Molecular Exclusion and Restricted Diffusion Processes in Molecular-Sieve Chromatography*

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From experimental measurements of equilibrium solute partitioning and of corresponding column elution volumes with Sephadex gels, there appear to be two primary molecular sieving effects responsible for the commonly observed variation of elution position with molecular size. The first is a simple exclusion effect presumably due to dimensional heterogeneity of the gel interstices. This effect is of primary importance in the more tightly cross-linked G-75 and G-100 gels. The second effect is a steric and frictional interaction of the solute molecule with the gel matrix and plays an important role in the more loosely cross-linked G-200 gels and possibly also in the agar gels. For these columns a method of calibration is derived, involving one adjustable parameter, the effective gel pore radius. This parameter is calculated from the effluent peak position of a macromolecule of known Stokes radius. A column calibrated by this method can then be used for determination of Stokes radii or diffusion coefficients of other macromolecules and an upper limit for the hydrated molecular weight can be obtained. For systems that conform to the theory a separation coefficient for any two macromolecules of known radii or free diffusion coefficients can be calculated for a column of any effective gel pore radius. This separation coefficient then determines the column size necessary to obtain a desired separation volume between effluent peaks of the two molecular species.

A powerful method for separation and characterization of macromolecules has recently been provided by means of chromatographic columns having bed materials that consist of small porous particles of starch, dextran, agar, or polyacrylamide gels. The general technique has been variously termed "gel filtration" (Porath and Flodin, 1959), "molecular-sieve chromatography" (Hjertén and Mosbach, 1962), "restricted-diffusion chromatography" (Steere and Ackers, 1962), and "exclusion chromatography" (Pedersen, 1962). The basis of the separations attained by these columns rests in the fact that access to the interior of the bed material bead is governed by the size of the solute molecule. Thus in its simplest form, the column operates by diffusional partition of a solute between an interior stationary phase and a mobile exterior phase without the intervention of specific adsorption of the solute to the bed matrix. Although empirical correlations of elution volumes with molecular weight (Andrews, 1962; Hjertén, 1962; Whitaker, 1963), sedimentation coefficient (Flodin and Killander, 1962; Roskes and Thompson, 1963) and molecular radius (Steere and Ackers, 1962) have been obtained, there has been to date no detailed formulation of the mechanism of operation of the molecular-sieve column in terms of fundamental processes.

The basic equation describing the operational parameters of the column, in the form given by Gelotte (1960) is:

$$V_e = V_o + K_D V_i \qquad (1)$$

The effluent peak volume V_e of a particle is related to the void volume of the column V_o (consisting of the total volume of liquid exterior to the gel phase) and the volume V_i of unbound solvent internal to the gel phase. The "distribution coefficient" K_D then characterizes the interaction between the gel and an effluent molecular species and is found to satisfy the conditions:

- $K_D = 0$ for a particle when totally excluded from the gel phase
- $K_D = 1$ for a particle that diffuses freely with no restriction within the gel matrix
- $0 < K_D < 1$ for a penetrant macromolecule in the absence of specific interaction
- $K_D > 1$ when specific interaction effects sufficiently retard the particle's elution velocity within the column

The formulation of any mechanism of operation for the column depends on an interpretation of K_D in terms of molecular processes. One such mechanism, outlined qualitatively by Flodin (1962), presumes the existence of microregions of excluded volume which are greater for the larger penetrant molecules than for the smaller ones. By this mechanism equilibrium partitioning occurs between mobile exterior liquid and the fractional regions of nonexcluded volume within the gel bed. In contrast to common partition chromatography this is viewed as a volume-controlled partitioning with concentrations interior to the gel pores essentially identical to those of the external liquid phase. The column distribution coefficient K_D then represents the fraction of the interior volume available for distribution of the solute. For this model then

$$K_D = \frac{V_p}{V_i} \qquad (2)$$

where V_p is the nonexcluded volume.

