DIFFUSION COEFFICIENT MEASUREMENT BY THE "STOP-FLOW" METHOD IN A 5% COLLAGEN GEL

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ABSTRACT We measured the translational bulk diffusion coefficient (D) of solute in a collagen gel column of 5% concentration (wt/wt) by a new, noninvasive method applicable to a wide range of solutes and gels. The system also enabled measurement of solute partition coefficients and convective flow velocity since the gel was contained within a chromatography column. The spread of diffusing solute in the gel column is measured during an interval of stopped flow in this method. Experimentally determined values of D/D° (free aqueous diffusion coefficient) ranged from 0.24 (^3H_2O) to 0.13 (ovalbumin) as anticipated by observations of other investigators from interstitium in heart and mesentery, but were significantly smaller than predicted by the widely used Ogston gel model with parameters extracted from partition coefficient data.

INTRODUCTION

Gels have long been considered to provide a barrier to the transport of solute (Friedman and Kraemer, 1930). We decided to measure solute diffusion coefficients in a collagen gel for the following reasons. Currently available theory regarding the barrier to diffusion of solutes in gels assumes that the gel phase comprises infinitely long, randomly oriented chains (Ogston et al., 1973), whereas collagen gel matrices are characterized by order (Walton and Blackwell, 1973). Further, we have independent evidence from partition coefficient studies that the Ogston gel model may not be applicable to collagen gels (Shaw and Schy, 1979*a*). Physiologists use this model to interpret transport studies in interstitial tissue on the basis of its demonstrated applicability to hyaluronate solutions despite the fact that collagen, not glycosaminoglycan, is the primary component of the interstitium and despite evidence that collagen is the major determinant of exclusion in the interstitium (Pearce and Laurent, 1977; Rutili, 1978; Fox and Wayland, 1979).

Various methods have been developed for measuring diffusion coefficients in gels, but these have limitations from our perspective. Some are invasive, (Shantz and Lauffer, 1962; Friedman and Kraemer, 1930), others give information on the diffusion coefficient alone (Shantz and Lauffer, 1962; Friedman and Kraemer, 1930; Ackers and Steere, 1962), or are indirect (McCabe, 1972; Comper and Preston, 1975 *a* and *b*). Diffusion coefficients of solutes in gel columns could be obtained in principle by measurement of solute concentration profiles in the gel column with optical-gel-column scanning techniques that are also useful for measuring other transport parameters (Ackers, 1975). However, application of those techniques to gel columns similar to the collagen gel column with which we are concerned is predicted to be very difficult owing to the opacity and inhomogeneity of the gel. This report describes a new method, the "stop-flow" method, without these limitations. A preliminary report of our results were given in Shaw and Schy, 1979*b*.

THEORY

A small solute-containing zone spreads as it is eluted through a gel column, owing to three processes: ordinary translational diffusion (Eq. 1), eddy diffusion, and local nonequilibria (Giddings, 1975). Giddings developed a theory for zone spreading on the basis that these processes are random and independent; this theory underlies our method of measuring diffusion coefficients in gels (Giddings, 1975).

Solute spreading around a planar cross-section of gel column, a composite of a stationary and a mobile phase, is measured by the variance, σ^2, of the axial concentration profile of the solute (Giddings, 1975):

$$\sigma^2 = 2Dt + Ld_p + 2LR(1 - R)vt_d + C_x. \quad (1)$$

The first term is the contribution to the variance from simple diffusion; the second and third terms arise from eddy diffusion and incomplete equilibration between the flow stream and the binding site ("local nonequilibria"), respectively. C_x is a term describing a small degree of coupling between the eddy diffusion (the second term) and the local nonequilibria (the third term) as was mentioned but not evaluated by Giddings (1975). In this expression, D is the bulk translational diffusion coefficient in the gel medium of the column, t is the time of column operation, L is the column length, d_p is the mean free path length between neighboring fluid eddies, R is the ratio of column void volume to solute elution volume, and t_d is the mean residence time of a solute molecule on the gel matrix.

We calculated the diffusion coefficient by comparing solute zone spreading in two experiments: (*a*) a control run in which buffer flowed continuously through the column, and the resulting solute variance is defined as σ_0^2, where

$$\sigma_0^2 = 2Dt + Ld_p + 2R(1 - R)vt_d + C_x, \quad (2)$$

and (2*b*) a stop-flow run identical in every way to the control run except that the flow through the column was interrupted for an interval, τ, of pure diffusion as the solute zone reached the column midpoint. In these experiments, τ was at least five times longer than the column running time, t. Fluid flow was maintained constant during column operation. The resulting variance, σ_1^2, in the stop-flow run is given by

$$\sigma_1^2 = 2D(t + \tau) + Ld_p + 2R(1 - R)vt_d + C_x, \quad (3)$$

where D, t, L, d_p, R, v, and t_d have magnitudes identical to those in the control run. Therefore, the diffusion coefficient can be calculated from

$$D = (\sigma_1^2 - \sigma_0^2)/2\tau. \quad (4)$$

See description in Procedures for operational definitions of σ_0^2 and σ_1^2.

