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Diffusion of proteins in Sepharose Cl-B gels

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ABSTRACT

The reduced diffusion coefficient, D/D_0, of fluorescein-labelled globular proteins in the agarose gels Sepharose Cl-2B, 4B and 6B were measured by the FRAP method. Comparison of the partition coefficients of the native and the labelled proteins in the gel showed that the fluorescein residues did not introduce new interactions between the solute and the gel matrix. D/D_0 decreased as a function of the Stokes radius. The variation of D/D_0 as a function of the partition coefficient of the proteins in the gel did not agree with a previously published prediction. This is in contrast with the diffusion of globular proteins in ACA34 gel, in which the sieving matrix is made of cross-linked polyacrylamide.

INTRODUCTION

The diffusion coefficient of a solute in gel beads is one of the factors that determine the efficiency of the separation of macromolecules by gel chromatography [1]. Only a few measurements of the diffusion of macromolecules in gels have been published until now [2–4]. In previous work, we determined the reduced coefficient, D/D_0, of the diffusion of fluorescein-labelled dextran fractions in Sephadex gel beads [5] and of fluorescein-labelled proteins in the ACA34 gel [6]. The technique used was the fluorescence recovery after photobleaching (FRAP) [5]. It was found that D/D_0 of the proteins in the gel AC34 decreased exponentially as the Stokes radius of the molecules increased. Further, D/D_0 varied as a function of the partition coefficient of the proteins in the gel, as predicted by the theory of Ogston *et al.* [7]. In this work, the same method was used for measuring the diffusion of fluorescein-labelled globular proteins in three Sepharose Cl-B gels.

The sieving properties of ACA34 gel are those of its polyacrylamide component, which means that the gel behaves as a microporous gel. On the other hand, the Sepharose Cl-B gels are essentially composed of agarose. The structure of agarose gels has been described as reticulated [8].

EXPERIMENTAL

The origin of the protein samples and of the fluorescein isothiocyanate isomer I (FITC) was as described previously [6]. Sepharose Cl-6B (Lot KI 38317), Cl-4B (Lot MB 01610) and Cl-2B (Lot 5204) were purchased from Pharmacia (Upsala, Sweden).

The technique of protein labelling with FITC has already been described, and the values of the labelling ratios of these samples were given [6]. Dextran blue was purchased from Pharmacia and DNA of phage lambda was purchased from Sigma.

The chromatography of proteins was performed on columns (90 × 1.35 cm) filled with the gels studied. Each column was equilibrated with buffer A [10 m*M* sodium phosphate–0.15 *M* sodium chloride–1 m*M* sodium azide (pH 8).

The partition coefficient was determined by the equation of Laurent and Killander [9]:

$$K_{AV} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

where V_e is the elution volume, V_t the volume of the gel bed in the column and V_0 the void volume. V_0 was determined by the chromatography of DNA of

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phage lambda. With Sepharose C1-6B dextran blue was also used.

Microfluorimetric measurements of the partition coefficient

The gel beads were immersed in a solution of a fluorescent protein, contained in a small optical cuvette which was placed on the microscope stage of the FRAP apparatus, as described [5]. The fluorimetric partition coefficient was defined by the following expression [5]:

$$K_f = F_B/F_f \quad (2)$$

where F_B and F_f are the fluorescences measured with the microscope–photometer assembly centred on a bead and on the free solvent respectively. K_f is equal to K_{AV} when the quantum efficiency of the macromolecule fluorescence is the same in the bead and in the free solvent. The diameter of the gel beads was in the range 100–150 μm. As mentioned previously, there was a stray fluorescence which came from the contribution of the fluorescent track of the laser beam in the solvent surrounding the bead [5,6]. The measured intensity, F'_B, was related to the corrected intensity by

$$F'_B = F_B + \alpha F_f$$

where α is a constant. If one puts

$$K'_f = F'_B/F_f$$

then

$$K_f = K'_f - \alpha(1 - K'_f) \quad (3)$$

We determined α by measuring the apparent fluorimetric partition coefficient of a high-molecular-weight fluorescein dextran excluded from the gel. The value of α was 0.04.