An experimental test for this mechanism is provided by direct measurement, under equilibrium conditions, of the partitioning of a series of macromolecules between the gel and an external liquid phase. For a system of total volume V_t at equilibrium, consisting of a gel phase and an external liquid phase of volume V_l, the penetrable volume is given by:

$$V_p = \frac{Q_p}{C_p} = \frac{Q_t - Q_l}{C_p} = \frac{Q_t - C_o V_l}{C_p} \qquad (3)$$

where Q_p is the amount of solute partitioned into the gel phase, Q_t is the total amount of solute in the

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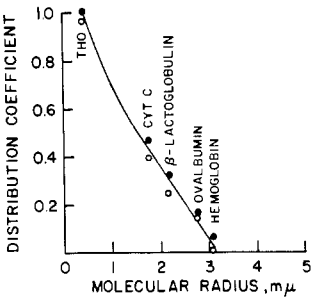

FIG. 1.—Comparison of equilibrium distribution coefficients (⊙) for Sephadex G-75 gels with corresponding column distribution coefficients (●). For calculation of column values the effluent volume of tobacco mosaic virus was taken as the column void volume V_o, and the internal volume V_i was taken as the difference between total column volume and V_o diminished by the percentage of the gel from which the tritium ion is excluded.

system, Q_l is the amount in the liquid phase external to the gel, C_p is the concentration within the pores of the gel, and C_o, that of the external liquid. Hence:

$$K_D = \frac{Q_t - C_o V_l}{C_p V_i} \qquad (4)$$

In order to relate the expression on the right (eq. 4) to experimentally measurable quantities, it is assumed in accord with this mechanism that $C_p = C_o$.[1] Then

$$K_D = \frac{Q_t/C_o - V_l}{V_i} \qquad (5)$$

Equation (5) provides a means of calculating a K_D interpreted as penetrable gel volume fraction from measurements of equilibrium solute partitioning.[2] For a column operating entirely by the molecular-exclusion principle, the parameter resulting from this calculation should be identical to the corresponding K_D of equation (1) which is derived from the column elution volume under nonequilibrium conditions and the relation

$$V_e - V_o = \frac{Q_t}{C_o} - V_l \qquad (6)$$

should be found. Data will be presented to show that, whereas for the Sephadex G-75 and G-100 gels such a correspondence is obtained, it does not hold for the more loosely cross-linked G-200 gels. For this material the differences in excluded volume are not sufficiently large to account for corresponding differences in column elution position. It is evident that a different mechanism must predominate in the G-200 gels.

This paper deals with the experimental measurement and interpretation of K_D and the formulation of a restricted diffusion mechanism for the operation of the molecular-sieve column in which K_D is interpreted as a kinetic parameter representing steric and frictional hindrance to diffusion. The Stokes radius of a macromolecule is then related to the operational parameters of the column. The theory provides a method for column calibration in terms of an effective gel pore size which can then be used to determine Stokes radii or diffusion coefficients of unknown macromolecules.

[1] The implications of this assumption for the measurements obtained in this investigation are discussed in a later section under RESULTS.

[2] The K_D used by Flodin is defined as the ratio of C_i, the effective solute concentration within the entire gel phase, to C_o. This expression for K_D can be obtained as a consequence of the present treatment: $Q_i = C_i V_i = C_o V_p$; $V_p/V_i = C_i/C_o = K_D$.

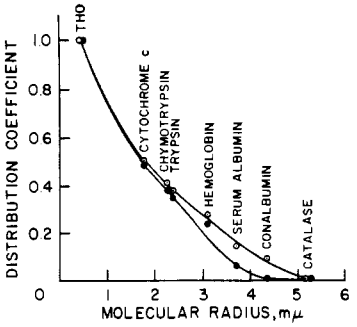

FIG. 2.—Column (●) and equilibrium (⊙) distribution coefficients for proteins on Sephadex G-100 gels.

