The use of gel chromatography for the determination of sizes and relative molecular masses of proteins

Interpretation of calibration curves in terms of gel-pore-size distribution

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The separation of proteins by gel-exclusion chromatography has been explained in terms of partitioning of the macromolecules within the gel by a distribution of pores of various radii. The assumption that the distribution of pore sizes is Gaussian has led to the prediction of a linear relationship between the molecular Stokes radius (R_s) of the protein and the function $\text{erf}^{-1}(1-K_D)$, where K_D is the partition coefficient [Ackers (1967) *J. Biol. Chem.* **242**, 3237–3238]. Since careful calibrations of classical (agarose and dextran) gels and h.p.l.c. gels have shown that such a linear relationship is not verified experimentally over a wide range of native protein sizes, we have reinvestigated the model of Ackers (above reference). We show that Ackers' (above reference) derivation is not valid except for a particular Gaussian distribution of pore sizes centred at the origin. Relaxation of this restriction to allow for other types of Gaussian distributions cannot account for the non-linear calibration curves that we have obtained. Instead we show that the pore-size distribution can be calculated from the experimentally determined function $K_D = f(R_s)$ and that this distribution is bimodal (non-Gaussian). One distribution is centred below 2 nm, whereas the mean value of the second one is around 6–8 nm. The minimum in this bimodal distribution corresponds, for some gels, to a region of poor resolution, which needs to be appreciated for the proper use of gel chromatography in the determination of molecular size.

INTRODUCTION

Gel-exclusion chromatography has been widely used not only to purify proteins but also to determine the sizes of macromolecules. It is now well established that, for classical gel chromatography and h.p.l.c., the elution behaviour of proteins is better related to their Stokes radii (R_s) than to their relative molecular masses (M_r) [see le Maire *et al.* (1986) and references cited therein]. If R_s varies in a predictable manner with M_r (for instance in a denaturing solvent such as in SDS or guanidinium chloride), this last parameter can be obtained directly (Fish *et al.*, 1970; Mann & Fish, 1972). This is not the case for native proteins in dilute aqueous buffers because such proteins vary in shape and hydration, and no unique relationship between M_r and R_s exists. Nevertheless, as originally pointed out by Siegel & Monty (1966), M_r values of native proteins can be obtained from the Stokes radii if the sedimentation coefficients are measured independently. This combination of gel-exclusion chromatography and sedimentation velocity, which does not require as high a degree of protein purity as does sedimentation equilibrium, is particularly useful for detergent-solubilized membrane proteins (Tanford *et al.*, 1974).

Determination of the R_s of an unknown protein by gel chromatography thus requires a proper calibration of the column with proteins of known Stokes radii [see, for

example, the lists of standards given by Horiike *et al.* (1983) and le Maire *et al.* (1980, 1986)]. An appropriate parameter used to characterize the elution position of a macromolecule is the partition coefficient, K_D , defined as:

$$K_D = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume of the protein, V_0 is the void volume of the column and V_t is the total volume of the column.

A model of the partitioning behaviour of a macromolecule in a gel-chromatography column has led to the prediction of a linear relationship between R_s and $\text{erf}^{-1}(1-K_D)$, where the function $\text{erf}^{-1}(x)$ is the inverse function of the probability integral:

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x e^{-x^2} dx$$

(Ackers, 1967, 1970; Vales & Ackers, 1979). A linear relationship was indeed reported for several gels (Nozaki *et al.*, 1976; Horiike *et al.*, 1982, 1983). If such a linear relationship always holds true, the calibration procedure is simplified, since only two standards are needed. But unfortunately, this is not the case: we have shown that if a large number of native proteins covering a wide range of Stokes radii are used to calibrate classical gels as well as h.p.l.c. columns the relationship between R_s and $\text{erf}^{-1}(1-K_D)$ is, in fact, non-linear (le Maire *et al.*, 1980,

Abbreviation used: C₁₂E₈, the non-ionic detergent 'dodecyl octa(ethylene glycol) monoether'.

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1986). We show here that there is an error in Ackers' (1967) derivation so that it is valid only under special conditions.

