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THERMODYNAMIC TREATMENT OF PARTITION EXPERIMENTS WITH  
SPECIAL REFERENCE TO MOLECULAR-SIEVE CHROMATOGRAPHY

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SUMMARY

Using thermodynamics an equation (eqn. 8) has been derived for the relationship between distribution coefficients and the parameters characterizing the solute (*e.g.* partial molal volume and molecular area) and the gel bed (*e.g.* pressure and interfacial tension). When active transport can be neglected the equation might also give a qualitative picture of the factors that determine the distribution of solutes between the internal space of a living cell and its surroundings. Introduction of a series of assumptions, which are all discussed, leads to simplified formulae (eqns. 14 and 15), which, in spite of their approximate nature, seem to be applicable in most experiments. The considerations herein also apply to regular partition experiments, including ALBERTSSON's aqueous two-phase systems, recalling that no pressure difference exists between two liquid phases, as between the interior of a gel and the surrounding medium.

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## INTRODUCTION

In molecular-sieve chromatography separation occurs on the basis of relative molecular size. It is therefore natural that a great deal of effort has been made to derive mathematical expressions for the relationship between chromatographic parameters (such as distribution coefficients and elution volumes) and parameters characterising the size of the molecules separated (for instance molecular weight and Stokes radius). Such mathematical treatments have been presented by PORATH<sup>1</sup>, SQUIRE<sup>2</sup>, LAURENT AND KILLANDER<sup>3</sup>, and ACKERS<sup>4</sup> and are reviewed by the present author in ref. 5. The various formulae which have been deduced are different as they are based upon different physical models for the separation mechanism. In this paper thermodynamic considerations are presented and therefore no assumptions about the separation mechanism are required. A series of simplifications must however be introduced in order to arrive at practicably useful expressions. These expressions (eqns. 14 and 15) are therefore very approximate.

## DERIVATION OF THE GENERAL FORMULA (EQN. 8)

We first consider, separately, the different terms which make up the final expression for the chemical potential  $g_i$  of a solute  $i$ .

(1) *Activity*

The activity  $a_i$  of a solute  $i$  is by definition related to the chemical potential  $g_i$  by the well-known equation

$$g_i = \mu_i = \mu_i^0 + RT \cdot \ln a_i \quad (1)$$

where  $\mu_i^0$  refers to the standard state;  $\mu_i$  is a function of temperature, pressure and the nature of the solvent. Putting  $a_i = f_i \cdot c_i$  ( $f_i$  = the activity coefficient;  $c_i$  = the concentration in moles/l) we obtain

$$g_i = \mu_i = \mu_i^0 + RT \cdot \ln f_i \cdot c_i \quad (2)$$

(2) *Temperature and pressure*

With variations in temperature  $T$  and pressure  $p$ ,  $g_i$  changes in accordance with the relation

$$dg_i = -s_i \cdot dT + v_i \cdot dp \quad (3)$$

where  $s_i$  is partial molal entropy and  $v_i$  is partial molal volume.

(3) *Interfacial tension*

The chemical potential is also a function of the interfacial tension  $\gamma_i$  for the interface between the solute  $i$  and the surrounding medium. If  $A_i$  is the area of  $N$  molecules ( $N$  = Avogadro number) of the solute  $i$  we obtain

$$dg_i = \gamma_i \cdot dA_i \quad (4)$$

(4) *Electric potential*

Assume that each molecule of the solute  $i$  has a net electronic charge  $Z_i$  ( $Z_i < 0$  for anions and  $> 0$  for cations) and that the molecules are located where the electric potential is  $\psi$ . The chemical potential for this system is then described by the expression

$$g_i = F \cdot Z_i \cdot \psi \quad (5)$$

where  $F$  is the Faraday constant.

(5) *Adsorption*

In the cases where there are other types of interaction between the solute  $i$  and the bed material (*i.e.* the gel particles) than the above electrostatic interaction, we get the following contribution to the chemical potential

$$g_i = -E_i \quad (6)$$

where  $E_i$  ( $> 0$  for adsorption) is the energy required to desorb  $N$  molecules of the solute  $i$ .

The total chemical potential is obtained by summation of eqns. 2-6 which gives

$$g_i = \mu_i^0 + RT \cdot \ln f_i \cdot c_i - \int_{T^0}^T s_i dT + \int_{p^0}^p v_i dp + A_i \cdot \gamma_i - A_i^0 \cdot \gamma_i^0 + \\ + F \cdot Z_i \cdot \psi - F \cdot Z_i^0 \cdot \psi^0 - (E_i - E_i^0) \quad (7)$$

where superscript  $^0$  refers to the standard state.

For any solute  $i$ , distributed between two phases  $s$  and  $m$ , the chemical potential in phase  $s$  is equal at equilibrium to the chemical potential in phase  $m$  ( $g_{i,s} = g_{i,m}$ ). Applying this law to a solute  $i$ , distributed between the stationary phase ( $s$ ) and the mobile phase ( $m$ ) of a chromatographic bed, we obtain the following relationship from eqn. 7 (to obtain a simpler formula we drop the subscripts  $i$ )

$$\begin{aligned} RT \cdot \ln K + \mu_s^0 - \mu_m^0 + v_s p_s - v_m p_m + A_s \gamma_s - A_m \gamma_m + F(Z_s \psi_s - Z_m \psi_m) - \\ - (E_s - E_m) + RT \cdot \ln \frac{f_s}{f_m} - (v_s p_s^0 - v_m p_m^0) - (A_s^0 \gamma_s^0 - A_m^0 \gamma_m^0) - \\ - F(Z_s^0 \psi_s^0 - Z_m^0 \psi_m^0) + (E_s^0 - E_m^0) = 0 \end{aligned} \quad (8)$$

where  $K$  is the distribution coefficient, defined by

$$K = \frac{c_s}{c_m} \quad (9)$$

#### *Selection of standard states*

The standard states may be defined in different ways. The following choice is convenient for the present treatment.

In the standard state for the solute in the mobile phase the concentration of the solute ( $c_m^0$ ) is such that  $a_m^0 = f_m^0 \cdot c_m^0 = 1$  ( $f_m = a_m/c_m \rightarrow 1$  when  $c_m \rightarrow 0$ ). The pressure is then  $p_m^0$  and the interfacial tension  $\gamma_m^0$ .

In the standard state the solute in the stationary phase is in equilibrium with the solute in the mobile phase, *i.e.*,  $g_s^0 = g_m^0$  or according to eqn. 7  $\mu_s^0 = \mu_m^0$ ; the solute in the stationary phase has at this equilibrium an activity which we put equal to 1. The pressure is then  $p_s^0$  ( $p_s^0 \neq p_m^0$ ) and the interfacial tension  $\gamma_s^0$  ( $\gamma_s^0 \neq \gamma_m^0$ ).

#### APPROXIMATIONS OF THE GENERAL EQN. 8

Eqn. 8 was derived under the assumption that the partial molal volume  $v$  is independent of the pressure. This assumption involves no limitations in practice. For example, for proteins  $v$  is changed by only about 1% when the pressure is changed by 1000 atm. (ref. 6). In many experiments the parameters  $v$ ,  $A$ , and  $Z$  for a given solute can be expected to have the same values in the two phases. With the above standard states ( $\mu_s^0 = \mu_m^0$ ) eqn. 8 then takes the form

$$\begin{aligned} RT \cdot \ln K + v[p_s - p_m - (p_s^0 - p_m^0)] + A[\gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)] + \\ + FZ[\psi_s - \psi_m - (\psi_s^0 - \psi_m^0)] - [E_s - E_m - (E_s^0 - E_m^0)] + RT \cdot \ln \frac{f_s}{f_m} = 0 \quad (10) \end{aligned}$$

As  $v$ ,  $A$ , and  $Z$  are treated as constants in eqn. 10, this equation is only valid if the solute molecules do not alter their structure or configuration, including the "thickness" and the nature of the double layer and the degree of hydration when they are transferred from one phase to the other. Globular proteins probably fulfil this requirement better than chain molecules with a loose structure (flexible polymers). In molecular-sieve chromatography on very tight gels (for instance Sephadex G-10 and Bio-Gel P-2) one may sometimes expect that the concentrations of the ions in

the eluting medium (the buffer) are not the same in the two phases. In such a case  $Z_s \neq Z_m$  (the net charge of the solute in the stationary phase differs from the net charge in the mobile phase) as the net charge  $Z$  is a function of the composition of the eluting medium. Under these conditions the fourth term in eqn. 10 should be replaced by  $F(Z_s\psi_s - Z_m\psi_m) - F(Z_s^0\psi_s^0 - Z_m^0\psi_m^0)$ . The net charge has, however, no influence on the  $K$  values as long as the gel matrix is neutral, *i.e.* when  $\psi = 0$ . It should be noted that the potential may differ from zero even if the gel polymer does not contain any charged groups, such as carboxylic groups, since certain ions in the eluting medium may have a greater tendency for interaction with the gel matrix than others. An example of this is shown by droplets of *n*-decanol which migrate in an electric field due to such a type of interaction<sup>7</sup>.

