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Diffusion of proteins in the chromatographic gel AcA-34

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ABSTRACT

The reduced diffusion of globular proteins in the gel AcA-34 was measured. The proteins were labelled with fluorescein isothiocyanate. The partition coefficient of the labelled proteins in the gel was equal to that of the native proteins. This result means that the fluorescein residues do not induce any specific interaction between the macromolecules and the gel matrix. The D/D_0 value was measured by alternately determining the fluorescence recovery after photobleaching curves of the labelled protein solution either in the free state or included in a gel bead. Both values of D/D_0, as a function of the Stokes radius of the proteins and the partition coefficient of the proteins in the gel, agree with the values predicted previously. These results suggest that the retardation of the diffusion of the proteins in the AcA-34 gel is due to the obstruction effect of the gel matrix.

INTRODUCTION

One of the factors determining the efficiency of the separation of macromolecules by gel chromatography is the diffusion coefficient of the solute in the gel beads which fill the chromatographic column [1]. There are some similarities between diffusion in the gel and other transport properties which operate in separation methods such as sedimentation in polymer solutions and electrophoresis [2]. Measurements of diffusion in chromatographic gels are still scarce [3,4] and the physical basis of the retardation of diffusion by a gel matrix is not yet firmly established [5]. For these reasons, measurements of diffusion in gels are needed.

In previous work, these authors measured the reduced coefficient of diffusion D/D_0 (where D and D_0 are the diffusion coefficients of the solute in the gel and in the free solvent, respectively) of fluorescein-labelled dextran fractions in Sephadex gel beads [6]. The technique used was fluorescence recovery after photobleaching (FRAP) [7].

In this method, a small region of a volume containing a mobile fluorescent molecule is exposed to a brief intense pulse of light, thereby causing an irreversible photochemical bleaching of the fluorophores. The diffusion coefficient is determined by measuring the rate of recovery of fluorescence which results from transport into the bleached region from the unirradiated surroundings.

In this work, the same method was used to measure the diffusion of

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fluorescein-labelled globular proteins in the gel AcA-34. The variation of D/D_0 was studied as a function of the Stokes radius of the protein molecules. The partition coefficient of these proteins in the gel was also determined and the variation of D/D_0 as a function of the partition coefficient was measured.

MATERIALS AND METHODS

Origin of the products

Bovine pancreas ribonuclease (ref. B-5112S), bovine pancreas chymotrypsinogen A (ref. C-4879), hen egg albumin (ref. A-2512), bovine serum albumin (BSA) (ref. A-7638), rabbit muscle aldolase (ref. A-7145) and bovine thyroid thyroglobulin (ref. T-1001) were purchased from Sigma. Catalase and ferritin were used from a gel filtration calibration kit from Pharmacia. Fluorescein isothiocyanate isomer I (FITC) was purchased from Molecular Probes (ref. F-143) and Sigma (ref. 7250). Dextran blue 2000 was from Pharmacia.

The Ultrogel AcA-34 was from Industrie Biologique Française (IBF) (lot 8379).

Macromolecule labelling

The proteins were labelled as follows: 50 mg of protein were dissolved in 2 ml of sodium hydrogencarbonate buffer solution at pH 9. A 2.6-ml volume of 1 mg/ml FITC in buffer solution A (10 m*M* sodium phosphate, 0.15 *M* sodium chloride, 1 m*M* sodium azide pH 8) was added. The mixed solution was stored at room temperature, away from light, for 5 h. The labelled protein was then dialysed against buffer A (pH 8) for 1–2 weeks in a cold room (4°C) with frequent changes of the dialysate. In the case of BSA, the following additional treatment was applied: the labelled protein was eluted on a Sephadex G-25 column with 20 m*M* acetic acid at pH 3 [8]. The solution was then dialysed for 24 h against buffer A with frequent changes of the dialysate.

The absence of free fluorescein in the preparations was checked by chromatography on a silica gel 60 plate (Merck ref. N-5724) with an eluent consisting of *n*-butanol–water–ethanol–acetic acid (50:20:20:10).

The labelling ratio was determined by spectrophotometry. The absorption coefficients of the fluorescein residues at 495 and 280 nm were taken as equal to 72 000 $cm^{-1}\ M^{-1}$ and 16 500 $cm^{-1}\ M^{-1}$, respectively [8]. The absorption coefficient of the proteins at 280 nm was taken from Fasman [9].

