOORE AND HENDRICKSON[40]
Sephadex	proteins	buffer	$\log V_e \sim \log M$ **	VAN THOAI *et al.*[41]
Sephadex	peptides	phenol–acetic acid–water	$K_d^{1}/_{3} \sim M^{1}/_{2}$	CARNEGIE[42]
Polyacrylamide	proteins	buffer	$K_d \sim \log M$	SUN AND SEHON[43]
Sephadex	proteins	buffer	$K_d^{1}/_{3} \sim M^{1}/_{3}$ / $K_d = f(r_s, r_t)$ / $K_d = f(a/r)$	SIEGEL AND MONTY[44]

* If $K_d \sim \log M$ is plotted, one also gets perfect straight lines.

** Calibration curve made up from only three results.

particles are represented by uniform cylindrical channels. He relates the STOKE radii (a) of macromolecules to the radius of such pores (r) using the RENKIN equation[46]. His standard curve connects the K_d value with a radius quotient (a/r). It may be that data[47,48] which agree with his theory very well are not very reliable as there is no correlation between the K_d value and the logarithm of the molecular weight, though THOMPSON and coworkers[49] have confirmed the ACKERS's treatment and their data also do not comply with the $K_d \sim \log M$ relationship. This latter proportionality, between elution volume and logarithm of molecular weight of globular proteins, has, however, been confirmed empirically in at least two dozen papers by different authors[6,10–32,35], using Sephadex gels in most cases, after GRANATH AND FLODIN[4] had derived it from their data with polysaccharides. To establish a calibration curve one simply has to measure the elution volume of some well known proteins. We consider it unsatisfactory, that none of the models, designed for the process of gel chromatography up till now, had any connection with this, the most frequently used relationship.*

CALIBRATION CURVES

As the calibration curves are linear over a considerable range they may be depicted by simple equations:

$$\log M = M_0 - \text{constant} \cdot (V_e/V_0); \quad \log M = M_0 - \text{constant} \cdot K_d \tag{1}$$

which are defined by the slope (constant) and by the point of intersection at the M-axis (M_0); we were interested to see if one could establish equations of general application to each type of Sephadex. If there are general equations, a calibration curve would no longer be required. We have therefore collected data from the literature which have been used for calibration purposes by different authors[8–10,12,14,17,36,38,50,51]. This could only be done with precision in cases where elution volumes or K_d values were tabulated and supplemented by column constants. Together with some new data, these values are presented in Table II.

By compensation calculation we determined the constants for the different gels using the method of least squares to obtain the straight lines from which all the data of Table II show a minimum deviation.

G-200: $\log M = 6.698 - 0.987 \cdot (V_e/V_0)$

$\log M = 5.731 - 2.16 \cdot K_d$ (1a)

G-100: $\log M = 5.941 - 0.847 \cdot (V_e/V_0)$

$\log M = 5.070 - 1.35 \cdot K_d$ (1b)

G-75: $\log M = 5.624 - 0.752 \cdot (V_e/V_0)$ (1c)

G-50: $\log M = 5.415 - 0.864 \cdot (V_e/V_0)$ (1d)

* *Note added in proof.* After the manuscript had been sent to the editor, a paper by ANDERSON AND STODDART[58] appeared, which tries to connect the current relationships by a mathematical treatment.