We now intend to introduce a series of assumptions in order to simplify eqn. 10 (in several experiments some of these assumptions involve so large an approximation that they cannot be considered as justified):

(a)  $\psi_s = \psi_m = \psi_s^0 = \psi_m^0 = 0$  and  $E_s = E_m = E_s^0 = E_m^0 = 0$

In this case, the solute does not show any electrostatic or other interaction with the gel bed. The condition  $E = 0$  is not fulfilled when low molecular weight aromatic compounds are chromatographed on tight gels. It should also be recalled that  $\psi$  may differ from zero even if the polymer is devoid of dissociable groups, as mentioned above.

(b)  $v = \bar{v} \cdot M = \text{constant} \cdot M$

Here  $\bar{v}$  is the partial specific volume and  $M$  is the molecular weight of the solute. The approximation that  $\bar{v}$  is constant is only justified when groups of solutes having similar chemical structures are considered, so-called isochemical substances. It is well known, for example, that most proteins have a partial specific volume close to  $0.74 \text{ cm}^3 \text{ g}^{-1}$  and carbohydrates close to  $0.60$ . The greater the similarity between the repeating units of a polymer solute, the better is the approximation; it is probably quite satisfactory for members of a homologous series.

(c)  $p_s - p_m = \text{constant for any given gel bed}$

This approximation is equivalent to the assumption that the pressure difference is determined by the nature and the composition (the concentration) of the gel polymer, but not of the solutes.

(d)  $\gamma_s - \gamma_m = \text{constant for isochemical solutes on any given gel bed}$

An indirect indication that the approximations (c) and (d) — and (f) below — may be justified is the observation that the  $K$  values vary only slightly with alterations in solute and buffer concentrations<sup>8,9</sup>; see also experiment III in Fig. 1.

(e) *Molecular area*

The difficulty of defining the area of a molecule is obvious, because neither the shape, nor the degree of solvation can be determined exactly. In the mathematical treatment we must assume that there is a sharp boundary between the solute and the surrounding medium, although in reality there is a smooth transition (similar assumptions are made in the current theoretical treatment of electrophoretic migration

where the conception of a "slipping plane" is introduced). We will now consider separately non-coiled chain molecules, globular polymers, and flexible polymer chains.

(e1)  $A = \text{constant} \cdot M$ . The constant is assumed to have the same value for isochemical substances. This proportionality may be a satisfactory approximation when the solute molecule is in the form of a non-coiled chain. Low molecular weight substances in a homologous series belong to this class of substances.

(e2)  $A = \text{constant} \cdot M^{2/3}$ . Here again the constant is assumed to have the same value for isochemical substances. This approximation is valid for solutes which can be considered spherical and therefore approximate for globular proteins. However, the same relation might also be approximately valid for flexible polymer chains. Before producing evidence for this it should be remembered that flexible polymers, for instance dextran chains, are "non free-draining" molecules: TANFORD<sup>10</sup> states that the solvent within the interior of a flexible polymer (perhaps within the radius of gyration) is trapped and is therefore essentially indistinguishable, from a hydrodynamic point of view, from a solvent which might be inherently combined with the polymer chain.

Following the treatment of ONCLEY<sup>11</sup> we write the volume  $V$  of a solvated molecule as

$$V = \frac{M}{N} (\bar{v} + \delta \cdot v_s) \quad (11)$$

where  $\delta$  is the number of grams of solvent per gram of dry macromolecular material and  $v_s$  is the specific volume of pure solvent. We now introduce the concept of an equivalent hydrodynamic sphere, which can be visualised as a solid sphere of radius  $a$  and with a volume equal to the volume of the solvated molecule. Accordingly

$$\frac{4}{3} \pi a^3 = \frac{M}{N} (\bar{v} + \delta \cdot v_s) \quad (12)$$

The area of the sphere will differ from the area of the macromolecule. The difference is, however, not large if the solute molecules are not too elongated, as numerical calculations show: if the macromolecules are cylindrical with radius  $r$  and height  $h = r, 2r,$  and  $4r$  the difference is 21, 14, and 20 %, respectively. We can therefore put  $A = C^0 \cdot 4\pi a^2 \cdot N$ , where  $C^0$  has a value fairly close to unity ( $a$  is not necessarily equal to the Stokes radius). Using eqn. 12 we then obtain

$$A = C^0 \cdot (4\pi N)^{1/3} \cdot 3^{2/3} \cdot (\bar{v} + \delta \cdot v_s)^{2/3} \cdot M^{2/3} \quad (13)$$

The parameters  $C^0$ ,  $\bar{v}$ , and  $\delta$  can be considered as constants for isochemical flexible polymer chains, *i.e.*,  $A = \text{constant} \cdot M^{2/3}$ , which is the same expression as that for globular proteins. The constant has, however, a considerably higher value for flexible polymers, because  $\delta$ , the degree of solvation, is much larger for these than for globular proteins. One should observe that for  $\delta = 0$  and  $C^0 = 1$  eqn. 13 gives the area of  $N$  rigid spheres.

(f)  $f_s/f_m = \text{constant}$

This assumption involves that the ratio between the activity coefficients in the

stationary and mobile phase has the same value for all isochemical solutes irrespective of their concentrations. This approximation might be valid for low solute concentrations.

#### Case (A)

When conditions (a), (b), (c), (d), (e1), and (f) are fulfilled, eqn. 10 can be simplified to

$$-\log K = C_1' \cdot M + C_0 \quad (14)$$

where  $C_1'$  is a sum of two terms, one similar to  $C_1$  (eqn. 16) and the other similar to  $C_2$  (eqn. 17).

#### Case (B)

When conditions (a), (b), (c), (d), (e2), and (f) are fulfilled, eqn. 10 can be simplified to

$$-\log K = C_1 \cdot M + C_2 \cdot M^{2/3} + C_0 \quad (15)$$

where

$$C_1 = \frac{\bar{v} \cdot [p_s - p_m - (p_s^0 - p_m^0)]}{2.3 \cdot RT} \quad (16)$$

$$C_2 = \frac{C_0 \cdot (4\pi N)^{1/3} \cdot 3^{2/3} \cdot \bar{v}^{2/3} (\bar{v} + \delta \cdot v_s)^{2/3} [\gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)]}{2.3 \cdot RT} \quad (17)$$

$$C_0 = \log \frac{f_s}{f_m} \quad (18)$$

We now make the assumption that in some experiments the constant  $C_2$  in eqn. 15 is so small that the term  $C_2 \cdot M^{2/3}$  is negligible. This equation then takes the form

$$-\log K = C_1 \cdot M + C_0 \quad (19)$$

In the cases when  $C_1$  can be put = 0, for instance in ALBERTSSON's two-phase systems<sup>12</sup>, eqn. 15 is reduced to

$$-\log K = C_2 \cdot M^{2/3} + C_0 \quad (20)$$

#### EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 14

The distribution coefficient  $K$  is calculated from the relationship

$$K = \frac{V_e - V_0}{V_t} \quad (21)$$

where

$V_e$  = the elution volume of the solute of interest,

$V_0$  = the void volume, and

$V_t$  = the volume of the solvent imbibed by the gel particles.

