A Quantitative Theory for Gel-Exclusion Chromatography

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(Received 20 January 1969)

It is generally recognized that there is an inverse relationship between molecular weight and the volume at which a protein is eluted from a gel-exclusion-chromatography column. Some attempts at placing this relationship on a quantitative basis are reviewed by Determann (1966) and by Joustra (1966).

In this communication a derivation of a relationship based on well-known principles of osmotic pressure is presented.

Theoretical. If we consider a horizontal section of infinitesimal thickness through a separating zone of a protein component in a gel-exclusion column, the osmotic pressure of the protein in the fluid surrounding the gel particles would be:

$$\pi_e = \frac{RTC_e}{M} + RTC_e^2B \quad (1)$$

where R , T and C_e are the gas constant, absolute temperature and the external concentration respectively. B is the second virial coefficient and M the molecular weight. B is related to the mutual exclusion of the protein molecules from a defined space. Further details of the virial coefficient may be obtained in Tanford (1963). The osmotic pressure of the protein fraction that diffused into the gel particles would be:

$$\pi_i = \frac{RTC_i}{M} + RTC_i^2B + RTC_iG \quad (2)$$

where C_i is the concentration of protein inside the gel particles. G in eqn. (2) is a factor related to the interference of the gel filaments with the Brownian motion of the protein molecules that diffused into the gel structure. This factor is of a similar nature to B in eqn. (1). The mutual interference or exclusion is between the fraction of concentration C_i and the filaments of the gel. It therefore is a function of C_iC_g , where C_g is the concentration of the gel-forming material. As it is not possible at the moment to give a theoretical value for this function the factor pertaining to the gel is expressed as G and it is assumed that G contains the energy factor RTC as the other members of the equation do. As shown below eqn. (2) is compatible with the formulations of Edmond, Farquhar, Dunstone &

Ogston (1968, eqns. 3 and 4). As there is no transfer of material when the system is at equilibrium:

$$\pi_e = \pi_i$$

Equating eqns. (1) and (2) and dividing throughout by RTC_i :

$$\frac{C_e}{MC_i} + \frac{C_e^2B}{C_i} = \frac{1}{M} + C_iB + G \quad (3)$$

But $C_i/C_e = K_d$, the distribution coefficient between the gel and the liquid phases.

On rearranging eqn. (3) it follows that:

$$\begin{aligned} G &= \frac{1}{M} \left(\frac{1}{K_d} - 1 \right) + B \left(\frac{C_e^2 - C_i^2}{C_i} \right) \\ &= \frac{1}{M} \left(\frac{1}{K_d} - 1 \right) + B \left(\frac{1}{K_d} - 1 \right) C_i \\ C_t &= C_e + C_i \end{aligned} \quad (4)$$

which indicates that G may be partly dependent on the concentration of the protein in the system. At low concentrations, which generally prevail in separating zones, the factor containing B may be disregarded as a first approximation and eqn. (4) may be written as

$$G = \frac{1}{M} \left(\frac{1}{K_d} - 1 \right) \quad (5)$$

To test the constancy of G a number of gel-exclusion-chromatography experiments were done

Table 1. *Determination of G for 10% agarose pearls (100 mesh)*

The elution medium was 66 mM-phosphate buffer, pH 7.1, containing NaCl (72 mM).

Protein	M (lit.)	K_d	10^5G
Cytochrome c	13000	0.695	3.37
Lactalbumin	16400	0.661	3.13
Chymotrypsinogen	25000	0.548	3.30
Ovalbumin	45000	0.415	3.31
Bovine serum albumin	68000	0.297	3.48
Average			3.28

with proteins of known molecular weights and of compact globular structure. The homogeneity of the proteins was confirmed by ultracentrifugation in the Spinco model E ultracentrifuge. The proteins used and the results obtained are listed in Table 1. K_d was calculated from the equation:

$$K_d = \frac{V_e - V_0}{V_i}$$

where V_e is the volume of fluid necessary to elute a component, V_0 is the void volume and V_i is the volume of liquid in the gel structure. For more details the brochures on Sephadex in gel filtration of Pharmacia, Uppsala, Sweden, may be consulted. Agarose pearls prepared according to the emulsification procedure of Hjertén (1964) were used as the exclusion agent. A 10% gel in pearl form and of 100 mesh was found suitable for the purpose. The proteins used were a generous gift from Seravac, Cape Town, South Africa. They were all freeze-dried, except ovalbumin, which was kept as a solution to prevent it from denaturing.

From Table 1 it is evident that G (3.28×10^{-5}) is essentially constant for 10% agarose pearls, indicating the correctness of eqn. (5).

In a similar test with 11%-chromium-tanned gelatin granules (Polson & Katz, 1968) it was found that this material had G 4.8×10^{-5} when tested with the proteins listed in Table 1. It was necessary to increase the electrolyte content of the elution fluid to between 0.6 and 1 M to obtain true exclusion that was uncomplicated by absorption effects on or in the chromium-tanned gel particles. The K_d value of cytochrome *c* was found to be as high as 3.1 in 0.14 M-NaCl, whereas the value decreased to $K_d = 0.69$ when the NaCl concentration was increased to 1 M.

The number of proteins investigated in this work is admittedly small, but there can be no reasonable doubt that other globular proteins with molecular weights between 13000 and 70000 would produce

the same G value provided that all adsorption effects are eliminated.

Filamentous molecules are known to have smaller K_d values than molecules of similar molecular weight but of globular structure. The present theory of gel exclusion will explain this behaviour, as one would expect more interference between rod-shaped molecules and the gel filaments.

It is noteworthy that eqn. (4) indicates a concentration-dependency of K_d . This offers a possible quantitative explanation for the small increase of K_d with concentration, a phenomenon observed by Winzor & Nichol (1965).

The molecular weights used in the calculation of G were selected from those listed by Determann (1966) and by Andrews (1966). As there is disagreement about some values, notably serum albumin, average values were used. The apparent remarkable constancy in G must therefore be regarded as fortuitous.

We do not propose that eqn. (5) be used for calculating molecular weights, as other methods such as ultracentrifugation are more accurate, but this communication is merely intended to offer an explanation for the mechanisms involved in gel exclusion.

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