TABLE II

MOLECULAR WEIGHTS (M.W.), STOKE RADII AND ELUTION CONSTANTS OF PROTEINS

No.	Protein	r (mμ)	Mol. wt. × 10^{-3}	G-200			G-100		G-75	
				K_d	V_e/V_0	R_{cyt}[m]	K_d	V_e/V_0	K_d	V_e/V_0
1	Cytochrome c	1.74[a]	13	0.76[b]	2.61[b]	1.00	0.70[b]	2.14[c]	—	1.99[c]
2	Ribonuclease	1.92[g]	13.6	0.76[d]	2.61[d]	1.04	—	—	0.35[h]	—
3	Methaemoglobin	—	17	0.68[d]	2.48[d]	—	—	—	—	—
4	Soybean trypsin-inhibitor	2.26[a]	21.5	—	—	1.20	—	1.93[b]	—	1.65[k]
5	α-Chymotrypsin	2.28[g]	22.5	—	—	1.18	0.54[b]	1.88[b]	0.28[f]	1.66[f]
6	Trypsin	2.41[a]	24	0.62[e]	2.30[e]	0.83	0.54[b]	1.84[b]	0.28[h]	1.71[c]
7	Pepsin	—	35.5	0.59[d]	2.26[d]	1.40	—	1.62[b]	0.25[h]	1.40[c]
8	α-Hydroxysteroid dehydrogenase	—	47	—	—	—	—	1.56[f]	—	—
9	Peroxidase-1	3.02[g]	40	—	—	1.50	—	1.57[b]	—	—
10	Ovalbumin	2.80[a]	45	—	2.08[l]	1.45[l]	0.29[l]	—	0.20[n]	1.35[n]
11	Phosphoglycerate mutase	—	64	—	—	—	—	—	—	1.21[b]
12	Serumalbumin (bovine)	3.61[a]	67	0.43[d]	1.90[d]	1.69	0.16[h]	1.32[b]	—	1.10[c]
13	Malate dehydrogenase	—	79	0.38[e]	1.80[e]	—	—	—	—	—
14	Enolase	—	80	—	—	—	—	1.29[b]	—	—
15	Creatinephosphate kinase	—	81	—	—	1.66	—	1.25[b]	—	1.09[b]
16	Transferrin	4.00[a]	88	0.32[h]	—	—	0.11[h]	—	—	—
17	Glyceraldehydephosphate dehydrogenase	4.30[a]	117	0.31[d]	1.66[d]	1.81	—	—	—	—
18	Serumalbumin (dimer)	—	134	0.26[d]	1.54[d]	—				
19	Aldolase (yeast)	—	147	0.27[d]	1.59[d]	1.89				
20	Alcohol dehydrogenase	4.55[a]	150	—	1.54[l]	2.00				
21	γ-Globulin (human)	5.22[a]	140	0.26[d]	1.61[d]	2.02[l]				
22	Catalase	—	225	—	1.38[l]	2.19				
23	γ-Globulin	—	200	—	1.44[l]	—				

				G-50[n]	
				K_d	V_e/V_0
2	Ribonuclease	1.92[g]	13.6	0.26	1.45
24	Chymotrypsinogen A	—	25	0.12	1.20
25	Limabean trypsin-inhibitor	—	8.4	0.42	1.72
26	Kallikrein-inhibitor	—	6.5	0.49	1.85

Superscripts: (a) *cf.* ref. 37; (b) new data included in this paper, obtained on columns of 300 to 150 ml and 2 cm width. The proteins were applied in 1–2 ml buffer and eluted (10–20 ml/h) with phosphate buffer pH 7.2 ($I = 0.075$) + 0.5 *M* NaCl; (c) *cf.* WHITAKER[10]; (d) *cf.* WIELAND *et al.*[8]; (e) *cf.* AURICCHIO AND BRUNI[9]; (f) *cf.* SQUIRE[38]; (g) *cf.* LAURENT AND KILLANDER[36]; (h) *cf.* DETERMANN AND GELOTTE[50]; (i) calculated from MORRIS[51]; (k) *cf.* ANDREWS[17]; (l) *cf.* LEACH AND O'SHEA[12]; (m) new data included in this paper, obtained by thin-layer chromatography (*cf.* DETERMANN AND MICHEL[52]) on Sephadex G-200 superfine; bed volume 0.05 × 20 × 40 cm, phosphate pH 6.6, $I = 0.2$; (n) *cf.* FRITZ *et al.*[14].

The calculated lines are drawn in Fig. 1 together with the data of Table II, from which they were derived. One sees from Fig. 1 that the data of different laboratories measured during the last three years using various batches of gel fit relatively well to a distinct straight line. Only the values of the careful investigations of ANDREWS[21] do not satisfy eqn. (1a). This is apparently due to the extensive swelling of the Sephadex G-200, used by him. This was recently discussed by SIEGEL AND MONTY[44].