EXPERIMENTAL

Materials

The collagen gel column was prepared and calibrated as described by Shaw and Schy (1979*a*). It was packed from purified collagen gel prepared from bovine Achilles tendon and cross-linked in glutaric dialdehyde. The degree of covalent cross-linking was ~2% and the overall concentration of collagen (5 ±

1%) (wt/wt). The column measured 1.6 × 36.4 cm. The void volume defined by elution of tobacco mosaic virus was 16.5 ml, and the total volume defined by elution of ^3H_2O was 36.5 ml. The column was equilibrated with 0.01 M phosphate buffer, pH 7.3, containing 0.15 M NaCl.

Diffusion coefficients were measured for the following solutes: FITC-dextrans (M_w 19,400 and M_n 17,400; M_w 2,900 and M_n 2,140), Ficoll (M_w 9,000 and M_n 8,000), ovalbumin and ^3H_2O. (Worthington Biochemical Corp., Freehold, N.J.) FITC-dextrans and Ficoll from K. A. Granath, Pharmacia AB, Uppsala, Sweden.

Apparatus

Eluant buffer was pumped through the column with a peristaltic pump (Gilson HP4, Gilson Medical Instruments, Middleton, Wis.). The concentration of UV-absorbing solutes in the buffer eluted from the column was minitored with a recording spectrophotometer (Gilson UV-RP); eluant containing polysaccharides was collected by fraction collector (Gilson "mini-escargot") for assay by the Anthrone technique (Scott and Melvin, 1953).

Procedures

The solute of interest was dissolved in 0.2 ml of buffer and applied to the column. The column was operated in the ascending flow mode. The mean flow rate, 4 ml/h, monitored periodically during a run, was found to vary by ± 3% at most. Experiments were conducted at 4°C.

The variance, σ^2, of the axial concentration profile of solute eluted from the column was obtained from (Cramer, 1946):

$$\sigma^2 = (Rv)^2 \int_{-\infty}^{\infty} (t - \bar{t})^2 c(t)\mathrm{d}t / \int_{-\infty}^{\infty} c(t)\mathrm{d}t \tag{5}$$

where $c(t)$ is the solute concentration in g/cm^3 monitored as a fraction of elution time t, in seconds, v is the velocity of eluting buffer in cm/s. amd R equals the column void volume divided by the elution volume of the solute. Keller and Giddings (1975) have shown that the product (Rv) is the convection velocity of the solute zone in the gel column. Eq. 6 defined $\bar{t}$, the mean time for solute elution:

$$\bar{t} = \int_{\infty}^{\infty} tc(t)\mathrm{d}t / \int_{\infty}^{\infty} c(t)\mathrm{d}t. \tag{6}$$

For a Gaussian concentration distribution, Eq. 5 reduces to

$$\sigma^2 = (Rv)^2 (W/vA)^2 = R^2W^2/A^2, \tag{7}$$

where W is half the width of the elution peak in cm^3 at 61% of peak maximum and A is the cross-sectional area of the column. For the more asymmetrical elution peaks, we compared the value of σ^2 calculated from Eqs. 5 and 7 and are found a difference of no more than 12%. It is always possible to calculate σ^2 with Eq. 5, but this, of course, is more time-consuming.

In the control run, the solute sample was applied to the column, pumped continuously through, and its elution volume and profile as functions of elution time were obtained. σ_0^2 was calculated numerically from Eqs. 5 or 6 or, where appropriate, from Eq. 7.

In the stop-flow run, a solute sample was applied to the column as in the control run, pumped to the midpoint of the column, defined as attained when the eluted buffer volume equaled half the elution volume of that solute in a control run, and the column was tied off for a period, τ, for pure diffusion of solute sample. At the end of the selected interval of diffusion, elution was resumed and continued until the sample peak was eluted from the outflow end of the column. From the elution profile, the parameter σ_1 was derived as discussed above for σ_0. The diffusion coefficient for the solute was then calculated from Eq. 4.

The experimentally determined values of diffusion coefficient D were divided by the values of free aqueous diffusion coefficient D^0 (Table I) for a comparison with predictions of the theory of Ogston et al., 1973.