In order to determine the constants in eqn. 14  $-\log K$  is plotted against  $M$ .

From the resulting straight line (Fig. 1)  $C_1'$  is obtained as the slope and  $C_0$  as the ordinate at the origin. For subsequent discussion we write  $C_1'$  as

$$C_1' = \frac{d(-\log K)}{dM} \quad (22)$$

#### EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 15

##### Alternative I

A smooth curve is drawn through the points obtained when  $-\log K$  is plotted against  $M$  (see Fig. 3, the solid lines). Derivation of eqn. 15 gives

$$\frac{d(-\log K)}{dM} = C_1 + \frac{2C_2}{3} \cdot \frac{1}{M} \quad (23a)$$

The numerical value of  $\frac{d(-\log K)}{dM}$  for a certain  $M$  value is obtained as the slope of the tangent to the curve for this  $M$  value.  $C_1$  is then obtained as the ordinate at the

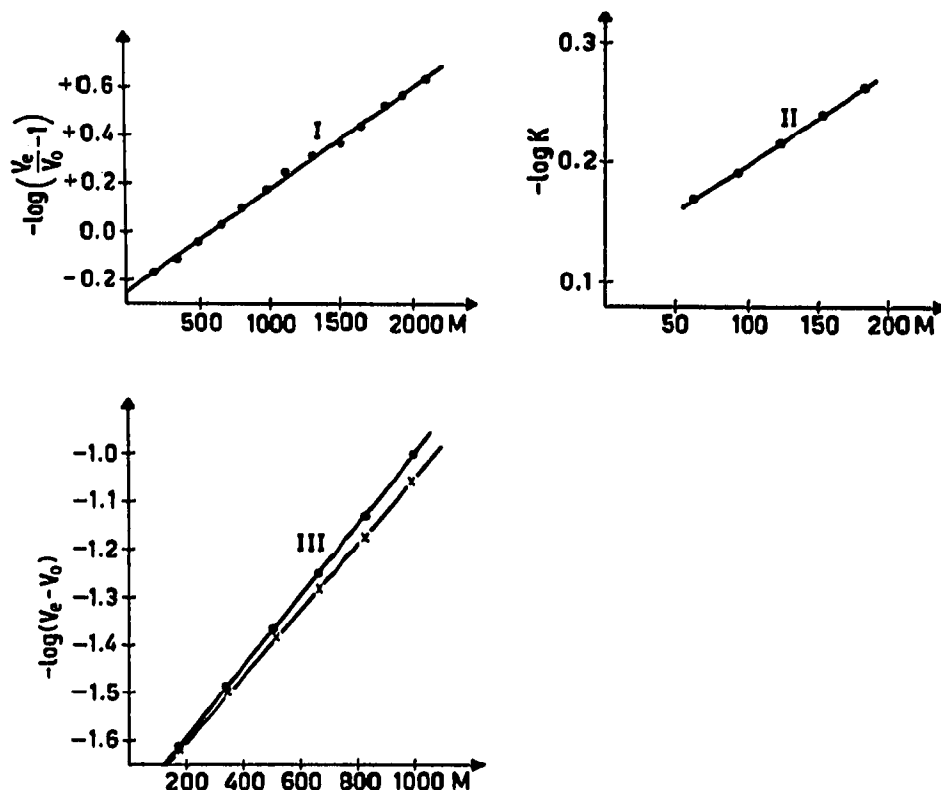


Fig. 1. Plots of  $-\log K$  ( $K$  = the distribution coefficient) against the molecular weight  $M$  of low molecular weight homologues (see eqns. 14 and 19). As only  $V_e/V_0$  values and  $V_e - V_0$  values but not  $K$  values, were reported in the experiments I and III, we have, for these experiments, plotted the parameters  $-\log [(V_e/V_0) - 1]$  and  $-\log (V_e - V_0)$ , which are related linearly to  $-\log K$ . I = oligosaccharides on Bio-Gel P-2 (ref. 29); II = polyhydric alcohols on a tightly cross-linked dextran gel (DVS 9; water regain 0.93 (ref. 26)); III = isomaltodextrins on Sephadex G-15 in distilled water ( $\times - \times$ ) and in 0.1 M Tris-HCl, pH 7.0, + 0.3 M NaCl ( $\bullet - \bullet$ ) (ref. 31). The references apply to papers from which the parameters plotted have been taken. We have also obtained a similar linear relationship when plotting published data from partition chromatography experiments on paper.

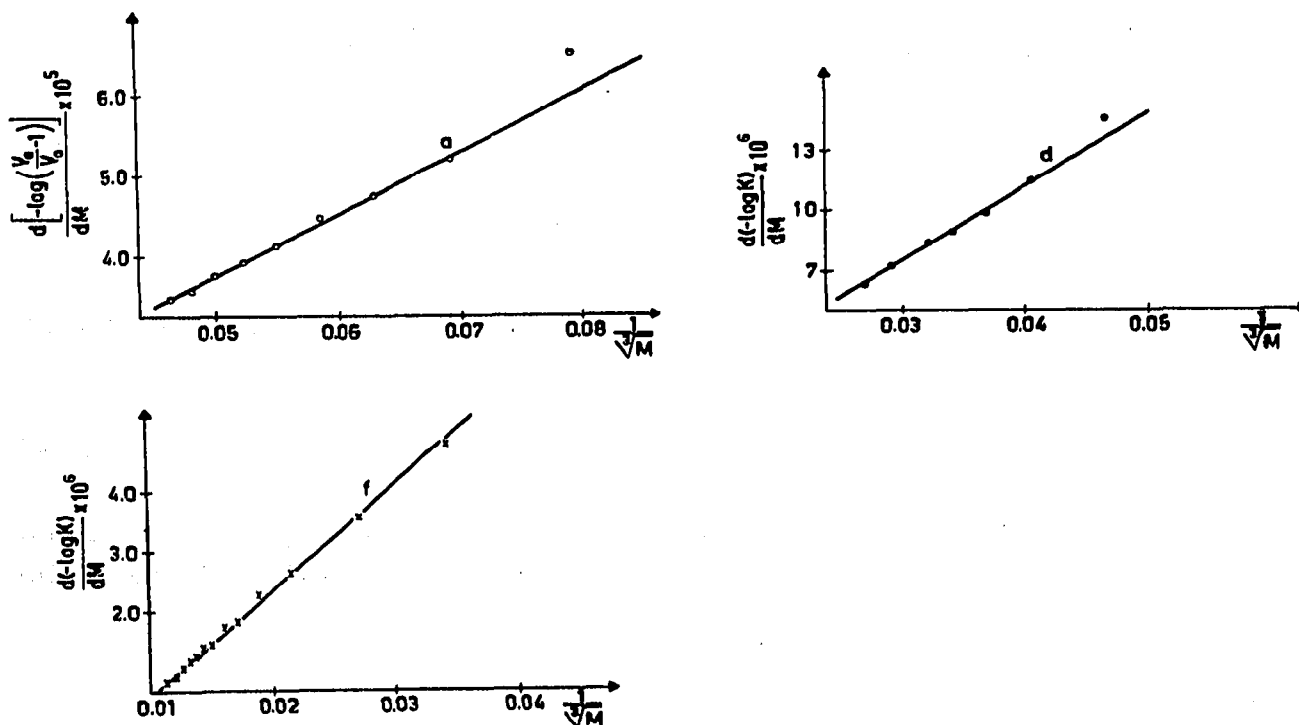


Fig. 2a. Plots of  $\frac{d(-\log K)}{dM}$  against  $1/\sqrt[3]{M}$  ( $K$  = the distribution coefficient;  $M$  = the molecular weight of *high* molecular weight *globular* or *flexible* polymers). (a) Polypeptides in a random coil conformation on a polyacrylamide gel, Bio-Gel P-150 (ref. 32). As only  $V_e/V_0$  values, but not  $K$  values, were reported for the experiment (a), we have in this case plotted the parameter

$$\frac{d \left[ -\log \left( \frac{V_e}{V_0} - 1 \right) \right]}{dM},$$

which is equal to  $\frac{d(-\log K)}{dM}$ . (d) Highly branched polysaccharides (ficoll fractions) on Sephadex G-200 (ref. 33). (f) Globular proteins on Sephadex G-200 (ref. 18). The linear relationship obtained is in accordance with eqn. 23a, which is the derived form of eqn. 15. These three experiments are found also in Figs. 3 and 4 with the same notations (a, d, f).