Nevertheless, the general features of Ackers' (1967) model are attractive in that they provide a simple framework for the understanding of gel chromatography without any assumption concerning a hypothetical structure of the gel pores. Accordingly, we have chosen to reinvestigate some of the features of this model on the basis of our previously published experimental data. We show that, in all cases, the data can be accounted for by the existence of a bimodal distribution of pore sizes in the gels.

MATERIALS AND METHODS

Chromatography

For classical gel chromatography we have used the data obtained on columns (1.5 cm × 90 cm) of Sepharose 6B-CL, 6B, 4B, and Sephacryl S-300, as previously published by le Maire *et al.* (1980). The buffers were: 0.01 M-Tes (pH 7.5)/0.1 M-KCl/0.1 mM-CaCl₂ for the Sepharose 6B-CL, 0.01 M-Taps (pH 8)/0.1 M-KCl/1 mM-EDTA/1 mM-NaN₃ for the Sepharose 6B, 0.01 M-Tes (pH 7.5)/0.05 M-NaCl/0.1 mM CaCl₂ for the Sephacryl S300, 0.01 M-phosphate (pH 7)/1 mM-NaN₃ for the Sepharose 4B. H.p.l.c. was carried out using a Beckman model 344 chromatography system equipped with a model 160 absorbance detector and a 280 nm filter. The data obtained by le Maire *et al.* (1986) from two types of columns (0.75 cm × 30 cm) were used: TSK 3000SW (silica-based support) and TSK 4000PW [poly(vinyl alcohol) matrix]. The buffer was 0.03 M-Hepes/0.01 M-imidazole/0.2 M-sodium acetate (pH 7.0)/0.1 mM-CaCl₂/C₁₂E₈ (0.5 mg/ml). Detergent was used because part of the previous work was performed with solubilized membrane proteins in their native state (le Maire *et al.*, 1986), and C₁₂E₈ is a particularly adequate detergent for this purpose (le Maire *et al.*, 1976; Møller *et al.*, 1986). In any case, low concentrations of non-denaturing detergents do not affect the elution behaviour of the water-soluble proteins (Nozaki *et al.*, 1976; le Maire *et al.*, 1980, 1986).

The standard proteins were of globular shape and covered the R_s range 1.7–8.6 nm (le Maire *et al.*, 1980, 1986); lists of these proteins, with their respective R_s values, are given in the Figure legends. As previously stated, the R_s values were ascertained in all cases by analytical ultracentrifugation (Beckman Model E or MSE Centriscan 75) performed on the eluted proteins. Cobalamin (C₆₃H₈₈O₁₄N₁₄PCo, M_r 1355) was used as a standard with a lower R_s than the proteins. X-ray-diffraction analysis characterizes this molecule as having an approximately spherical shape (Hodgkin *et al.*, 1956). The effective R_s was taken as 0.8–0.9 nm (8–9 Å) from X-ray diffraction structure and a hydrodynamic estimation based on a partial specific volume of 0.72 cm³/g as estimated from the mass/proton ratio (Møller *et al.*, 1986) and frictional ratio ($f/f_{\min}$) of 1.1–1.2. The calibrations used did not include the solubilized membrane proteins, whose behaviour on columns is slightly different from that of the water-soluble proteins. As markers of V_0 we used macromolecules, either Blue Dextran or large DNA fragments, whereas V_t was measured by using dithiothreitol, NaNO₃, LiNO₃ or ³H₂O.

Numerical treatment

The function $K_D = f(R_s)$ was approximated by fitting the experimental points with a polynomial, using a computer program written for an Apple II microcomputer by Dr. N. Kellershon and based on the method of orthogonal polynomials (Sørensen & Schack, 1972). The program offers the possibility of weighting differently certain points in order to get a better fit of the curve with the experimental data. From this polynomial the pore-size distribution ($-dK_D/dR_s$; see the next subsection) is then easily calculated.