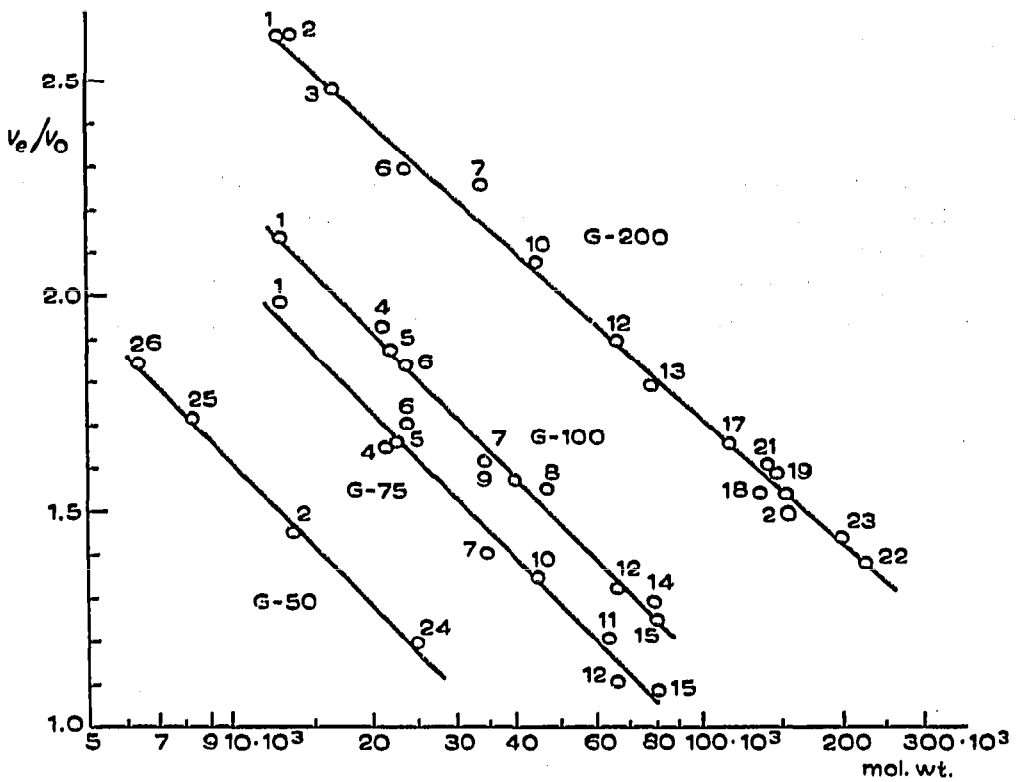

Fig. 1. Elution data of Table II together with calibration curves calculated therefrom. The numbers refer to those given in Table II.

Similar equations, valid only for their own data, have been calculated by WHITAKER[10] and by LEACH AND O'SHEA[12]. Our equations are of course only valid for a distinct range of molecular weights, which is to be found in Fig. 1. Assuming linearity up to the upper limit, one should be able to calculate (by inserting $V_e/V_0 = 1$ in eqn. (1)) the exclusion limits of the different gels:

G-50 = 35,000	G-100 = 125,000
G-75 = 75,000	G-200 = 500,000

These values correlate relatively well with the values from experiments with globular proteins. It is probable that the constants of eqn. (1) will depend on temperature; apparently the differences are relatively small[10,12]. Further systematic investigation is needed in this respect.

During thin-layer chromatography[17,53,54] it was found advantageous to use the linear relationship between the migration referred to a standard protein and the

logarithm of the molecular weight[51,52]. We have made use of cytochrome c as it is an intensively coloured calibration protein, and have tested the behaviour of some of the proteins shown in Table II together with some additional enzymes on thin layers of Sephadex G-200. The result is an improved diagram (Fig. 2) as compared with an older one (*cf.* Fig. 5 in ref. 52). By analogy to eqn. (1) it follows

$$\log M = \log M_0 + \text{constant} \cdot R_{cyt} \qquad (2)$$

and by compensation calculation:

$$\log M = 3.024 + 1.092 \cdot R_{cyt} \qquad (2a)$$

It is not certain if this is an equation of universal validity, as the experiments were only done with one batch of Sephadex G-200, superfine, in our laboratory.