EXPERIMENTAL

Measurements of the solute equilibrium distribution coefficients for Sephadex G-75, G-100, and G-200 gels were made with a series of macromolecules that were selected to span the range of elution volumes obtainable on the corresponding columns. Each column was made from the same batch of Sephadex as those used for the equilibrium experiments. Corresponding column distribution coefficients were obtained by measurement of elution volumes for samples of the same protein solutions for which the equilibrium distribution values were measured. Pyrophosphate buffer (0.1 M sodium pyrophosphate, *p*H 8.5) was used in all experiments. The dry gel was allowed to swell with buffer for 75 hours prior to packing of the columns. Glass columns 1.1 × 52.7 cm were fitted with a disk of porous polyethylene above the rubber stopper at the bottom and a disk of filter paper at the top. Each of the following proteins was suspended in and dialyzed against the buffer overnight: cytochrome c (Sigma), human carbon monoxide-hemoglobin, bovine serum albumin (Sigma), catalase (Sigma), ferritin (Sigma), southern bean mosaic virus (SBMV) and tobacco mosaic virus (TMV) (highly purified virus preparations supplied by R. L. Steere of the Plant Virology Laboratory, USDA, Beltsville, Md.), trypsin (Worthington), β-lactoglobulin (Sigma), ovalbumin (Sigma), and α-chymotrypsin (Sigma). The penetration experiments were carried out as follows. To each 50-ml volumetric flask was added 1.0 g of dry Sephadex G-200 powder or 2 g of G-75 or G-100, and 35.0 ml of buffer. The gel was allowed to swell at room temperature for 72 hours with occasional shaking. At the end of this time a 5.0-ml protein solution containing approximately 50 mg of solute was introduced and additional buffer was added to a total volume of 50.0. ml. Each flask was shaken periodically and allowed to stand at room temperature for an equilibration period of 24 hours. No detectable concentration differences were found for identically prepared flasks when equilibration was allowed to proceed for 1, 5, 24, and 36 hours. A period of 24 hours, therefore, represents a more than adequate equilibration period. After equilibration the gel was allowed to settle and a sample of liquid was withdrawn from the top of the flask and centrifuged 10 minutes at 500 *g* (to remove any small quantities of gel granules), and the optical density was measured at 280 mμ and 260 mμ by means of a Beckman DU spectrophotometer. In making these measurements each sample was read against a control solution from a flask to which only Sephadex and

buffer, but no protein, had been added, and an identical equilibration time allowed. Interference by ultraviolet-absorbing material released from the gel with time was thus avoided. Experiments with sodium chloride and tritiated water were carried out similarly to the protein measurements. Tritium was assayed by means of a Packard Tri-Carb liquid scintillation counter and sodium chloride by silver nitrate titration.

Equilibrium K_D values were calculated according to equation (5). V_l, the liquid volume exterior to the gel phase, was taken as the distribution volume for TMV, $(Q_t/C_o)_{TMV}$ and V_i, the internal volume, was taken as the difference between this value and the corresponding distribution volume for tritiated water $(Q_t/C_o)_{THO}$. Resulting K_D values for the different molecular species are given in Figures 1, 2, and 3 along with the nonequilibrium K_D values obtained from the column elution volumes. In making the column determinations, each protein sample consisted of 0.2 ml of supernatant liquid from an equilibrium determination. Thus the initial concentrations for the column determinations were the same as the equilibrium concentrations in the penetration experiments (generally about 1 mg/ml). Effluent peak positions were determined by means of a Vanguard 1056 A ultraviolet analyzer, recording at 220 mμ. From these data it is seen that both kinds of K_D are correlated with molecular Stokes radii of the protein species employed.

A series of experiments were performed with the G-200 gels to test for effects of protein concentration on column elution volumes and equilibrium partition coefficients. These experiments were performed identically to those described above with the exception that initial protein concentrations added were 0.1, 0.5, 1.0, and 50.0 mg/ml. Within the limits of experimental error these experiments yielded both column and equilibrium distribution coefficients that were identical to those shown in Figure 3.