Measurement of the reduced diffusion coefficient

The relative diffusion coefficient of labelled proteins in a gel bead was determined by FRAP, as already described [5,6]. FRAP curves were recorded with the microscope alternately centred on a gel bead and on the surrounding solvent. These curves were analysed with the following function [10]:

$$F(t) = \frac{F_0 + F_\infty\, t/t_{\frac{1}{2}}}{1 + t/t_{\frac{1}{2}}} \quad (4)$$

where F_0 and F_∞ are the fluorescence intensities emitted immediately after the bleaching pulse and at a infite time after bleaching, respectively, and $t_{\frac{1}{2}}$ is the half-time of the fluorescence recovery.

The bleaching fraction was defined as

$$B = (F_i - F_0)/F_i \quad (5)$$

and the fraction of freely diffusing molecules (or recovery fraction), by

$$L = (F_\infty - F_0)/(F_i - F_0) \quad (6)$$

where F_i is the prebleaching fluorescence intensity. The reduced diffusion coefficient was obtained by applying the following relationship [5,6]:

$$\frac{D}{D_0} = \frac{(t_{\frac{1}{2}})_f}{(t_{\frac{1}{2}})_b} \cdot \frac{\beta_b}{\beta_f} \quad (7)$$

where D and D_0 are the diffusion coefficients of the solute in the gel and in the free solvent, respectively, $(t_{\frac{1}{2}})_f$ and $(t_{\frac{1}{2}})_b$ are the half-times of the fluorescence recovery and β_f and β_b are factors (depending on B) [10] measured in the free solvent and in the bead, respectively. Eqn. 4 is a very good approximation of the theoretical function of Axelrod *et al.* [11] provided that $B < 0.7$, which always applied in our experiments. Eqn. 7 then gives accurately the ratio D/D_0. The values of β were given as function of B by Yguerabide *et al.* [10].

We have shown that the influence of the stray fluorescence on the FRAP curves determined with the microscope centred on the bead depended on the value of K_{AV} [6]. In this work, this stray fluorescence was negligible as K_{AV} was $\geqslant 0.3$ in all instances [6].

RESULTS

Chromatography of proteins on a gel column

Six native proteins, namely ribonuclease A, α-chymotrysinogen A, ovalbumin, bovine serum albumin, aldolase and thyroglobulin, were chromatographed on columns of Sepharose C1-2B, 4B and 6B. These proteins were labelled with fluorescein isothiocyanate and purified as described previously [6]. The labelled proteins were also chromatographed on the Sepharose C1-6B column. Table I shows that the partition coefficients of a labelled and of the corresponding native protein were identical within the experimental accuracy.

TABLE I

PARTITION COEFFICIENTS OF NATIVE AND LABELLED PROTEINS IN SEPHAROSE CL-6B GEL

Protein	K_{AV}	
	Native	Labelled
Ribonuclease	0.80	0.87
Chymotrypsinogen A	0.71	0.76
Ovalbumin	0.58	0.57
Aldolase	0.49	0.50
Thyroglobulin	0.29	0.31

In addition, we measured the microfluorimetric partition coefficient K_f of the labelled proteins on the three Sepharose gels, and found that it was equal to the K_{AV} of the parent native protein (Figs. 1–3). These results showed that the fluorescein residues did not induce any interaction between the labelled proteins and the gel matrix.

Determination of the reduced diffusion coefficient of proteins in a bead of Sepharose gel

This was performed by FRAP as described under Experimental. The bleaching fraction was about 0.5. After its fitting, eqn. 4 satisfactorily reproduced the experimental curves. In every instance we found that the fraction of fluorescence recovery L was equal to 1. This showed that there was no slow exchange between a free species and a species bound to the gel matrix. The reduced diffusion coefficient D/D_0 was determined as described above.