origin and  $C_2$  as  $3/2$  times the slope of the straight line obtained when  $\frac{d(-\log K)}{dM}$  is plotted against  $1/\sqrt[3]{M}$  (see Fig. 2a); if a curved line is obtained for a certain molecular weight range, eqn. 15 is not valid in this range. The constant  $C_0$  is equal to  $-\log K$  for  $M$  equal to zero. If such an extrapolation cannot be performed accurately it might be better to make an estimation from the equation

$$3C_0 = -\log K_1 - \log K_2 - \log K_3 - C_1M_1 - C_1M_2 - C_1M_3 - C_2M_1^{2/3} - C_2M_2^{2/3} - C_2M_3^{2/3} \quad (24)$$

where  $C_1$  and  $C_2$  have been determined graphically as described above;  $K_i$  ( $i = 1, 2, 3$ ) corresponds to the molecular weight  $M_i$ .

#### Alternative II

The drawback of the above method of plotting is that it requires that the derivative  $\frac{d(-\log K)}{dM}$  can be determined relatively accurately, *i.e.* the shape of the curve obtained when  $-\log K$  is plotted against  $M$  must be accurately known. As this is



seldom the case we have plotted  $(-\log K - C_0)/M$  for most experiments instead of  $\frac{d(-\log K)}{dM}$  against  $1/\sqrt[3]{M}$  where  $C_0$  has previously been graphically determined after extrapolation, as described above. This plotting technique should also give a straight line which is evident from eqn. 15, written in the form

$$\frac{-\log K - C_0}{M} = C_1 + \frac{C_2}{\sqrt[3]{M}} \quad (23b)$$

Some examples are given in Fig. 2b. From these lines,  $C_1$  and  $C_2$  can be determined as the ordinate at the origin and the slope, respectively.

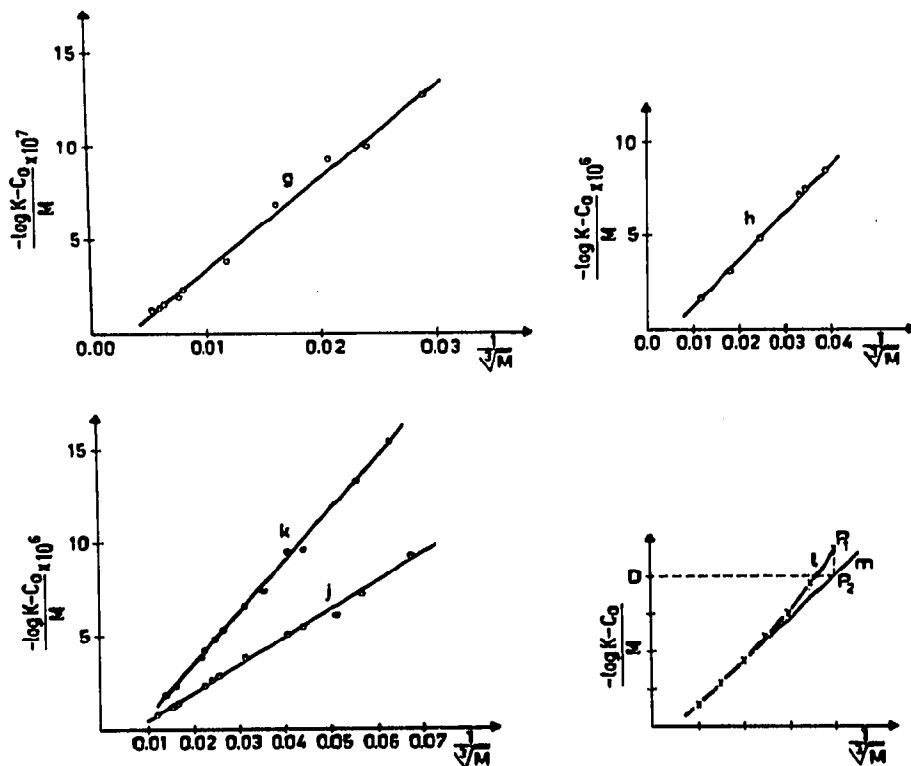


Fig. 2b. Plots of  $(-\log K - C_0)/M$  against  $1/\sqrt[3]{M}$  for high molecular weight *globular* or *flexible* polymers (see eqn. 23b). (g) Polysaccharides (dextran) on an agarose gel, Sepharose 2 B (ref. 35). (j) Polysaccharides (dextran) on an agarose gel, Sepharose 4 B (ref. 35). (k) Polysaccharides (dextran) on an agarose gel, Sepharose 6 B (ref. 35). (h) Proteins on a polyacrylamide gel, T = 6.5%, C = 15% (ref. 36). (l, m) An outline of the procedure for the calculation of  $C_0$  according to the formula  $(-\log K - C_0)/M = D$ . The experiments (h) and (g) are also referred to in Fig. 4.

If the value of  $C_0$  is not accurately known a line will result, which is straight for high molecular weights and curved for low molecular weights (line l in Fig. 2b). This is due to the fact that small errors in  $C_0$  may cause grave errors in  $(-\log K - C_0)/M$  for low  $-\log K$  values (low molecular weights) but not for high  $-\log K$  values (high molecular weights). However, it is possible to calculate a more correct  $C_0$  value from the expression  $(-\log K - C_0)/M = D$ , where  $D$  is the ordinate for a point  $P_2$  on the extrapolated straight line m in Fig. 2b; the points  $P_1$  and  $P_2$  in this figure have the same abscissa. A new plot of  $(-\log K - C_0)/M$  (with this new  $C_0$  value) against  $1/\sqrt[3]{M}$  is made. If this second plot does not yield a straight line,

another  $C_0$  value is calculated from the new  $D$  value that this plot gives. This procedure is repeated until a  $C_0$  value is obtained such that all the points scatter around a straight line.

#### VERIFICATION OF THE FORMULAE 14 AND 15

The experimental data were next investigated as to whether they fit the formulae derived, as this is a necessary condition for the correctness of the formulae (it is, however, not a sufficient condition).

##### (a) Low molecular weight homologues (eqn. 14)

Eqn. 14 requires that a plot of  $-\log K$  against  $M$  results in a straight line. Fig. 1 shows that this relationship is obtained.