A second series of experiments was performed with the G-200 column in an effort to vary the column flow rate. A stopcock attached to the bottom of the column permitted control of the flow between 1.5 and 6.0 ml/hour. The upper limit was imposed by packing the flaccid G-200 beads. Over this range of flow rates it was not possible to detect differences in elution volumes. The general insensitivity of elution position to flow rate for columns of this type is generally recognized (Flodin, 1962; Tiselius *et al.*, 1963).

The equilibrium distribution coefficients were found to be independent of the total volume and proportion of gel phase when measurements were made with a 12-ml system having a V_i of 4.0 ml and with a 25-ml system for which V_i was 14.7 ml.

Results

It is evident from the data of Figures 1 and 2 that for the more tightly cross-linked G-75 and G-100 gels the equilibrium distribution ratios closely parallel the column distribution coefficients and that these gels therefore conform to equation (6). For the G-75 gels (Fig. 1) the column values are slightly greater than the equilibrium values. Although the exact cause of this effect is not evident, it is known that special solute-matrix interaction effects may be enhanced in the more tightly cross-linked dextran gels. It is possible that adsorptive or other special effects in the column may be involved.

It is seen (Fig. 3) that a marked difference exists between the behavior of the G-200 gels and the more tightly cross-linked gels, and that the equilibrium solute distribution ratios do not parallel the variation in corresponding column K_D values with molecular

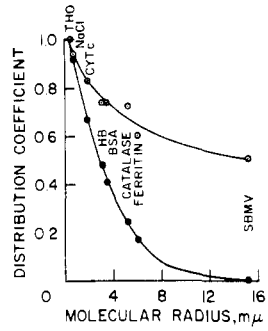

FIG. 3.—Column (●) and equilibrium (⊙) distribution coefficients for proteins on Sephadex G-200 gels.

size. These data show that, within the particle size range for which column separations occur, the equilibrium molecular exclusion effect is more sensitive to molecular size in the G-75 and G-100 gels than in the G-200 gels. This would suggest the presence of greater dimensional heterogeneity for the interstices of the more tightly cross-linked gels. For the G-100 gels the relation between static and column distribution coefficients is intermediate between that for the G-75 and G-200 gels. It appears more similar to the G-75 with equilibrium coefficients only slightly higher than the corresponding column coefficients. This greater similarity of behavior is in accord with the fact that these gels are closely similar in degree of cross-linking.

The implications of the assumption that solute concentrations within the gel pores are essentially the same as those of the external liquid phase can be assessed by consideration of the experimental results obtained. The parameter calculated to represent the penetrable volume fraction in the static experiments (eq. 5) is $Q_p/C_oV_i = V_pC_p/V_iC_o$. Equation 6 then, in slightly different form, can be written[3]

$$K_D = \frac{V_e - V_o}{V_i} = \frac{V_p C_p}{V_i C_o} \qquad (6a)$$

[3] If K_D is defined simply as the extent of solute equilibration with the gel phase, Q_p/V_iC_o, equation (1) is derived from this definition in the following way: For a thin section of the column having height δx, void volume $\alpha\delta x$, and internal gel volume $\delta V_i = \beta\delta x$ we have

$$K_D = \frac{Q_p}{V_i C} = \frac{Q}{\beta C}$$

where Q is the amount of solute within the gel per unit column length and C is the solute concentration within the void volume. Then, if V is the volume of liquid that has passed into the column, the conservation of mass requires that

$$\left(\frac{\partial C}{\partial x}\right)_v + \xi\left(\frac{\partial C}{\partial V}\right)_x = 0$$