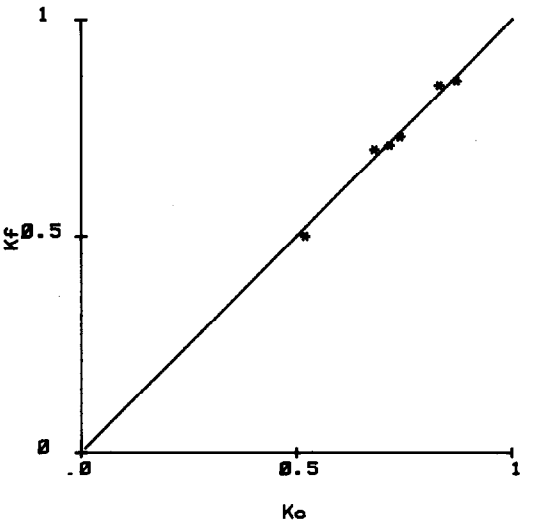

Fig. 2. Variation of the K_f as a function of the K_{AV} of native proteins in Sepharose Cl-4B gel.

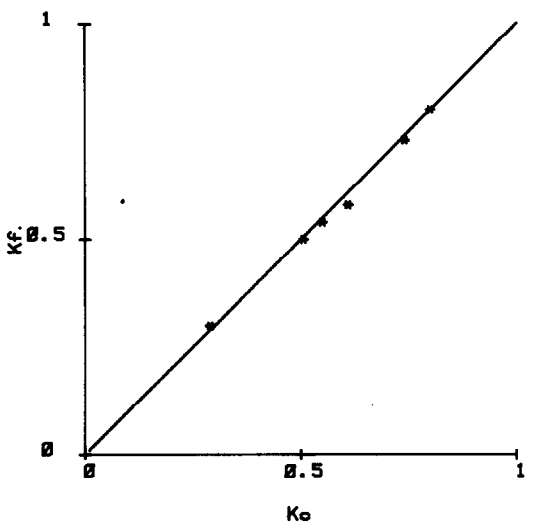

Fig. 1. Variation of the microfluorimetric partition coefficient K_f of labelled proteins as a function of the chromatographic partition coefficient K_{AV} of the native proteins in Sepharose Cl-6B gel.

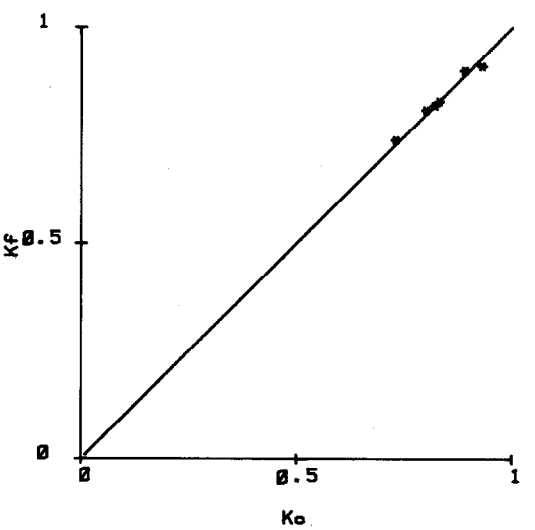

Fig. 3. Variation of the K_f as a function of the K_{AV} of native proteins in Sepharose Cl-2B gel.

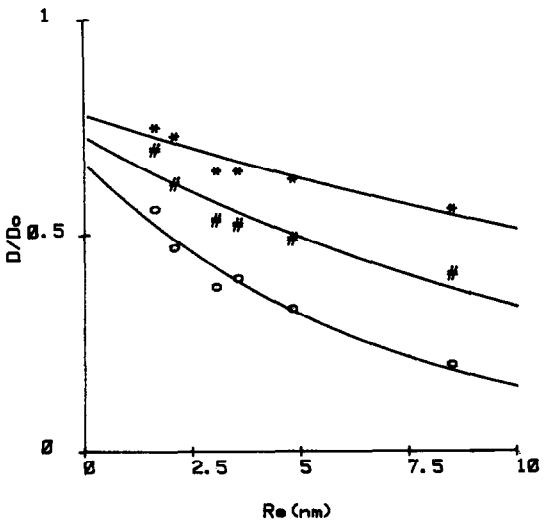

Fig. 4. Variation of the reduced diffusion coefficient of the fluorescein-labelled proteins as a function of their Stokes radius in (○) Sepharose Cl-6B, (#) Cl-4B and (*) Cl-2B. The continuous curves represent the fitted eqn. 9.