TABLE I
DIFFUSION COEFFICIENTS, 5% COLLAGEN GEL

Solute	r_s, A°*	M_w‡	M_n‡	K_{av}§	D^0 (4°C) $\times 10^5$ cm^2/s	Reference for D^0*	D/D^0		
							Experimental‖ (see column 6 for D^0)	Ogston prediction¶ (Eq. 8)	Ratio of experimental to predicted
^3H_2O	0.94	18	18	1.0	1.38	**	0.24	0.85	0.28
Glucose	3.7	180	180	1	0.35	‡‡	0.19	0.82	0.23
FITC-3	14.0	2,900	2,140	0.97	0.093	§§	0.17 ± 0.02	0.71	0.24
FITC-20	34.0	19,400	17,400	0.66	0.038	‡‡	0.17	0.53	0.32
Ficoll-9	20.5	9000	8000	0.83	0.064	‖‖	0.17	0.65	0.26
Ovalbumin	27.6	45,000	45,000	0.74	0.046	¶¶	0.13	0.58	0.22
								Mean	0.26 ± 0.04

*Stokes' law was used in calculating the radius, r_s, from the free diffusion coefficient, D^0; $r_s = RT/(6\Pi\eta D^0)$. Each value of D^0 was interpolated from data obtained from the corresponding reference and converted to 4°C with Stokes' law.
‡M_w is the weight-averaged molecular weight. M_n is the number-averaged molecular weight. Values for FITC-3, FITC-20, and Ficoll-9 were obtained from Granath (personal communication).
§Partition coefficient K_{av} is given by $(V_e - V_0)/(V_t - V_0)$, where V_e, V_t and V_0 are the elution volume of the solute and total and void volumes of the column (Laurent and Killander, 1964).
‖The diffusion coefficient of FITC-3 is the mean of five determinations at different diffusion intervals. The other results were obtained from single observations.
¶Calculated from Eq. 8 with r_r (9.1×10^{-8} cm) and L (7.2×10^{11} cm^{-2}) obtained by Shaw and Schy (1979).
**Eisenberg and Kauzmann, 1969.
‡‡Longsworth, 1963.
§§Amu, 1979.
‖‖Laurent and Granath, 1967.
¶¶Tanford, 1961.

According to the Ogston theory, the ratio of D/D^0 is predicted by

$$D/D^0 = \exp[-\pi^{1/2} L^{1/2} (r_r + r_s)], \tag{8}$$

where L is the concentration in terms of length (cm/cm^3 of gel) of chains of radius r_r, *and* r_s is the Stokes radius of the solute. r_r and L are obtained by fitting partition coefficient (K_{av}) data from noninteracting solutes of known Stokes radius (Table I) in the gel by the expression (Laurent and Killander, 1964; Ogston, 1958):

$$K_{av} = \exp - [\pi L(r_r + r_s)^2]. \tag{9}$$

Included in the assumptions underlying this model are: the solute and gel do not interact; the gel can be represented by a solution of randomly oriented, infinitely long chains; and the partition coefficients, K_{av}, of noninteracting solutes can be fit by Eq. 9.

RESULTS AND CONCLUSIONS

The results of five observations on FITC-3 (see Table I) taken at differing diffusion intervals in the collagen gel column are plotted in Fig. 1. The diffusion coefficient for FITC-3, calculated from the slope of the line, is equal to $(1.6 \pm 0.2) \times 10^{-7}$ cm^2/s. The results from observations on all molecular species studied are given in Table I. Confidence in the results for species where only a single observation was taken is less than that for FITC-3; taken together, however, the data of Table I illustrate that the gel provides a substantial barrier to diffusion, even for smaller molecular species. The mean of experimental values of D/D^0 divided by

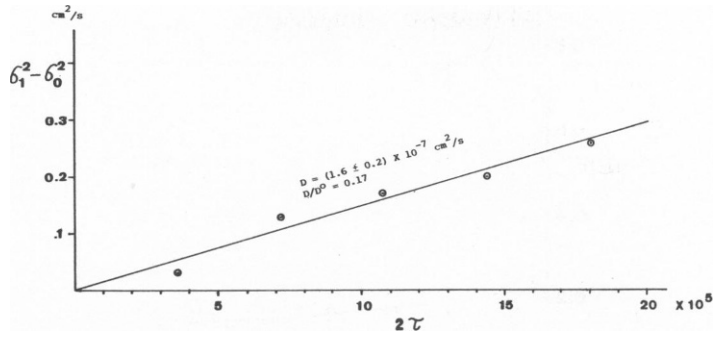

FIGURE 1 Dispersion from the control state vs. diffusion time (see Eq. 4). Data are for the diffusion of FITC-3 on the 5% collagen column. FITC-dextran, M_w 3,000, D^0 9.3 × 10^{-7} cm^2/S at 4°C; 5% collagen gel column. Time, t, is in seconds.

predicted values is 0.26 ± 0.04. Our data imply that the Ogston model for diffusion does not work for these solutes in the 5% collagen gel.