where x is distance from the top of the column and $\xi = \alpha + \beta K_D$. Since we consider only the center (peak) of the solute zone and neglect zone spreading, the initial column load can be represented by a Dirac delta function, $D(x)$. The solution of the above conservation equation subject to the conditions: $V = 0, C = D(X)$; $V = 0, x = 0, C = 0$ is given by: $C = D(x - V/\xi)$. We have therefore the result that for any given volume V that has flowed through the column, the solute is distributed at the position:

$$X_v = \frac{V}{\alpha + \beta K_D}$$

Since $\alpha = V_o/l$, $\beta = V_i/l$ where l is the column's length

$$\frac{X_v}{l} = \frac{V}{V_o + K_D V_i}$$

when $X_v = l$, $V = V_e$ and the equation becomes

$$V_e = V_o + K_D V_i$$

Since for the more highly cross-linked gels conformity to this equation is obtained, it follows that, if $C_p \neq C_o$, $K_D \neq V_p/V_i$, and the excluded volume mechanism would not be valid for these columns. Thus the validity of this mechanism is dependent on the assumption of equal concentrations as well as the conformity of the gels to equation (6). For the G-200 gels (Fig. 2) equation (6a) must be replaced by the inequality:

$$\frac{V_e - V_o}{V_i} < \frac{V_p C_p}{V_i C_o} \qquad (7)$$

and the discrepancy between the terms of this inequality is seen (Fig. 3) to increase with molecular size. Consequently, if penetrable volume fractions do indeed equal column K_D values for the different molecular species, the variations in penetrable volume fraction are compensated by just the inverse variation in concentration ratios. This would require that the larger molecular species have the higher concentrations within the gel. On the contrary, it is the smaller protein molecules such as lysozyme, ribonuclease, and cytochrome c, that tend to exhibit marked adsorptive interactions in chromatography of this type. Even if such an anomalous concentration effect were to exist within the gel it would exactly cancel the operation of the excluded volume mechanism. It would appear that, since elution positions cannot be attributed to penetrable gel volume, a process other than simple molecular exclusion must govern column solute retention. For these columns, a mechanism based on steric and frictional hindrance to molecular diffusion within the gel matrix will be formulated and applied to the column data.

The restricted diffusion mechanism for the G-200 gel is based on a model in which the fluid regions within the gel particles are represented by uniform cylindrical channels. Such a model cannot, of course, represent the more complicated true structure of the gel matrix. However, its use can lead to a scheme for approximate prediction of the behavior of the real system. Each gel particle within the column presents a diffusion barrier to molecules in the exterior mobile fluid phase. For a penetrating molecular species this barrier can be characterized by two types of interaction: (1) If the molecular radius a is smaller than the pore radius r, the molecule can penetrate the gel only if its center passes within a virtual pore of radius $r - a$. The probability of penetration for a large molecular species in the region of a pore is therefore less than the corresponding probability for a small species where this steric hindrance is characterized by a larger virtual pore radius. (2) If a molecule does enter the channel, it encounters increased hydrodynamic frictional resistance to motion and therefore has a lower diffusion coefficient than in free solution. This type of diffusional restriction is also greater for a large molecular species than for a small one. Partitioning of a molecular species between liquid phases interior and exterior to the gel is then governed by these two types of diffusional restriction, which can be quantitated in the following way.

The Renkin equation (Renkin, 1955) relates the Stokes radius a of a macromolecule diffusing within a restrictive barrier of effective pore radius r to the equivalent free cross-sectional pore area A_o and effective actual area A_R available for diffusion:

$$\frac{A_R}{A_o} = \left(1 - \frac{a}{r}\right)^2\left[1 - 2.104\frac{a}{r} + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right] \qquad (8)$$

The first factor on the right is the steric term (Ferry, 1936). The second was derived on theoretical grounds by Faxén as a correction to Stokes' law for the case where the dimensions of the chamber through which a particle is moving are of the same order of magnitude as those of the particle itself (Faxén, 1922). The equation has been found to approximate the steric and frictional hindrance to diffusional migration within a number of systems for which the porous model employed in its derivation cannot be considered tenable as a structural representation (Goldstein and Solomon, 1960; Durbin, 1960).