Dextran 2000 from Pharmacia (molecular weight 2 000 000) was labelled with FITC by the method of De Belder and Granath [10]. Fractions of fluorescein dextran, excluded from the AcA-34 gels, were obtained by chromatography on the gel Sepharose Cl-2B.

Gel chromatography

Native and labelled proteins were chromatographed on a column (90 × 1.35 cm) filled with the AcA-34 gel. The column was equilibrated with buffer A. A 2-ml volume of a solution of a protein at a concentration of 5 mg/ml in the same buffer solution was injected onto the column. The volume of a fraction collected at the bottom of the column was about 2 ml; the exact volume was determined by weight. The flow-rate was 5–7 ml/h. The void volume, V_0, was determined by the elution of Dextran blue 2000 and absorption measurements at 305 nm. The elution diagrams of

the native and labelled proteins were obtained by measuring the absorbance of each fraction at 280 and 495 nm, respectively.

The partition coefficient of a protein sample (K_{AV}) was determined according to the formula of Laurent and Killander [11]:

$$K_{AV} = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

where V_t is the volume of the gel bed in the column and V_e the elution volume of the fraction situated at the peak of the elution diagram. V_t was determined from the height and diameter of the column.

Measurements of the reduced coefficient of diffusion

For these measurements the labelled protein solution was obtained by eluting the main chromatographic fraction of the AcA-34 column in a Sephadex G-25 column.

These measurements were performed with the FRAP apparatus previously described [6]. The laser beam was horizontal and crossed the vertical microscope axis at right-angles, in the object plane.

The gel beads were immersed in a solution of fluorescent macromolecules contained in a small glass cuvette. The FRAP curves were measured with the laser beam crossing a single gel bead and the surrounding solvent alternately. Both measurements were repeated from seven to twenty times.

The FRAP curves were fitted with the function of Yguerabide *et al.* [12]:

$$\mathrm{F}(t) = \frac{F_0 + F_\infty t/t_{1/2}}{1 + t/t_{1/2}} \tag{2}$$

where F_0, F_∞, are, respectively, the fluorescence intensities emitted immediately after the bleaching pulse and at an infinite time after bleaching. $t_{1/2}$ is the half-time of recovery and t is time.

Eqn. 2 was fitted by the non-linear least-squares method of Marquardt [23] on an IBM-PS2 computer.

The fraction of freely diffusing molecules (or recovery fraction), L, was calculated by the following formula:

$$L = (F_\infty - F_0)/(F_i - F_0) \tag{3}$$

where F_i is the intensity before bleaching.

The bleaching fraction (B) is defined as:

$$B = (F_i - F_0)/F_i \tag{4}$$

Eqn. 2 is an approximation of the function of Axelrod *et al.* [7] which correctly describes that function when the bleaching fraction is smaller than 0.85 [12].

The reduced diffusion coefficient of a protein in a gel was obtained by applying the following relationship [6]:

$$\left(\frac{D}{D_0}\right) = \left(\frac{(t_{1/2})_0}{t_{1/2}}\right) \cdot \left(\frac{\beta}{\beta_0}\right) \tag{5}$$

where D and D_0 are the coefficients of diffusion in the gel and in the free solvent, respectively; $t_{1/2}$, $(t_{1/2})_0$ are the half-times of recovery measured on the bead and on the surrounding solution, respectively; and the factor β or β_0 depends on the bleaching fraction of the molecules included in the bead or in the solvent, respectively. The values of β and β_0 have been determined by comparing the function of Axelrod *et al.* [7] with eqn. 2 [12].

It has already been noted that the fluorescence was not zero for fluorescein dextran fractions excluded from a gel [6]. This stray fluorescence came from the fluorescence track of the laser beam in the free solution surrounding the bead. This stray fluorescence did not influence the value of D/D_0 measured for the protein samples, except for the thyroglobulin sample which had a very low partition coefficient in the gel.

In the thyroglobulin sample the fluorescence of the macromolecules included in a bead was small and the stray fluorescence was therefore relatively important. To take this contribution into account, the FRAP measured on the bead immersed in a thyroglobulin solution was analysed with a sum of two terms, as follows:

$$\mathrm{F}(t) = \frac{F_0^1 + F_\infty^1 t/t_1}{1 + t/t_1} + \frac{F_0^2 + F_\infty^2 t/t_2}{1 + t/t_2} \tag{6}$$

where the sub- and superscripts 1 and 2 refer to the molecules surrounding the bead (stray fluorescence) and to the fluorescence of the molecules included in the gel, respectively.