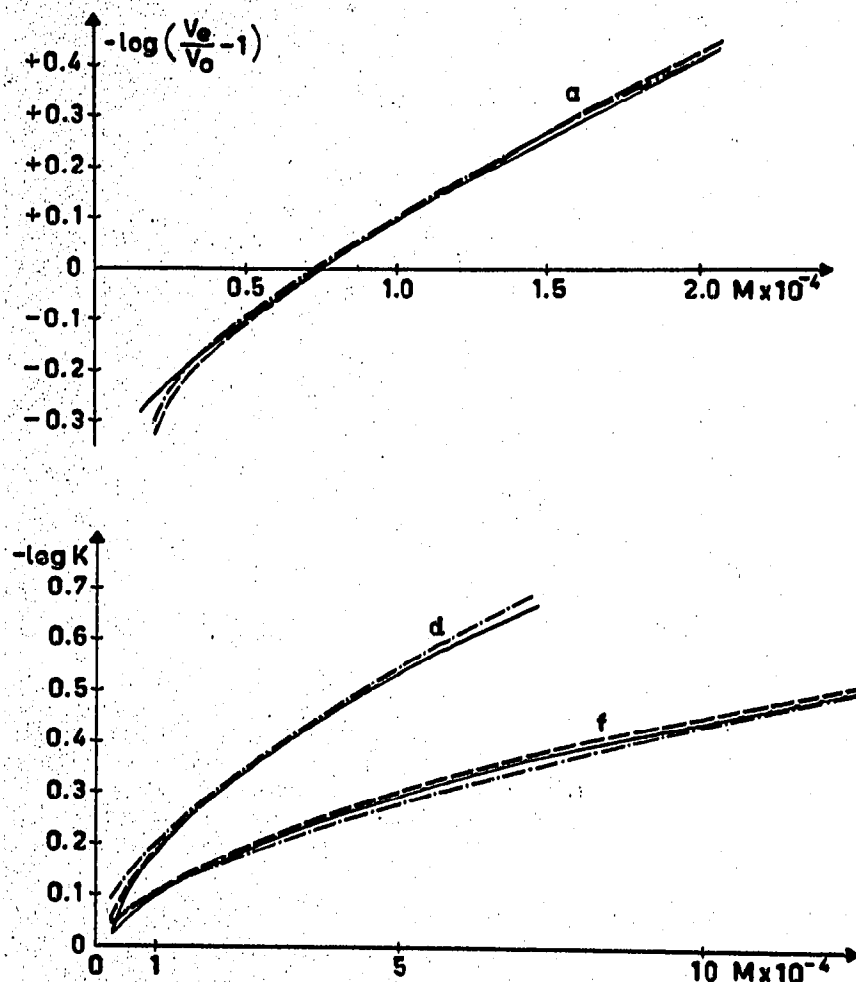


Fig. 3. Plots of  $-\log K$  and  $-\log [(V_e/V_0) - 1]$  against  $M$  for the experiments (a), (d), and (f), referred to in Fig. 2a (and Fig. 4). As only  $V_e/V_0$  values, but not  $K$  values were reported for the experiment (a), we have, for this experiment, plotted the parameter  $-\log [(V_e/V_0) - 1]$ , which is related linearly to  $-\log K$ . (—) experimental curve; (---) theoretical curve:  $-\log K = C_1 \cdot M + C_2 \cdot M^{2/3} + C_0$  (eqn. 15). The constants  $C_1$ ,  $C_2$  and  $C_0$  have been estimated as described under EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 15. (-·-·-) approximate curve:  $-\log K = R \cdot M^{2/3} + S$  (eqn. 25).  $R$  and  $S$  have been determined as described under VERIFICATION OF THE FORMULAE 14 AND 15.

TABLE I

APPROXIMATE VALUES OF SOME CHROMATOGRAPHIC PARAMETERS FOR GELS WITH AN OPEN STRUCTURE, SUITABLE FOR THE SEPARATION OF HIGH MOLECULAR WEIGHT SUBSTANCES

The constants  $C_1$ ,  $C_2$ , and  $C_0$  refer to eqn. 15:  $-\log K = C_1 \cdot M + C_2 \cdot M^{2/3} + C_0$ . The values of  $\Delta\Delta p = p_s - p_m - (p_s^0 - p_m^0)$ ,  $\Delta\Delta y = \gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)$ , and  $f_s/f_m$  have been estimated from eqs. 16, 17, and 18, respectively.  $\Delta\Delta y$  is calculated under the assumption that  $C^0 = 1$  and  $\delta = 0$ . This assumption might be justified for globular proteins (for ovalbumin  $\delta = 0.18$  (ref. 38)). However, as the polysaccharides and polypeptides, referred to in this table, are more or less in a random coil conformation, their  $\delta$  values are very high and unknown. We cannot therefore calculate  $\Delta\Delta p$  and  $\Delta\Delta y$  for these polymers. The data used for the calculation of the different parameters are taken from the references given. All values listed in the table are so extremely approximate, that they only reflect the order of magnitude.

Solute	Solvent	Gel type	Reference	$C_1 \times 10^6$ (moles <sup>-1</sup> g)	$C_2 \times 10^4$ (moles <sup>-3/2</sup> g <sup>3/2</sup> )	$C_0$	$\Delta\Delta p$ (atm)	$\Delta\Delta y$ (dyne cm <sup>-1</sup> )	$f_s$ $f_m$
Polysaccharides									
Dextran fractions	0.3% NaCl	Sephacrose 2B	35	-0.3	+0.5	+0.053			1.1
Dextran fractions	0.3% NaCl	Sephacrose 4B	35	-1	+1	+0.032			1.1
Dextran fractions	0.3% NaCl	Sephacrose 6B	35	-3	+3	+0.021			1.1
Ficoll fractions	0.2% NaCl	Sephadex G-200	33	-4	+6	-0.043			0.91
Proteins	0.05 M Tris-HCl, pH 7.5 + 0.1 M NaCl	Sephadex G-200	18	-1	+3	-0.007	-0.1	+0.1	0.98
Proteins	0.5% NaCl	Polyacrylamide, T = 6.5%, C = 15%	36	-2	+3	+0.049	-0.2	+0.1	1.1
Polypeptides (random coil conformation)	0.05 M Tris-HCl, pH 7.5 + 1 M CaCl <sub>2</sub>	Bio-Gel P-150	32	-0.3	+12	<sup>a</sup>			-

<sup>a</sup> The  $C_0$  value (and the  $f_s/f_m$  value) could not be calculated for this experiment as only  $V_e$  and  $V_0$  values, but not  $K$  values, have been reported.