As one compares the elution behaviour of globular proteins on gel columns with their molecular weight, systematically, it is assumed that molecules under investigation are similar in frictional ratio and partial specific volume (*cf. e.g.* refs. 6, 10,

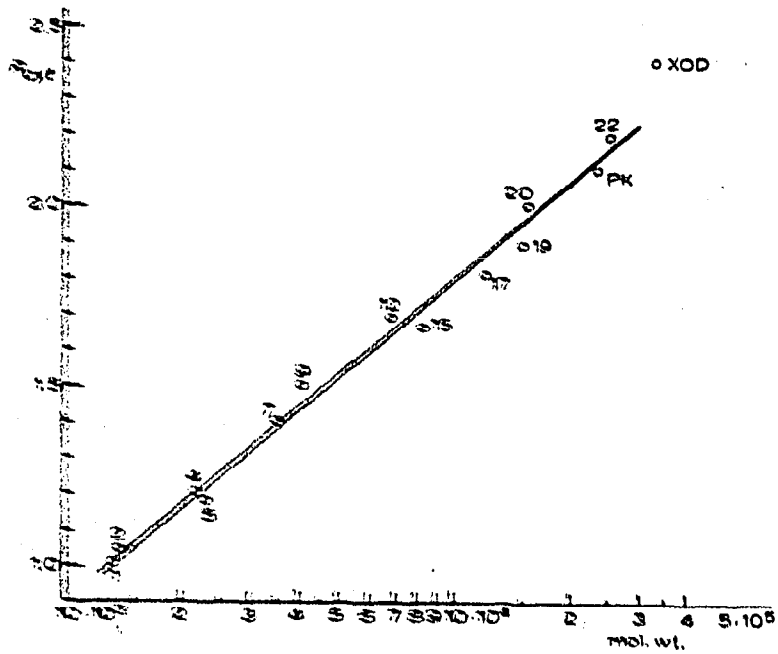

Fig. 2. Standard curve for molecular weight estimation by thin-layer gel chromatography of proteins from Table II and of phosphocreatine kinase (PK, mol.wt. $230 \cdot 10^3$) and of xanthine oxidase (XOD, mol.wt. $300 \cdot 10^3$) with Sephadex G-200, superfine.

27, 28, 37). In three very special cases SIEGEL AND MONTY[44] stated that it is not the molecular weight, but the STOKES radius which governs the elution position. Their results with ferritin, urease and fibrinogen are partially in conflict with ANDREWS' data[38]. Fibrinogen contains a considerable amount of carbohydrate and thus eqn. (1) does not apply[38,39]; also there is no general agreement on the molecular weight of ferritin (iron complex) or urease[38].

Normally one can assume the molecular weight to be proportional to the cube

of the radius, which transforms eqns. (1) and (2) to

$$\log r = r_0 - \text{constant} \cdot (V_e/V_0) \tag{3}$$

$$\log r = r_0 + \text{constant} \cdot R_{cyt} \tag{3a}$$

In Fig. 3 we have compared the STOKE radii from Table II with the elution constants (V_e/V_0 or R_{cyt}). A linear correlation was found for Sephadex G-100 and a fairly good one for G-75. Concerning the proportionality between R_{cyt} and log r on Sephadex G-200, the postulate of eqn. (3a) is perfectly fulfilled.