The variation of D/D_0 is plotted as a function of the Stokes radius of the protein in Fig. 4 and as a function of K_{AV} in Fig. 5.

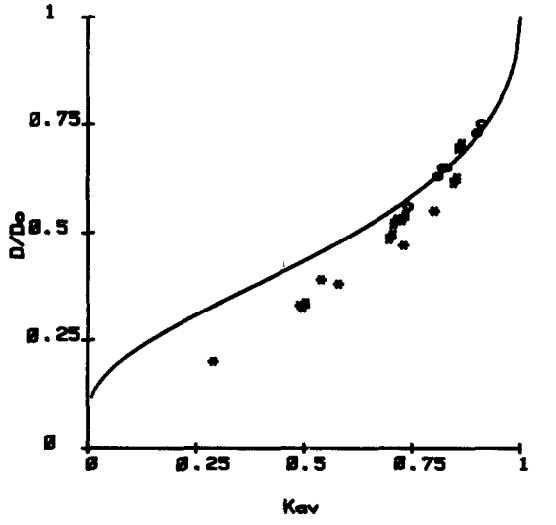

Fig. 5. Variation of the reduced diffusion coefficient of the proteins as a function of their partition coefficient in (○) Sepharose Cl-2B, (#) Cl-4B and (*) Cl-6B. The continuous curve represents eqn. 12.

DISCUSSION

The partition coefficient measurements showed that the fluorescein residues do not induce interactions between the labelled proteins and the matrix of the Sepharose Cl-B gels. Therefore, the diffusion coefficient of the labelled proteins determined by FRAP is equal to the diffusion coefficient of the parent native proteins.

For solute molecules which do not bind to the gel matrix, there are two possible causes of their diffusion retardation by the gel: the hydrodynamic effect and the obstruction effect [4]. The present theories of diffusion in gels assume that the solute molecules are spherical. These theories rest on simplified models of the gel matrix and lead to expressions of D/D_0 as functions of the molecular radius.

We first tried to apply the equation of Faxen derived from a hydrodynamic theory [12]. It did not represent our data satisfactorily.

The gel model of Ogston [13] consists of a random network of straight fibres. The partition coefficient of spherical molecules between the gel and the free solution is given by the following equation [9]:

$$K_{AV} = \exp\left[-\pi l\,(R_f + R_s)^2\right] \tag{8}$$

where R_f is the radius of the cross-section, l the linear concentration of the fibres and R_s is the Stokes radius of the solute molecule. The same matrix model was used in Ogston *et al.*'s theory of diffusion in gels [7]. In addition, the retardation was ascribed to the obstruction effect. The equation proposed by Ogston *et al.* can be written as:

$$D/D_0 = A \exp\left(-BR_s\right) \tag{9}$$

where

$$A = \exp\left[-(\pi l)^{\frac{1}{2}} R_f\right] \tag{10}$$

$$B = (\pi l)^{\frac{1}{2}}$$

We fitted the parameters A and B of eqn. 9 to our experimental data by the non-linear least-squares method. The parameter values are given in Table II and the corresponding calculated curves are shown in Fig. 4.

From A and B, R_f and l could be calculated by eqn. 10. Finally, the average radius of the cavities of the gels could be calculated according to the following equation [13,14]:

$$R_c = 0.5\, l^{-\frac{1}{2}} - R_f \tag{11}$$

TABLE II

DIFFUSION OF GLOBULAR PROTEINS IN SEPHAROSE C1-B GELS

Parameter values of eqns. 9, 10 and 11 fitted to the experimental variation of D/D_0 as a function of R_s.