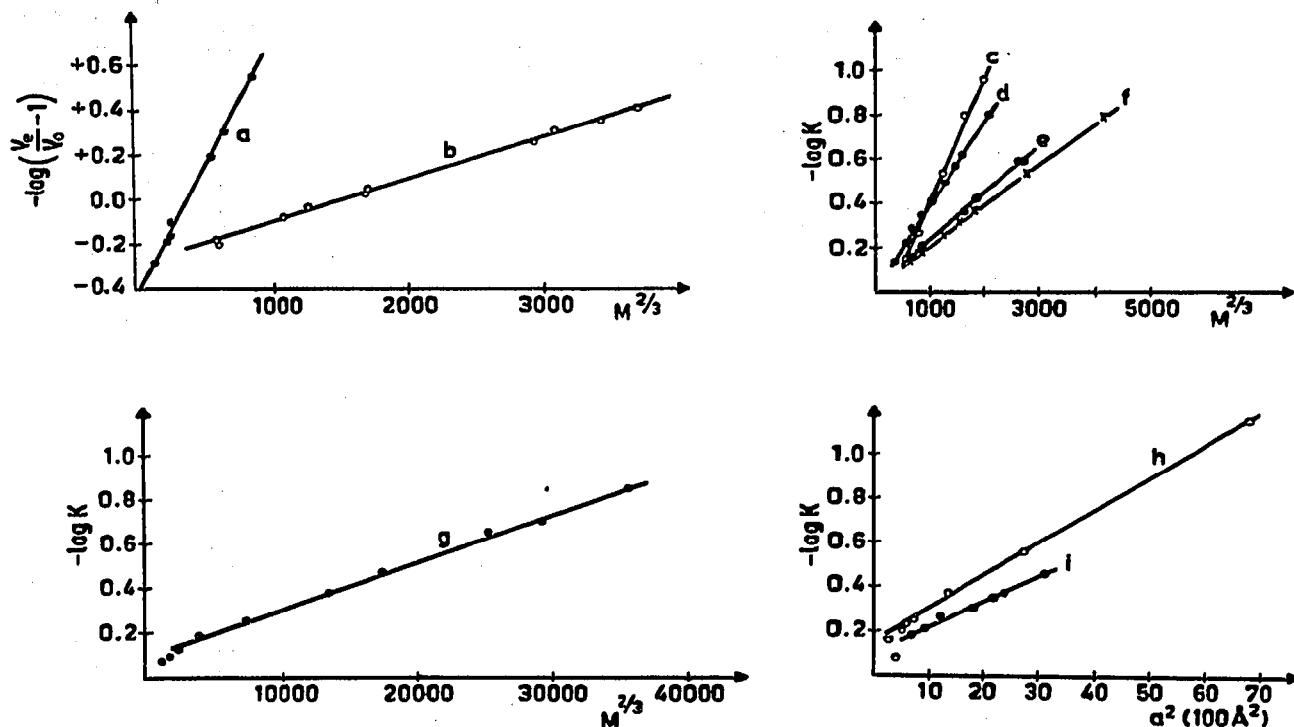


Fig. 4. Plots of  $-\log K$  ( $K$  = the distribution coefficient) against  $M^{2/3}$  ( $M$  = the molecular weight of high molecular weight globular or flexible polymers) or  $a^3$  ( $a$  = Stokes radius) (see eqn. 25). As only  $V_e/V_0$  values, but not  $K$  values, were reported for the experiments (a) and (b), we have plotted the parameter  $-\log [(V_e/V_0) - 1]$  for these experiments, which is related linearly to  $-\log K$ . (a) Polypeptides on a polyacrylamide gel, Bio-Gel P-150 (ref. 32). (b) Proteins on Sephadex G-200 (ref. 19). (c) Proteins on Sephadex G-100 (ref. 34). (d) Polysaccharides on Sephadex G-200 (ref. 33). (e) Proteins on Sephadex G-200 (ref. 34). (f) Proteins on Sephadex G-200 (ref. 18). (g) Polysaccharides on an agarose gel, Sepharose 2B (ref. 35). (h) Proteins on a polyacrylamide gel, T = 6.5%, C = 15% (ref. 36). (i) Polysaccharides on a 6% agarose gel<sup>37</sup>. The references apply to papers from which the plotted parameters have been taken. As indicated in the diagrams, the points scatter around straight lines for the major part of the separation range. Such linear relationships are not to be expected in all experiments with proteins or polysaccharides, as eqn. 25 is only a formal approximation of the more generally applicable eqn. 15; thus, a straight line is not obtained when  $-\log K$  is plotted against  $M^{2/3}$  for experiments (j) and (k), referred to in Fig. 2b.

(b) High molecular weight globular proteins and flexible polysaccharides (eqn. 15)

A necessary condition for eqn. 15 to be valid is that a plot of  $\frac{d(-\log K)}{dM}$  or  $(-\log K - C_0)/M$  against  $1/\sqrt[3]{M}$  gives a straight line (eqns. 23a and 23b). Using chromatographic data from the literature we have obtained such a relationship in most cases. Some examples are given in Figs. 2a and 2b.

The constants  $C_0$ ,  $C_1$ , and  $C_2$  in eqn. 15 were determined as described in the previous sections (in some experiments alternative I was used and in others alternative II). A graphic representation of eqn. 15 with these values of the constants is shown in Fig. 3 (the broken curves). These curves are in good agreement with the experimental curves (solid lines). Figs. 2a and 2b show that  $C_1 \neq 0$ ; furthermore,  $C_1$  is not sufficiently small compared with  $C_2$  (Table I) that the term  $C_1M$  in eqn. 15 can be neglected in comparison with the term  $C_2M^{2/3}$  for molecular weights over ca. 1000. Consequently, eqn. 20 which corresponds to eqn. 15 for  $C_1 = 0$  is not applicable to molecular-sieve chromatography. This statement is at first a little surprising with

respect to the linear relationship often obtained in plots of  $-\log K$  against  $M^{2/3}$  (see Fig. 4). However, this empirical relationship

$$-\log K = R \cdot M^{2/3} + S \quad (25)$$

can be considered a formal approximation of eqn. 15, as it appears to be possible to find such values of  $R$  and  $S$  that  $-\log K = C_1 M + C_2 M^{2/3} + C_0$  is virtually equal to  $R \cdot M^{2/3} + S$  (the constants  $R$  and  $S$  have no evident physical significance). Examples of the feasibility of approximating eqn. 15 with eqn. 25 satisfactorily are given in Fig. 3 (the  $R$  and  $S$  values are those obtained graphically from the straight line obtained in plots of  $-\log K$  against  $M^{2/3}$ ). As eqn. 25 is applicable in several experiments it is not surprising that a plot of  $-\log K$  against  $a^2$  ( $a$  = Stokes radius) also often results in a straight line (see Fig. 4, experiments h and i).

## DISCUSSION

### *A comparison between the present treatment and previous ones*

The present theoretical treatment of molecular-sieve chromatography is a thermodynamic one and thus differs fundamentally from those of many other authors<sup>1-4</sup>, in that no physical model is needed to explain the separations obtained. These previous hypotheses of molecular-sieve chromatography are based upon different physical models, which have a common factor in that the sieving properties of the gel columns are assumed to be due to steric exclusion and/or restricted diffusion. An inspection of eqn. 10 shows that thermodynamic considerations provide another explanation; namely that in ideal molecular-sieve chromatography ( $\psi = E = 0$ ) the size-sieving properties of gels are due to: (a) differences in pressure between the gel phase and the mobile liquid phase, and (b) alterations in the interfacial tension of the solute when it moves from one phase to the other. In a recently published communication POLSON AND KATZ<sup>13</sup> advanced the hypothesis that chromatographic molecular sieving may partly be explained by osmotic effects. From this point of view a paper by EDMOND *et al.*<sup>14</sup> on the osmotic behaviour of dextran gels is of interest.

In most of the previous hypotheses it has been assumed that the solute concentrations are the *same* in the mobile phase and the part of the stationary phase that is available to the solute. In thermodynamic treatment the solute concentration in the stationary phase (the gel grains) is assumed to be different from the concentration in the mobile phase and it is not necessary to assume a non-uniform distribution of the solute in the gel grain. This fundamental dissimilarity is reflected in the definition of the distribution coefficients: in the present treatment the distribution coefficient  $K$  is equal to the ratio between the concentrations in the stationary and the mobile phase (eqn. 9), while in the previous treatments  $K$  is equal to the fraction of the inner volume  $V_i$  that is available to the solute. (LAURENT<sup>37</sup> has defined  $K$  in a somewhat different way.)

Although it has been almost generally accepted that solute molecules of both low and high molecular weight substances do penetrate the *whole* gel particle, experimental evidence is still lacking. From the thermodynamic point of view the penetration depth is not of interest: no assumptions about the dimensions of the stationary phase are required.

Many experiments have been reported in which solutes are not eluted according to molecular weight. This elution order is, however, not always to be expected — not even in the absence of interaction between solute and gel polymer — since it is the

partial molal volume  $v$ , the area  $A$  of the solute molecules and the interfacial tension  $\gamma$  between the solute molecules and the surrounding medium that primarily determine the distribution coefficients as eqn. 10 shows; it is only when certain conditions, mentioned under APPROXIMATIONS OF THE GENERAL EQN. 8, are fulfilled, that there is a positive correlation between elution volume and molecular weight (as for instance for globular proteins and for solutes of a homologous series: eqns. 15 and 14). In an extensive study of the behaviour of amino acids on Sephadex G-10, EAKER AND PORATH<sup>15</sup> observed that the distribution coefficients of these solutes were markedly changed with alteration in buffer composition. Several explanations were given by the authors. It is also probable that these changes in the  $K$  values may in part result from Donnan effects (see below) and alterations in the above parameters ( $v$ ,  $A$ ,  $\gamma$ ): these effects are more pronounced in experiments with low molecular weight ions, such as amino acids, on tight gels.

Some authors have stated that the physical molecular sieving model used for the derivation of an equation is correct if the experimental data fit the equation. Such a verification is no proof, it is only a necessary, but not a sufficient condition, for the correctness of the model.