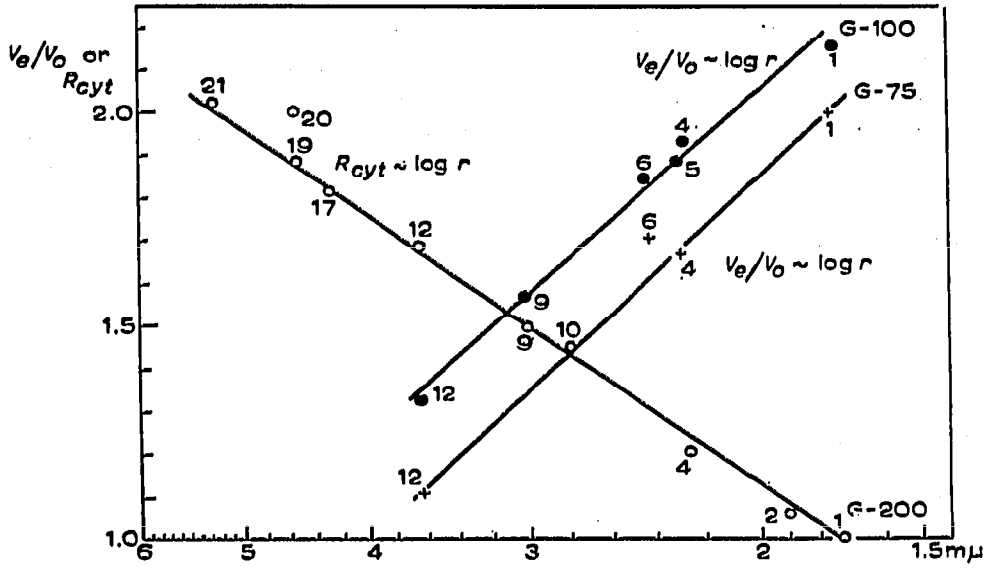

Fig. 3. The correlation between the elution constants (V_e/V_0 or R_{cyt}) and the logarithm of the STOKE radii of the different proteins of Table II.

MATHEMATICAL TREATMENT

As far as the results of more than a dozen publications are concerned, one can assume the validity of eqn. (1) for the behaviour of globular proteins on columns of porous dextran gels. We have now tried to find out, if one can make any general statement about mechanisms for gel chromatography described by this equation. The exponential form of eqn. (1):

$$M = \text{constant} \cdot \text{e} - \text{constant} \cdot (V_e/V_0) \tag{4}$$

was differentiated

$$\frac{\mathrm{d}M}{\mathrm{d}(V_e/V_0)} = -\text{constant} \cdot M$$

and transformed subsequently to give

$$\frac{\mathrm{d}M}{M} = -\text{constant} \cdot \mathrm{d}(V_e/V_0) \tag{5a}$$

From eqn. (3) it follows

$$\frac{dr}{r} = -\text{constant} \cdot d(V_e/V_0) \tag{5b}$$

Eqns. (5) illustrate the fact that within the limits of validity of eqn. (1), the relative change of molecular size is linearly proportional to a change in elution volume. One may now try to substitute the differential change in the elution constant by other quantities, which are connected with the proposed mechanism for the gel chromatography process. To date two different concepts have been published as discussed in the introduction. The kinetic mechanism by ACKERS[37] and the (quasi) equilibrium exclusion mechanism of all other workers (*cf.* refs. 3, 7, 36). In the latter case it is considered that, dependent on the size of the solutes, the separation is effected by differences in the availability of the solvent in the gel phase (V_i). If molecules of a certain size will only enter a fractional part (K_d) of this volume, the elution volume may be replaced by this available volume (V_{av}). One has to use the defining equation of the elution volume[3], $V_e = V_0 + K_d V_i = V_0 + V_{av}$. The elution constant in eqns. (5) may now be substituted by a number of equivalent values:

$$\frac{V_e}{V_0} \sim \frac{V_e}{V_t} \sim K_d = \frac{V_{a_v}}{V_i} \tag{6}$$

For one type of molecular size the available volume of a given gel bed (V_{av}) is assumed to consist of a number (n_i) of single pores which have the volumes A_i:

$$V_{a_v} = \sum A_i = V_t \cdot n \cdot A \tag{7}$$

The average volume of an individual pore (A) is connected to a real pore by $A = \sum n_i A_i / n_i$; V_t is the total volume of the gel bed; n represents the number of individual pores per unit of volume. Combination of eqns. (6) and (7) yields

$$\frac{V_{a_v}}{V_i} = \frac{V_t}{V_i} \cdot n \cdot A = \text{constant} \cdot n \cdot A \cdot \frac{V_e}{V_0} \tag{8}$$

and

$$\frac{dM}{M} = -\text{constant} \cdot n \cdot dA \tag{9}$$

Since n is also constant for a given gel, it may be combined with the former constant to give a new value; in this case it follows that $d(V_e/V_0) \sim dA$. As n is the number of available average pores, combined with V_t/V_i it is a function of the gel density d, which is tabulated for the Sephadex gels in the advertising technical brochures and is easily estimated with the aid of a pycnometer[57]. The most simple connection between (constant $\cdot n$) and d would also be a linear proportionality:

$$\text{constant} \cdot n = \text{constant} - \text{constant} \cdot d \tag{10}$$

assuming an equivalent pore size distribution in all gels. The negative sign represents the decreasing available volume by increasing the density. Eqn. (9) then becomes

$$dM/M = -(\text{constant} - \text{constant} \cdot d) \cdot dA = -(\text{constant} - \text{constant} \cdot d) \cdot d(V_e/V_0)$$