Sepharose gel	A	B (nm^{-1})	$l \times 10^4$ (nm^{-2})	R_f (nm)	R_c (nm)
C1-2B	0.78 ± 0.03	0.042 ± 0.008	5.6	5.9	31
C1-4B	0.73 ± 0.03	0.078 ± 0.016	19.3	4.0	16
C1-6B	0.67 ± 0.05	0.15 ± 0.02	72.6	2.7	8

These parameters are also given in Table II.

The theory of Cukier [15] is based on hydrodynamic interactions. The results can also be expressed by eqn. 9 in which $A = 1$. This last prediction is obviously not verified by our data (see Table II). The theory of Altenberg and Tirrel [16] takes both the hydrodynamic and the obstruction effects into account. In this theory, the gel model is constituted of fixed spheres randomly distributed in a viscous fluid.

By fitting the theoretical equation of these authors to our experimental data, we found that the hydrodynamic terms were negligible. The function of Altenberg and Tirrel did not fit our data as well as eqn. 9.

Taken together, these analyses suggest that the obstruction effect may play an important role in the diffusion retardation of proteins in Sepharose C1-B gels.

A relationship between the reduced diffusion coefficient and the partition coefficient can be obtained from eqn. 8–10 [7]:

$$D/D_0 = \exp\left[-(\ln K_{AV})^{\frac{1}{2}}\right] \quad (12)$$

The curve representing the theoretical function 12 is shown in Fig. 5. It can be seen that the points representing the experimental data corresponding to large values of K_{AV} ($K_{AV} > 0.8$) are on the theoretical curve. For smaller values of K_{AV}, the experimental points are below the theoretical curve. The difference between the experimentral and theoretical points increases when K_{AV} decreases. For a given K_{AV}, the diffusion is more retarded than is predicted by the theory.

In contrast, we found in previous work that eqn. 12 represented the diffusion measurements of proteins in ACA34 gel fairly well [6]. The sieving properties of the ACA34 gel are ascribed to its polyacrylamide component. The fibres of polyacrylamide gels are composed of single or a small number of polymer molecules [14]. These fibres are cross-linked by covalent bonds. On the other hand, the fibres of Sepharose consist of aggregates of a large number of agarose molecules linked by hydrogen bonds [17–19]. The structure has been described as reticulated [8].

Ogston's model of gels seems more appropriate to describe the sieving properties of microporous gels such as polyacrylamide gels and cross-linked dextran gels than reticulated gels such as agarose [8,9,14,20]. This may explain why eqn. 12 describes the results of experiments with ACA34 gel better than those with sepharose C1-B gels.

The diffusion of dextran fractions with a Stokes radius between 33 and 100 nm have been studied in gels containing 0.3–4% of agarose using the quasi-elastic laser light-scattering method (QELS) [2]. For gels containing 2% and 4% of agarose (equal to the nominal concentrations of Sepharose C1-2B and C1-4B, respectively), the variation of D/D_0 as a function of the partition coefficient is qualitatively similar to the experimental data in Fig. 5, that is the experimental points are on the curve representing eqn. 12 for high partition coefficients and below this curve for small partition coefficients. With the FRAP method we also obtained similar results by measuring the diffusion of fluorescein-labelled dextran in Sepharose C1-B (results not shown).

It should be noted, however, that the diffusion coefficient of a solute in gels may depend of the probe distance associated with the method of measurement [21]. In QELS, the probe distance is a few tenths of a micrometre whereas in our FRAP measurements it is of the order of 20 μm. Further exper-

iments may reveal systematic differences between the diffusion coefficients determined by these techniques and help to reach a better understanding of the diffusion mechanism in gels. In any case, FRAP measurement of proteins in chromatographic gels appears to be a useful and simple technique.

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