#### *Estimation of the molecular size of a solute by the approximate eqns. 14 and 15*

The transformation of the general eqn. 8 into eqns. 14, 15, 19, and 20 involves a series of approximations, mentioned above in section APPROXIMATIONS OF THE GENERAL EQN. 8. It should also be stressed that all the equations presented in this paper are based upon the assumption that both the mobile and stationary phase are homogeneous. This might be a simplification when the stationary phase is a granular gel: microheterogeneities in the gel may mean that the parameters  $p$ ,  $\gamma$ ,  $\psi$ , and  $E$  in eqn. 8 have different values in different regions of the gel grain. The approximate nature of eqns. 14 and 15 is thus obvious. The molecular weight estimated by these equations — or by any other of the previously published formulae<sup>1-4</sup> — must therefore be regarded as tentative, particularly when the shape of the molecule is unknown. An example of how large the errors may become is given by the case of globular proteins which, after denaturation by urea to a random coil conformation, migrate close to the void volume on a 6% agarose gel (Bio-Gel A-5 m), while the same proteins in their native, globular state require almost double this volume for elution<sup>16</sup>.

Without attempting a rigorous explanation of this "anomaly" we will call attention to the fact that the transition of a macromolecule from a globular to a random coil state involves an enormous increase in the area of the molecule (the amount of solvent in the macromolecule may exceed the amount of the dry macromolecular material by a factor of 100 (ref. 10), which means that  $\delta$  in eqn. 13 — and consequently the area  $A$  — is much larger for denatured than for native proteins). As  $\gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0) > 0$  according to Table I an increase in  $A$  in eqn. 10 corresponds to a decrease in  $K$  provided that the other terms do not affect  $K$  in an opposite direction, *i.e.* a randomly coiled molecule will migrate with a lower  $K$  value than a globular molecule of the same molecular weight. This could also explain the high chromatographic migration rates of linear polyethylene glycol molecules as compared with globular proteins of the same molecular weight<sup>17</sup>. It is thus evident that the constants in the various proposed equations for the relation between  $K$  values and molecular size have different values for different kinds of solutes, depending on their nature (for

instance, whether they are proteins or carbohydrates) and shape (for instance, whether they are linear, globular, or randomly coiled). A molecular size calculated by these equations may consequently be entirely erroneous. The same uncertainty is, of course, associated with the use of empirical calibration diagrams. Different authors have proposed plotting different parameters in order to obtain a straight line in such diagrams<sup>1-4, 18</sup>. The present treatment and Figs. 1 and 4 indicate that there is a rather high probability that a straight line will be obtained if  $-\log K$  (or  $-\log [(V_e/V_0) - 1]$ , or  $-\log (V_e - V_0)$ ) is plotted against  $M$  for low molecular weight compounds and against  $M^{2/3}$  for both globular and flexible macromolecules, provided the solutes of interest are isochemical substances, for instance homologues or polymers where the repeating units are similar. It is, however, irrelevant which plotting techniques are used, because in practice none of them will give a straight line in all cases (nor is it necessary that the calibration curve is a straight line).

It has been proposed<sup>10</sup> that the estimation of the molecular weight of a protein of an unknown shape should be performed in the presence of urea or guanidine hydrochloride in order to transform this protein and the reference proteins to the same conformation (random coil).

*The dependence of solute concentration, temperature, ionic strength, and Donnan effects on the distribution coefficients*

WINZOR AND NICHOL have found that the elution volumes or  $K$  values are somewhat dependent on the solute concentration<sup>8</sup>. This is explained by eqn. 8, as the partial molal volume, the pressure, the interfacial tension, and the activity coefficients vary with the concentration of the solute. Eqn. 8 also shows that the  $K$  values are somewhat temperature and ionic strength dependent, which has been experimentally verified in refs. 9 and 19 and in experiment III in Fig. 1. Molecular-sieve chromatography should therefore not be used, as has been proposed<sup>9</sup>, for studying the effects of ionic strength and temperature on the shape of the solute molecules. NICHOL *et al.*<sup>20</sup> have reported that Donnan effects — although small — can be experimentally established in molecular-sieve chromatography of charged macromolecules, which means that the terms of the form  $FZ\psi$  in eqn. 8 may sometimes play a certain role, even if they are negligible in most experiments. The factors  $Z$  and  $\psi$  are discussed under APPROXIMATIONS OF THE GENERAL EQN. 8. It is to be expected that the Donnan effects are more pronounced when small molecules are chromatographed on gels with low water regain (for instance Sephadex G-10 and Bio-Gel P-2).

*The Brønsted formula*

In 1962 HJERTÉN AND MOSBACH advanced different hypotheses to explain the molecular-sieving action of polyacrylamide gels<sup>21</sup>. One of the hypotheses was based upon the assumption that the solutes are distributed between the stationary and mobile phases in a gel bed according to the Brønsted formula<sup>22</sup>

$$K = e^{-\frac{\lambda}{hT}} \quad (26)$$

where:

$K$  = the distribution coefficient,

$\lambda$  = a parameter which depends on the molecular size of the solute,

$k$  = the Boltzmann constant, and

$T$  = the absolute temperature.

This formula is obtained directly from the assumption that the solute molecules partition between the two phases according to the Boltzmann distribution formula.

ALBERTSSON<sup>12</sup> has proved Brønsted's statement<sup>22</sup> that  $\lambda$  for macromolecules in a two-phase system is proportional to the area of the solute. For such solutes eqn. 26 can be written

$$-\log K = \text{constant} \times A \quad (27)$$

which is a special case of eqn. 10 when all terms, except the first and the third, can be neglected (the constant has the same value for "isochemical" substances). For a spherical molecule one thus obtains the relation

$$-\log K = \text{constant} \times M^{2/3} \quad (28)$$

which corresponds to eqn. 20 for  $C_0 = 0$ .

In many experiments we have found that a plot of  $-\log K$  against  $M^{2/3}$  gives a straight line (Fig. 4). In some cases it passes through the origin in accordance with eqn. 28; examples are found in Fig. 4, experiments d, e, and f. However, eqn. 28 also requires that a straight line through the origin is obtained when  $(\frac{d-\log K}{dM})$  is plotted against  $1/\sqrt[3]{M}$ . As in practice this line does not pass through the origin (which an extrapolation of the lines in Fig. 2a indicates), it is more correct to consider eqn. 28 a formal approximation of eqn. 15; the arguments are the same as those mentioned under VERIFICATION OF THE FORMULAE 14 AND 15 where it was proved that eqn. 25 is an approximation of eqn. 15. The reasons why the Brønsted formula is only approximately valid for chromatographic molecular sieving are as follows: (a) the interactions between the solute and the surrounding medium that give rise to a term containing activity coefficients ( $C_0$  in eqn. 15) have been neglected; (b) the formula has been derived for conventional two phase systems where the pressure in the top phase is the same as in the bottom phase, while the "swelling pressure" in a gel particle causes a pressure difference between the interior and exterior of the gel particle<sup>10</sup>, resulting in the term  $C_1 \cdot M$  in eqn. 15.

According to Brønsted,  $\lambda$  for low molecular weight substances is proportional to the molecular weight  $M$  of the solute<sup>22</sup>. Eqn. 26 then takes the form

$$-\log K = \text{constant} \times M \quad (29)$$

This equation can be considered a special case of eqn. 19. The latter equation written in the form  $-\log K = \text{constant} \times v + C_0$ , where  $v$  is the partial molal volume, has been used in the treatment of the behaviour of neutral low molecular weight substances on ion exchange resins<sup>23</sup>, and has been found to conform to experimental data for cellodextrins on Sephadex G-25 (ref. 24). (See also ref. 25.)