By an analogous integration and logarithmic process one finds:

$$\log M = M_0 - (\text{constant} - \text{constant} \cdot d) \frac{V_e}{V_0} \qquad (11)$$

equivalent to eqn. (1). Eqn. (11), within its empirical limits would be of general validity. Indeed the constants can be calculated from eqns. (1a–1c). Fig. 4 relates the

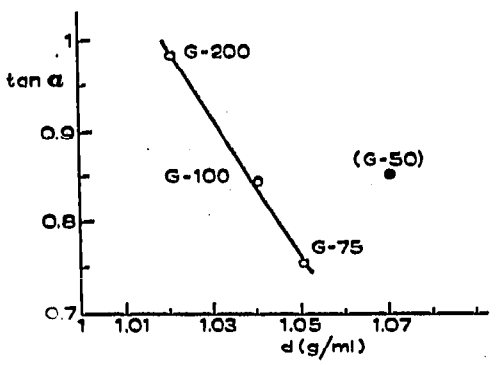

Fig. 4. Relation between the slope of the lines of Fig. 1 and the gel dens ty.

slope of the eqns. (1) with the wet density d of the pertinent gel. The data for Sephadex G-200, G-100 and G-75 lie well on a straight line, whereas the values for the G-50 do not. The reason may be that we used only limited data from one laboratory to establish eqn. (1d). But if this value is authentic, it might be based on a different pore size distribution in the denser gel. The line in Fig. 4 is described by the equation $\tan \alpha = \text{constant} = 6.062 - 5.00 \cdot d$. Insertion in eqn. (1) results in

$$\log M = M_0 - (6.062 - 5.00 \cdot d) \frac{V_e}{V_0} \qquad (11a)$$

valid for the Sephadex gels in the range G-200 to G-75. Eqn. (11a) describes the elution behaviour of globular proteins as a function of the gel density d. The estimation of the molecular weight of an unknown globular protein with a bed of porous gel of known density is now performed very easily, simply by comparing its reduced elution volume (V_e/V_0) with that of one known protein. In view of the fact that there is one equation, describing the elution of macromolecules from the Sephadex gels G-75 to G-200, it is very likely, that the separation in the three gels with different density is governed by the same mechanism. This seems to be a severe objection to the ACKERS concept[37].

The general validity of eqn. (11) which was derived without any geometric model concerning the microstructure in the gel can be taken as a proof of the only basic assumption during its derivation: *i.e.* that only a fraction of the gel phase is available to a molecule of given size.

SUMMARY

Nearly thirty papers in the literature, concerning the chromatographic behaviour of macromolecules on columns of porous gels demonstrate a linear relation

between the elution volume and the logarithm of the molecular weight. The current theoretical treatments of gel chromatography do not explain this relationship.

Data from ten recent papers were collected, recalculated and, together with some new data, it was found that the behaviour of globular proteins on Sephadex gels can be described by the special equations:

$$\text{G-75:} \quad \log M = 5.624 - 0.752 \cdot (V_e/V_0)$$

$$\text{G-100:} \quad \log M = 5.941 - 0.847 \cdot (V_e/V_0)$$

$$\text{G-200:} \quad \log M = 6.698 - 0.987 \cdot (V_e/V_0)$$

A mathematical treatment of the gel chromatography process, based on these empirical equations and avoiding assumptions about the microstructure of the gel, strengthens the concept of the exclusion mechanism. It results in the general equation:

$$\log M = \log M_0 - (6.062 - 5.00 \cdot d) \cdot (V_e/V_0)$$

covering the field of very porous gels (Sephadex G-75 to G-200). From experiments with globular proteins on colums of any gel their molecular weight may be determined, if—besides the void volume (V_0)—the wet density of the gel (d) and the elution pattern of only one test protein is known.

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