Eqn. 6 is equivalent to eqn. 7:

$$\mathrm{F}(t) = C_1\left(\frac{F_0 + F_\infty t/t_1}{1 + t/t_1}\right) + (1 - C_1)\left(\frac{F_0 + F_\infty t/t_2}{1 + t/t_2}\right) \tag{7}$$

where

$$F_0 = F_0^1 + F_0^2$$

$$F_\infty = F_\infty^1 + F_\infty^2$$

$$C_1 = \frac{L_1}{L}\frac{B_1}{B}\frac{F_i^1}{F_i} \tag{8}$$

F_i^1, L_1 and B_1 are the prebleaching intensity, the recovery fraction and the bleaching fraction of the stray fluorescence.

It was found experimentally that L_1 and L were equal to 1 and that B_1 was approximately equal to B. Therefore eqn. 8 becomes:

$$C_1 = F_i^1/F_i \tag{9}$$

t_2 is the parameter which allows the calculation of D/D_0 (eqn. 5). t_2 was obtained by fitting eqn. 7 to the experimental FRAP. C_1 and t_1 were considered as fixed parameters and were determined as follows.

Gel beads were immersed in a solution of a fluorescent dextran fraction of high molecular weight which was excluded from the gel. The prebleaching fluorescences F_B on a bead and F_f in the free solution were measured successively. The same measurements were made on a bead immersed in a labelled thyroglobulin solution. C_1 was then given by the following relationship:

$$C_1 = \left(\frac{F_B}{F_f}\right)_e \Big/ \left(\frac{F_B}{F_f}\right)_p \tag{10}$$

where e and p refer to the dextran and protein solutions, respectively.

To obtain t_1 the half-time of recovery of FRAP curves measured on a bead and in the free solution of the high-molecular-weight dextran were measured successively. If α is the ratio of these half-times, t_1 is equal to the product of α by the $t_{1/2}$ value measured in the free solution of the labelled thyroglobulin.

RESULTS

Chromatography of proteins on a gel column

Eight native proteins, namely ribonuclease A, α-chymotrypsinogen A, ovalbumin, BSA, aldolase, catalase, ferritin and thyroglobulin were chromatographed on a column of AcA-34 gel. The partition coefficients, K_{AV}, of these proteins were calculated according to eqn. 1.

The proteins were labelled with FITC and purified. The study of labelled ferritin and catalase was not continued as a denaturation of the proteins occurring during the preparation.

Five of these labelled samples were also chromatographed on the same column. Table I shows that the partition coefficients of the labelled and native proteins were equal within the range of experimental accuracy.

For a macromolecule which does not bind to the gel matrix, the partition coefficient between the gel and the surrounding solution is equal to the volume fraction available to the macromolecule in the gel [11]. In the Ogston model the gel is made up

TABLE I

COMPARISON OF THE PARTITION COEFFICIENTS OF THE NATIVE AND LABELLED PROTEINS

Proteins	Labelling ratio	K_{AV}	
		Native	Labelled
Ribonuclease A	0.56	0.91	0.89
Chymotrypsinogen A	1.86	0.81	0.79
Ovalbumin	1.39	0.63	0.62
Serum albumin	1.9	0.52	–
Aldolase	4.8	0.40	0.37
Thyroglobulin	17	0.08	0.1

of a random network of straight fibres, and the partition coefficient of a spherical molecule can be written as [11,13]:

$$K_{AV} = \exp[-\pi l(R + R_f)^2] \tag{11}$$

where l is the total length of fibres per unit volume, R is the radius of the molecule and R_f is the radius of a fibre.

Eqn. 11 was fitted to the variation of the K_{AV} value of proteins in the AcA-34 gel as a function of the protein Stokes radius R_S (see Fig. 1). The values of R_S were taken from ref. 14. The following parameter values were obtained by the method of non-linear least-squares:

$$\begin{aligned} l &= 1.05 \pm 0.16\,\text{nm}^{-2} \\ R_f &= 0.5 \pm 0.3\,\text{nm} \end{aligned} \tag{12}$$

The AcA-34 gel consists of 3% polyacrylamide and 4% agarose gels [15]. Its sieving properties are expected to be those of the polyacrylamide component, the agarose component essentially giving the rigidity to the gel beads [16]. This is confirmed by comparing the l and R_f values of eqn. 12 to those obtained by protein chromatography on polyacrylamide [17]. This comparison shows that the AcA-34 gel has the sieving properties of a polyacrylamide gel with a low bisacrylamide content.