THEORY AND RESULTS

Ackers' (1967) derivation is not valid for all Gaussian distributions

Let us first summarize the model of Ackers (1967). If the column is operating close to equilibrium conditions, and the protein does not interact with the gel, K_D can be written as:

$$K_D = \frac{V_e - V_0}{V_t - V_0} = V_p/V_i$$

where V_i is the internal volume of the column and V_p is the gel volume that is accessible to the solute. Ackers' (1967) model is based on three assumptions. The first two assumptions are: (1) the micro-regions within the gel are heterogeneous in size; (2) the individual regions may be characterized by the radius, a , of the largest molecule that it can accommodate. Before coming to the third assumption let us define with Ackers (1967) a function $\Phi(a)$, such that $\Phi(a) \cdot da = dV_p/V_i$ is the increase in volume fraction penetrable by solute molecules when their radius decreases from a to $a - da$. Thus $\Phi(a)$ represents a frequency distribution of pore sizes. The fraction of the gel volume that is inaccessible to a species of radius a is the surface under the distribution curve Φ , from pore size zero to pore size a ; conversely, the fraction of the gel volume that can be occupied by a species of radius a is the surface under the curve Φ , extending outward from a .

$$K_D(a) = \int_a^\infty \Phi(a) \cdot da = 1 - \int_0^a \Phi(a) \cdot da \quad (1)$$

The third assumption of the model is that the distribution of pore size $\Phi(a)$ is a Gaussian probability curve. If a_0 is the maximum value of the distribution and if b_0 is the standard deviation, from eqn. (1) Ackers (1967) derives the formula:

$$R_s = a_0 + b_0 \cdot \text{erf}^{-1}(1 - K_D) = a_0 + b_0 \cdot \text{erfc}^{-1}(K_D) \quad (2)$$

where erfc is the error function complement and R_s replaces a (which is reasonably valid for globular proteins). In fact, there is an error in Ackers' (1967) derivation of eqn. (2). The general form of a Gaussian distribution is given by:

$$y = \frac{A}{\sqrt{2\pi}b_0} e^{-\frac{(x-a_0)^2}{2b_0^2}} \quad (3)$$

where A is fixed by the normalization condition:

$$\int_0^\infty y \cdot dx = 1$$

(1) for $a_0 = 0$, the normalization condition gives $A = 2$. From eqn. (1) one gets:

$$R_s = \sqrt{2} \cdot b_0 \cdot \text{erfc}^{-1}(K_D) \quad (4)$$

(2) for $a_0 > 0$, the normalization condition gives:

$$A = \frac{2}{1 + \operatorname{erf}\left(\frac{a_0}{\sqrt{2} b_0}\right)}$$

(5)

From eqn. (1), one gets:
for $R_S \geq a_0$:

$$K_D = 1 - \frac{A}{2} \left[\frac{2}{\sqrt{\pi}} \int_0^{a_0/\sqrt{2} b_0} e^{-x^2} dx + \frac{2}{\sqrt{\pi}} \int_0^{(R_S - a_0)/\sqrt{2} b_0} e^{-x^2} dx \right]$$

(6a)

for $R_S \leq a_0$:

$$K_D = 1 - \frac{A}{2} \left[\frac{2}{\sqrt{\pi}} \int_0^{a_0/\sqrt{2} b_0} e^{-x^2} dx - \frac{2}{\sqrt{\pi}} \int_0^{(a_0 - R_S)/\sqrt{2} b_0} e^{-x^2} dx \right]$$

(6b)

which leads to:
for $R_S \geq a_0$:

$$R_S = a_0 + b_0 \cdot \sqrt{2} \cdot \operatorname{erfc}^{-1} [K_D (1 + \operatorname{erf} a_0/\sqrt{2} \cdot b_0)]$$

(7a)

for $R_S \leq a_0$:

$$R_S = a_0 - b_0 \cdot \sqrt{2} \cdot \operatorname{erf}^{-1} [K_D (1 + \operatorname{erf} a_0/\sqrt{2} \cdot b_0) - 1]$$

(7b)

Fig. 1. Comparisons between theoretical and experimental calibration curves based on the assumption of a Gaussian distribution of pore sizes