### Homologous series

If it is assumed that eqn. 14, or eqn. 19, is applicable for substances which are members of a homologous series. For the pairs  $n$  and  $n + 1$  in such a series we get

$$-\log K_n = C_1' \cdot M_n + C_0$$

$$-\log K_{n+1} = C_1' \cdot M_{n+1} + C_0$$

or

$$-\Delta(\log K_n) = C_1' \cdot \Delta M \quad (30)$$



As this equation is valid for any  $n$  value one can conclude that the difference between the  $\log K$  values for any two consecutive members of a homologous series is constant. MARS DEN<sup>26</sup> has arrived at the same conclusion using an assumption that MARTIN<sup>27</sup> employed in his theoretical studies on partition chromatography, namely that the free energy required to transport a molecule from one solvent to another is the sum of the free energies for transport of the individual constituent groups of the molecule. MARS DEN<sup>26</sup> also verified that  $\Delta(\log K)$  values for two consecutive members of a series of polyols were approximately constant.

The above conclusion that the difference between the  $\log K$  values for any two consecutive members of a homologous series is constant is evident also from eqn. 22 and is in agreement with MARTIN's statement<sup>27</sup> that "the addition of a group X changes the partition coefficient by a given factor depending on the nature of the group, and on the pair of phases employed, *but not on the rest of the molecules*". MARTIN considers this "prediction contrary to usual expectation. It is usually felt that the formation of a derivative of greatly increased molecular weight will "swamp" any differences that exist and will render separation more difficult. This, however, is not to be expected if such a derivative be chosen that the same pair of phases can be employed while still maintaining convenient values for the partition coefficients". However, MARTIN has not taken into account that the resolution of two solutes is a function not only of the  $\Delta K$  values but also of the  $K$  values (see refs. 28 and 5), the resolution in general decreasing with a decrease in  $K$  values. In the case of molecular-sieve chromatography one may therefore expect the resolution to decrease when one goes up through a series of homologues, which is in agreement with what is found in practice (see for instance Fig. 3, ref. 29). This decrease in resolution with diminishing  $K$  values (*i.e.* with increasing values of the molecular weights  $M$ ) is still more pronounced for solutes (such as many proteins) whose chromatographic behaviour is determined by eqn. 15 instead of eqn. 14. This is evident from eqn. 23a which shows that  $\frac{d(-\log K)}{dM}$  is not constant (as in eqn. 22), but decreases with  $M$  ( $C_2 > 0$  according to Table I).

These considerations may partly explain why the resolution of proteins in molecular sieving is far inferior to that of low molecular weight homologues.

#### *Chromatographic parameters (Tables I and II)*

In Table I we have listed the values of the constants  $C_1$ ,  $C_2$ , and  $C_0$  for high molecular weight substances on loose gels, estimated as described under EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 15; the corresponding values of  $\Delta\Delta p = p_s - p_m - (p_s^0 - p_m^0)$ ,  $\Delta\Delta\gamma = \gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)$ , and  $f_s/f_m$  obtained from eqns. 16, 17, and 18 are also given.

The negative value of  $p_s - p_m - (p_s^0 - p_m^0)$  means that the difference in pressure between the stationary and mobile phase is larger in the standard state than in the actual experiment; the pressure in the gel must always be larger than outside<sup>10</sup>. According to Table I  $\Delta\Delta\gamma$  has a value of 0.1 dyne  $\text{cm}^{-1}$ . Direct measurements of the interfacial tension between two phases of aqueous polymers have given values of 0.003–0.1 dyne  $\text{cm}^{-1}$  (ref. 30). As expected the activity coefficient of a solute has about the same value in the stationary and in the mobile phase ( $f_s/f_m \sim 1$ ).

In Table II chromatographic data from experiments with low molecular weight homologues on tight gels are collected. The constants  $C_1'$ ,  $C_1$ , and  $C_0$  are those in

TABLE II

APPROXIMATE VALUES OF SOME CHROMATOGRAPHIC PARAMETERS FOR TIGHT GELS, SUITABLE FOR THE SEPARATION OF LOW MOLECULAR WEIGHT SUBSTANCES (HOMOLOGUES)

The constants  $C_1'$ ,  $C_1$ , and  $C_0$  are those in the formula  $-\log K = C_1' \cdot M + C_0$  (eqn. 14) and  $-\log K = C_1 \cdot M + C_0$  (eqn. 19).  $C_1'$  and  $C_1$  have the same numerical value but a different physical meaning. The values of  $\Delta Ap = p_s - p_m - (p_s^0 - p_m^0)$  refer to the alternative situation where eqn. 19 is applicable. The data used for the calculation of the different parameters are taken from the references given. All values listed in the table are so extremely approximate, that they only reflect the order of magnitude.

Solute	Solvent	Gel type	Reference	$C_1' \times 10^4$ ( $= C_1 \times 10^4$ ) (moles <sup>-1</sup> g)	$C_0$	$\Delta Ap$ (atm)	$f_e$  $f_m$
Polyhydric alcohols	0.05 M Tris-HCl, pH 7.6	Dextran gel, DVS 9	26	4	0.14	(+30)	1.4
Oligosaccharides	Water	Bio-Gel P-2	29	4	<sup>a</sup> —	(+30)	—
Isomaltodextrins	Water	Sephadex G-15	31	6	<sup>a</sup> —	(+50)	—
Isomaltodextrins	0.1 M Tris-HCl, pH 7.0 + 0.3 M NaCl	Sephadex G-15	31	7	<sup>a</sup> —	(+50)	—

<sup>a</sup>  $C_0$  values (and  $f_e/f_m$  values) could not be calculated for these experiments as only  $V_e$  and  $V_0$  values but not  $K$  values are reported.

eqns. 14 and 19. Knowledge of the value of  $C_1'$  does not permit calculation of the values of  $\Delta\Delta p$  and  $\Delta\Delta\gamma$ . The values of  $\Delta\Delta p$  given in Table II do not therefore refer to eqn. 14 but to eqn. 19. As it is difficult to decide if the latter equation is applicable, the  $\Delta\Delta p$  values have been put in parentheses. Fortunately eqns. 14 and 19 are formally very similar inasmuch as they predict that a straight line should be obtained when  $-\log K$  is plotted against  $M$  — a relationship which we have obtained experimentally in all the cases we have studied. It should be noted, however, that eqn. 14 and eqn. 19 give a different physical interpretation of the slope of the straight line. The ratio between the activity coefficients  $f_s/f_m$  seems to differ more from unity for hard gels (Table II) than for loose gels (Table I). This finding is to be expected.

#### *Verification of the formulae*

From Figs. 1–3 it is evident that the experimental data conform to the formulae derived. Another condition for the validity of the derived formulae is that they give values of  $\Delta\Delta\gamma$  and  $f_s/f_m$  which have the expected order of magnitude (see the above section *Chromatographic parameters*).

#### *Applicability of the thermodynamic considerations in areas other than chromatography*

The thermodynamic considerations presented in this paper have been centred around molecular-sieve chromatography but will — with due modifications — also apply to conventional two phase partition experiments, for instance those described by ALBERTSSON<sup>12</sup> from whose theoretical treatment of aqueous two-phase systems the author has obtained many ideas. In these systems one may put  $p_s = p_m$  (ref. 10); accordingly the constant  $C_1$  (eqn. 16) has the value zero, *i.e.* formulae similar to eqns. 14 and 20 are applicable (the constant  $C_1'$  in eqn. 14 consists in this case of only one term).

All the considerations throughout this paper are based upon the assumption of equilibrium between two phases and cannot therefore be used with any exactness for living systems. The treatment may, however, yield a qualitative picture of the solute distribution across a cell membrane when active transport can be neglected. Thus eqn. 10 shows that the concentration of a solute (positively charged) within the particles (for instance mitochondria or bacteria) can be extremely small if the electric potential and the pressure in the particle is sufficiently high and/or the interfacial tension  $\gamma$  for a solute is higher on the inside than the outside of the particle (see also ref. 26). Similar considerations might be applicable to fluids streaming in the semi-permeable capillaries in living tissue. It may be mentioned that the pressure in tightly cross-linked resin particles used for ion-exchange chromatography has been estimated to several hundred atm<sup>23</sup>.

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