Determination of the reduced diffusion coefficient of proteins in a AcA-34 gel bead

This was performed by FRAP as described under Materials and methods. Eqn. 2 satisfactorily reproduced the experimental curves after curve-fitting. In every case it was found that the fraction of fluorescence recovery L was equal to 1. This showed that

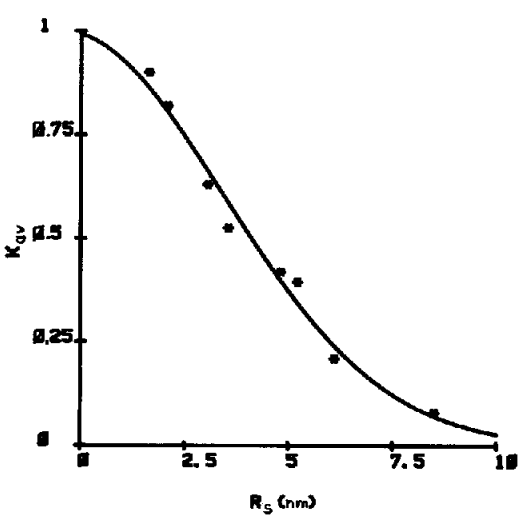

Fig. 1. Partition coefficient of globular proteins in the AcA-34 gel as a function of the Stokes radius of the protein. The continuous line represents eqn. 11 after curve-fitting.

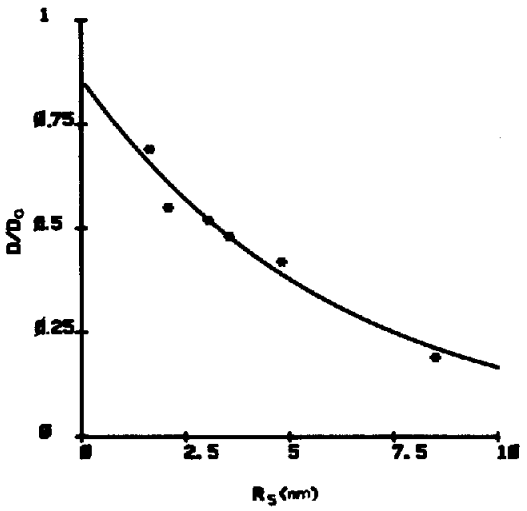

Fig. 2. Reduced diffusion coefficient of fluorescein-labelled proteins in the AcA-34 gel as a function of the Stokes radius of the protein. The continuous line represents eqn. 13 after curve-fitting.

there was no interaction between the proteins and the gel matrix, leading to a slow exchange between the free and the bound species. The reduced coefficient of diffusion was determined by eqn. 5.

In the case of thyroglobulin, which had a small K_{AV} value, there was a significant contribution of the stray fluorescence, and consequently the FRAP curves were fitted by eqn. 7.

The variation of D/D_0 as a function of R_S is plotted in Fig. 2, and as a function of K_{AV} in Fig. 3.

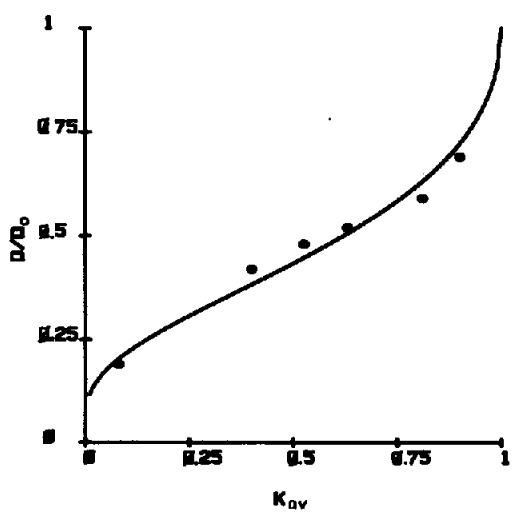

Fig. 3. Reduced diffusion coefficient of fluorescein-labelled proteins in AcA-34 gel as a function of the protein partition coefficient in the same gel. The continuous line represents eqn. 15.

DISCUSSION

The FRAP method is a convenient and relatively simple method for measuring the diffusion of fluorescent molecules in a single gel bead. This method was previously used to measure the diffusion of fluorescein dextran fractions in Sephadex gels [6]. The diffusion of labelled proteins in Sepharose was also measured [18].