The left-hand part of the Figure depicts the variation of Stokes radius (expressed as R_s/b_0) as a function of $\operatorname{erf}^{-1}(1 - K_D)$ for various values of a_0/b_0 ($a_0 > 0$). The broken line illustrates that curves are displaced upwards if the pore distribution stops before zero, corresponding to a minimum effective pore radius of the gel, $a_{\min.}$, as shown in the inset (see the text; in the example given, $a_0/b_0 = 3$ and $a_{\min.} = b_0$). The right-hand part of the Figure shows calibration curves based on water-soluble globular proteins (essentially the data of le Maire *et al.*, 1980) and cobalamin for Sepharose 4B (×), Sepharose 6B-CL (●) and Sephacryl S-300 (○). The following standards, which may be identified in the Figure by their R_s values, were used.

Protein	Source	R_s (nm)
Cobalamin	—	0.85
Cytochrome c	Horse heart	1.7
Ribonuclease II-A	Bovine pancreas	1.75
Myoglobin	Horse skeletal muscle	1.9
Haemoglobin	Human	2.4
Ovalbumin	Chicken egg	2.8
Alkaline phosphatase	<i>Escherichia coli</i>	3.3
Albumin	Bovine serum	3.5
Transferrin (iron-free)	Human	3.6
Aldolase	Rabbit muscle	4.6
Catalase	Ox liver	5.2
Aspartate transcarbamylase	<i>Escherichia coli</i>	6.0
Ferritin	Horse spleen	6.3
β -Galactosidase	<i>Escherichia coli</i>	6.9
Thyroglobulin	Ox thyroid	8.6

Fig. 2. Calibration curves and pore-size distribution for classical gel columns

(a), (b) and (c) show K_D plotted against native-protein Stokes radius (R_S) for, respectively, Sepharose 6B-CL, 6B and 4B. The points refer to data reported by le Maire *et al.* (1980), except that ferritin was added in (a) and (c) and ribonuclease in (c). Proteins may be identified on the basis of their R_S values (see the legend of Fig. 1) and that of NADPH: sulphite reductase from *Escherichia coli*, an additional standard protein (R_S 8.65 nm). The continuous line is the fit obtained with a polynomial. (a'), (b') and (c') show pore-size distributions (Φ) as a function of R_S , for Sepharose 6B-CL, 6B and 4B respectively, obtained as the derivative (multiplied by -1) of the polynomials in (a), (b) and (c).

It can be seen that R_S is not a linear function of $\text{erfc}^{-1}(K_D)$, except for $a_0 = 0$ or in the limit where a_0/b_0 tends to zero.

(3) In a similar way it can be shown that $a_0 < 0$ also leads to a non-linear relation between R_S and $\text{erfc}^{-1}(K_D)$.

Nozaki *et al.* (1976) have already mentioned that eqn. (2) is only valid for $a_0 = 0$. This situation is not likely to be encountered in practice, since low- M_r molecules of different size, such as $^3\text{H}_2\text{O}$ and vitamin B_{12} , are observed to be eluted at exactly the same position on agarose and dextran gels. Apparently for such substances the gel matrix does not provide sufficiently small pores or pore recesses to discriminate between the size of these molecules. As a result a distribution function can only be defined for $R_S \geq a_{\min.}$, where $a_{\min.}$ denotes the smallest effective pore size of the gel. In that case the relevant equations are still (7a) and (7b), with the exception that $\text{erf } a_0/\sqrt{2} \cdot b_0$ becomes $\text{erf } (a_0 - a_{\min.})/\sqrt{2} \cdot b_0$. That $a_0 \neq 0$ can also be inferred from results plotted according to eqn. (2), which most often show that lines drawn through the points intersect the ordinate above zero [e.g. Fig. 2 of Ackers (1967)].