Before the observations were taken on FITC-3, the gel was removed from the column, divided into two equal volumes, and packed into two columns for determination of the effect of column repacking on the measured diffusion coefficient. The mean value of D was the same in both columns, but the degree of scatter in the values of $(\sigma_0^2 - \sigma_0^2)$ differed between the two columns, the scatter for the column not plotted in Fig. 1 being about two times greater than for the plotted data. Thus, repacking a column does not change the average diffusion characteristics but may make the column more difficult to study.

DISCUSSION

Measurements of diffusion of interstitially confined tracers in tissue have already anticipated the magnitude of our diffusion data in the collagen gel: Fox and Wayland (1979) report average values for D/D^0 of 0.25 and 0.20 for FITC-dextrans (M_w 3,400 and 19,000, respectively) in mesentery; Safford and Bassingthwaighte (1977) report 0.23 for sucrose in heart muscle, and Safford et al. (1978) 0.25 for water in heart muscle.

Our diffusion data do not, however, agree with the prediction of the Ogston model (Ogston et al., 1973), nor does this model successfully predict partition coefficient data for this type of collagen gel (Shaw and Schy, 1979*a*). Based on parameters derived from measured values of partition coefficient K_{av}, the Ogston model predicts values of D/D^0 four times larger than the measured values (Fig. 2). To fit the Ogston model to the diffusion data, one must increase the radius, r_f, of the gel fiber in Eq. 8 from the value 9.1 × 10^{-8} cm implied by the partition coefficient data to 1.0 × 10^{-6} cm, or assume that values of D^0 in the "free fluid phase" of the gel be reduced to 0.26 of true aqueous values. Each of these assumed manipulations would simulate modes of interaction within the system (gel matrix-solute-solvent), e.g., binding. Partition coefficient data did not give evidence of binding between the gel matrix and solutes including those used here (Shaw and Schy, 1979*a*). Possibly the diffusion measurement is more sensitive than the partition measurement to binding within the gel. The possibility also exists that some solvent is structured by the gel matrix so that there is no true free fluid phase. Westover and Dresden (1974) report evidence from nuclear magnetic resonance studies that

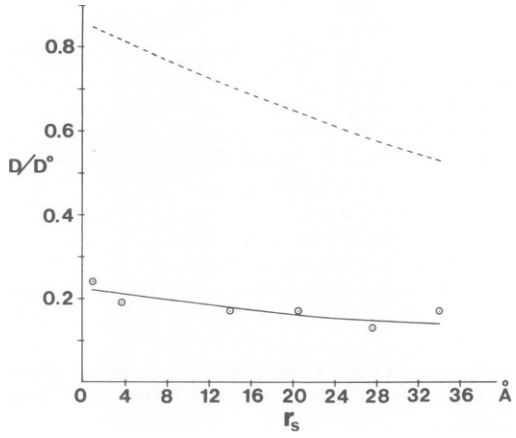

FIGURE 2 Experimental data. (---) Ogston model (Eq. 8) with parameters r_s and L derived from partition coefficients (K_{av}) (Table I and Shaw and Schy, 1979): $L(7 \times 10^{11}$ cm/cm^3) and $r_s(9.1 \times 10^{-8}$ cm). Ogston model with r_s adjusted to fit the data: $L(7 \times 10^{11}$ cm/cm^3) and $r_s(1.0 \times 10^{-6}$ cm).

there is "bound" water in collagen gels but it is not clear that the extent of this binding is sufficient to explain our observations (Fig. 2).

Alternatively, a solute with value of D/D^0 smaller than expected from the measured value of its partition coefficient may be encountering significant trapping within dead-end pores of the gel. Binding and sequestration of solute by a gel are kinetically equivalent and create apparently accessible space within the gel not contributing to translational diffusion (Goodknight and Fatt, 1961).

A major disadvantage of the stop-flow method for measuring the diffusion coefficient is the length of time involved for a complete study. The collagen gel with which we were working has an irregular bead size and a degree of inhomogeneity in cross-linking (Shaw and Schy, 1979*a*) and probably represents one of the more difficult gels to study. For gels with greater uniformity, this problem would be less severe since scatter in the data would be reduced and, consequently, useful information would be provided with shorter diffusion times and fewer observations.

The gel chromatogrophy column is principally used for separation of molecular compounds, although it can also be used for studies of properties of gels and gel-solute interactions, effects of gels on chemical equilibria, and binding phenomena (Giddings, 1970; Ackers and Thompson, 1965; Hummel and Dreyer, 1962). In the present study yet another application of this system was demonstrated.

The authors would like to acknowledge the critical reading of Dr. Wylie I. Lee of the Center of Bioengineering and the Department of Biological Structure and Dr. James B. Bassingthwaighte of the Center of Bioengineering, University of Washington, Seattle. The research was supported by National Institutes of Health grant GM-24990.

Received for publication 20 March 1980 and in revised form 24 October 1980.

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