It is necessary to label the macromolecules with covalently linked fluorescent residues. The diffusion coefficient of the parent non-labelled molecules in the gel will be equal to that of the labelled molecules if the fluorescent residues do not induce additional interactions with the gel matrix. This condition may be checked by chromatography as described earlier.

The fluorescent track produced by the laser beam in the free solution surrounding the bead brings about a stray fluorescence which becomes relatively important when the partition coefficient of the macromolecules in the gel is small (<0.1). Consequently the determination of the diffusion coefficient in the gel of such macromolecules becomes complicated and inaccurate.

It was found that the stray fluorescence decreased when the diameter of the bead increased. This stray fluorescence can also be decreased by reducing the diameter of the microscope photometer diaphragm. The volume of the illuminated region in the bead must be small compared to the total volume of the bead to avoid boundary effects on the diffusion [19].

From these considerations, it is concluded that there is a minimum diameter under which the diffusion coefficient in the gel becomes difficult to measure. This limiting size depends on the optical configuration of the laser beam and of the fluorescence measurement system. With this optical arrangement the bead diameter should not be smaller than 50 μm.

It was found that the partition coefficient of the fluorescein-labelled proteins in AcA-34 was equal to the partition coefficient of the native proteins. This shows that the fluorescein residues did not induce any interaction of the macromolecules with the gel matrix. As already discussed, the chromatographic properties of the protein in AcA-34 are explained by the sieve properties of its polyacrylamide component which does not contain charged groups. Furthermore, the salt concentration used in the elution buffer will decrease the electrostatic interactions. Fig. 2 shows that there was a regular decrease of D/D_0 as R_S increased, which cannot be explained by hydrophobic or hydrogen bonding. Such interactions are expected to depend on the primary, secondary and tertiary structure of the protein molecules and not on their molecular size. Two main effects may cause this behaviour [5]: (1) the hydrodynamic effect, in which the gel fibres increase the hydrodynamic drag exerted by the solvent on the solute molecule; and (2) the obstruction effect, in which the presence of impenetrable, immobile polymer molecules increases the path length of the solute diffusion. Several theoretical works deal with these physical principles and provide mathematical expressions of D/D_0 as a function of the radius of the solute molecules [20–22].

The parameters of these expressions were fitted to the experimental variation of D/D_0 as a function of the Stokes radius of the protein molecules. These calculations were performed by the non-linear least-squares method of Marquardt [23]. In this method the parameter values are determined which minimize χ_2, the sum of the square of the differences between the predicted and experimental D/D_0 values.

None of these theoretical formulae were satisfactory. After fitting them to the data the value of χ_2 remained high. In addition, the values of the parameters were not in agreement with their physical meanings. These discrepancies may be partly ascribed to the crudeness of the matrix models on which the calculations were based.

The matrix model of Ogston [13], which was used to derive eqn. 9 [11], appears to be more realistic. The same model supplied the basis of the theory of diffusion in gels [2]. According to this theory, the reduced diffusion coefficient may be written as follows:

$$\frac{D}{D_0} = A\exp(-BR) \tag{13}$$

where

$$A = \exp[-(\pi l R_f)^{1/2}]$$
$$B = (\pi l)^{1/2} \tag{14}$$

The curve representing eqn. 13 with A and B fitted to these experimental data is represented in Fig. 2. The χ_2 value is better than any value obtained with the other theoretical expressions quoted earlier. The values of A and B are in fairly good agreement with the values calculated from eqn. 14 when replacing l and R_f by the values obtained from the K_{AV} data (eqn. 12).

Combining eqns. 13 and 14 leads to the relationship [2]:

$$\frac{D}{D_0} = \exp[(-\ln K_{AV})^{1/2}] \tag{15}$$

As seen in Fig. 3, this function describes the experimental results reasonably well.

It may be concluded that:

(1) The partition coefficient of globular proteins in the AcA-34 gel is described well by the Laurent and Killander equation (eqn. 11).

(2) The variation of the reduced diffusion coefficient of the proteins in this gel, as a function of R_S, agrees with the prediction of Ogston *et al.* [2] (eqns. 13 and 14). The variation of D/D_0 as a function of K_{AV} is also in agreement with the theory (eqn. 15).

According to this theory the diffusion coefficient of a solute is decreased by the obstruction effect exerted by the gel matrix. Therefore these results suggest that the obstruction effect is an important cause of the diffusion retardation of the globular proteins in the AcA-34 gel.

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