In order to evaluate if deviations from linearity can be attributed to normal distributions with $a_0 > 0$, we have compared theoretical curves with those determined by experiment (Fig. 1). For any given value of a_0/b_0 a unique relationship exists between R_S/b_0 and $\text{erf}^{-1}(1 - K_D)$ that can be calculated either from eqns. (6a) and (6b) or (7a) and (7b). The result of such calculations is shown in the left side of Fig. 1, whereas actual data for R_S versus $\text{erf}^{-1}(1 - K_D)$ on various gels are shown on the right side of Fig. 1, drawn to about the same scale. It is seen that in no case can the non-linear course of the experimental curves be accounted for by a normal distribution with $a_0 > 0$. This is true whatever scale is chosen for R_S/b_0 (not shown). In particular the theoretical curves do not reproduce the steep rise in the middle region of the plot. Extending the assumptions to take into account the probable existence of an $a_{\min.}$, where the pore-size distribution function stops (see inset to Fig. 1), only affects the initial part of the curves. This is demonstrated by the broken curve in Fig. 1 for $a_0/b_0 = 3$ and $a_{\min.} = b_0$. Similarly, for $a_0 < 0$, no correspondence with actual data can be obtained (not shown).

Fig. 3. Calibration curves and pore-size distributions for h.p.l.c.

(a) and (b) show K_D plotted against native-protein Stokes radius (R_s) respectively for TSK 3000 SW and TSK 4000 PW. The points refer to data given by le Maire *et al.* (1986). Proteins may be identified on the basis of their R_s values (see the legend to Fig. 1) and those of the following additional standard proteins: trypsin inhibitor from soybean, R_s 2.2 nm; β -lactoglobulin A and B from bovine milk, R_s 2.75 nm; tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*, R_s 4.6 nm. The continuous line is the fit obtained with a polynomial. (a') and (b') show pore-size distribution (Φ) as a function of R_s , for TSK 3000 SW and TSK 4000 PW respectively, obtained as the derivative (multiplied by -1) of the polynomials in (a) and (b).

Direct determination of pore-size distribution

As an alternative to assumptions *a priori*, one may empirically determine gel pore distributions from the experimental data. To do so let us first examine the relation between K_D and R_s for various agarose gels (Figs. 2a, 2b and 2c). In the middle portion of the plot even large variations in Stokes radius do not produce appreciable changes in K_D . Such a behaviour suggests that there are few pore sizes in the gel that match with the size of the proteins that are eluted in this region. The pore-size distribution can be estimated by taking the first derivative of eqn. (1) and replacing a with R_s to obtain:

$$dK_D/dR_s = -\Phi(R_s)$$

Thus the desired distribution function, Φ , is obtained from the derivative of $K_D = f(R_s)$. In order to calculate the derivative of K_D with respect to R_s we have fitted the experimental points with a polynomial whose derivative can then be formally calculated. The continuous lines shown in Figs. 2(a), 2(b) and 2(c) are the fit obtained with a polynomial of degree 4 or 5, over the range from $R_s = 1.7$ nm to $R_s = 8.6$ or 6.9 nm. These are the lowest degrees of polynomials which give a reasonable fit to the data as judged from visual inspection. Figs. 2(a'), 2(b') and 2(c') show in each case the function Φ , obtained as the derivative of the polynomial with respect to R_s (multiplied by -1). It should be noted that at high R_s values there are few calibration points and thus it is difficult to obtain a proper fit in this region; this leads in some cases (Figs. 2c' and 3a') to negative values of Φ , which are meaningless.

Provided that the basic assumptions that we made (equilibrium conditions, no gel-protein interactions) are

valid, the function Φ thus actually represents for each column the pore-size distribution. It is obvious that none of these functions are Gaussian in nature. At least two sets of pore size are defined by the data, with a trough in the 3–4 nm region.

Fig. 3 shows application of the same kind of analysis to h.p.l.c. data. These also give evidence of a bimodal distribution of pore size. However, the trough is not as pronounced as for classical gels, which in part could account for the better resolving power of h.p.l.c. as against classical gels.

DISCUSSION

Many models of gel-exclusion chromatography have been proposed (Ackers, 1967; Yau *et al.*, 1978). Ackers' (1967) model, based on the concept of heterogeneity of pore sizes, has the merit of relating K_D and R_s in a physically simple way. The assumption of a normal distribution of pore sizes led him to predict a linear relationship between R_s and $\text{erf}^{-1}(1 - K_D)$. We have shown, however, that a linear relationship is only true for a particular Gaussian distribution with the maximum of the distribution coinciding with the origin of the plot.

Retaining the basic aspects of Ackers' (1967) model and starting from an experimental determination of the relationship between R_s and K_D , we have been able to calculate the pore-size distribution for various gels. A non-Gaussian distribution was obtained in all cases. Although each type of column gave a different distribution, the overall shape was the same. Our results are best described in terms of the superimposition of two distributions of pore sizes centred on different mean values, the smaller one being outside the protein

calibration range and the larger one being in the 6–8 nm region. In other words, the gel seem to consist of two sets of pore sizes whose distributions overlap to various extents (Figs. 2 and 3). In fact, the presence of two sets of pore sizes was already suggested on intuitive grounds for classical gels (le Maire *et al.*, 1980). The existence of a large population of pores at less than 2 nm is not surprising; for example, agarose is thought to occur as a network of intersecting bundles of double helices which define the boundaries of the pores (Arnott *et al.*, 1974). Optical analysis and microscopic examination reveal the presence of regions of high concentrations of agarose polysaccharide which may correspond to a set of smaller pores (Pines & Prines, 1973). The larger pores may correspond to regions of relatively low concentration of polysaccharide.

Up to this point we have only considered the simplest and most common case in gel filtration, i.e. when there is no (or very little) gel–protein interaction. In fact there are instances of interaction leading to retardation of the eluted protein, presumably mainly as the result of electrostatic interaction between positively charged groups on basic proteins and residual negative charges on, e.g., agarose and Sephacryl gels. This interaction tends to an increase in the measured K_D . Contrarily, negative interactions (apparent repulsions) have been observed for negatively charged proteins at low ionic strength and for detergent-solubilized membrane proteins (le Maire *et al.*, 1980, 1986), giving rise to a lower K_D than expected on the basis of their known Stokes radii. In all cases of interaction, the shape of the function Φ does not any longer represent only a pore size distribution. For example, a maximum of this function at low R_s might be an indication of increasing gel–protein interaction with decreasing M_r , superimposed on the normal exclusion behaviour. This could arise, e.g., because of closer interaction between small molecules with charges located at the interior of the gel particles. On the other hand, for a larger molecule, binding affinity could be increased because of increased electrostatic interaction on the surface of the gel, due to a larger number of charged groups on the macromolecule. If the resultant interaction, positive or negative, remains effectively the same for a series of standards, i.e. independent of R_s , the function Φ remains the expression of an apparent pore size distribution, with the pore size being respectively larger and smaller than that expected purely on the basis of steric hindrance.

Failing to realize the biphasic behaviour of chromatographic gels, and relying on the elution behaviour of a few standard proteins, may result in misleading values for R_s , particularly in the middle region of the plot, which otherwise would be considered to be the optimal range. It may also result in less-clear-cut separations than expected. For instance, it is not possible, according to our experience, to separate on classical gels the $C_{12}E_8$ -solubilized monomer from the dimer of two membrane proteins, the sarcoplasmic-reticulum Ca^{2+} -ATPase (le Maire & Møller, 1986) and lactose permease of *Escherichia coli* (Houssin *et al.*, 1985). This is probably attributable to elution of both species (monomer and dimer) in the plateau region of agarose and dextran gels (Figs. 2a–2c).

The non-Gaussian distribution also implies that the plot R_s versus $\text{erf}^{-1}(1 - K_D)$, which is still used by many laboratories (e.g. Horiike *et al.*, 1983; Robinson & Talbert, 1986), including ours (le Maire *et al.*, 1986), is not justified and does not present any advantage over the direct plot R_s versus K_D . Thus a complete calibration is necessary for each column. This is easily performed in the case of h.p.l.c., which should be increasingly used in the future. Finally the approach outlined here, because it allows an easy visualization of the distribution of pore sizes, could be of some help to gel manufacturers. Indeed it would be desirable to design a gel with a single pore-size distribution for better resolution over a wide range of protein sizes.

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