Where specific interaction effects such as adsorption and ion exchange are minimized, a modified form of equation (8) has been shown to approximate the diffusional restriction encountered by several macromolecules during migration through thin agar gel membranes (Ackers and Steere, 1962). Its use can be extended to the case of a molecular-sieve chromatographic column composed of granulated agar or dextran gels such as are currently employed in many preparative and analytical procedures.

It is proposed that the effective solute distribution ratio is governed by steric and frictional hindrance to diffusion and that K_D is equal to the ratio A_R/A_o, of restricted to free areas available for diffusion. A means of testing this hypothesis is provided by combining equations (1) and (8). The relation is then written:

$$\frac{V_e - V_o}{V_i} = \left(1 - \frac{a}{r}\right)^2\left[1 - 2.104\frac{a}{r} + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right] \qquad (9)$$

For particles of known Stokes radius a the effective pore radius r within the gel is determined from the

TABLE I

VALUES OF r CALCULATED FROM SEPHADEX G-200 DATA[a]

Molecular Species	a (mμ)	r Calcd (mμ)
A. Data of Roskes and Thompson (1963)		
γ-Globulin	5.22[b]	20.9
Serum albumin (human)	3.62[c]	20.3
Hemoglobin (human)	3.08[d]	19.9
Mean effective pore radius		20.4
Mean deviation		1.8%
B. Data of Rogers and Thompson (1963)		
L-Glutamate dehydrogenase	6.40	18.6
Ferritin	6.00[e]	18.8
Catalase	5.22[f]	18.4
γ-Globulin	5.22[b]	18.2
Yeast alcohol dehydrogenase	4.55[g]	19.0
Glyceraldehyde-3-phosphate dehydrogenase	4.30[h]	19.2
Transferrin	4.00[e]	18.8
Lactic dehydrogenase (rat liver)	3.74[i]	15.1
Bovine mercaptalbumin	3.70[e]	18.6
Human serum albumin	3.61[c]	20.8
Human hemoglobin	3.08[d]	19.4
Cytochrome c	1.74[j]	18.3
Mean effective pore radius		18.7
Mean deviation		4.6%

[a] Stokes radii were calculated from free-diffusion coefficients except for the papilloma and f2 viruses in which electron micrographic particle diameters were used, and ferritin for which low-angle X-ray scattering measurements were used. [b] Nichol and Deutsch (1948). [c] Baldwin *et al.* (1955). [d] Lamm and Polson (1936). [e] Thompson (1956). [f] Sumner and Gralen (1938). [g] Hayes and Velick (1954). [h] Fox and Dandliker (1956). [i] Davisson *et al.* (1953). [j] Atlas *et al.* (1952).

TABLE II
VALUES OF r CALCULATED FROM AGAR GEL DATA

A. Data of Andrews (1962)

Molecular Species	Thyroglobulin	γ-Globulin	Bovine Serum Albumin	Lactalbumin		
a (mμ)	8.1[a]	5.22[b]	3.70[c]	2.02[a]		
Gel (%)	Values of r Calculated by Equation (9)				Mean r	Mean Deviation (%)
2.5	64.8	62.9	51.4		59.7	9.3
5	33.7	34.6	28.9	27.7	31.2	9.4
7	24.2	24.6	20.9	24.3	23.5	5.5
9	18.0	18.8	16.4	18.7	18.0	4.4
12	12.5	12.6	12.2	11.7	12.2	2.4

B. Data of Steere and Ackers (1962)

Molecular Species	Papilloma Virus	Southern Bean Mosaic Virus	f2 Coliphage	Ferritin	Hemoglobin		
a (mμ)	22.5[d]	15.4[e]	11.0[f]	6.0[g]	2.44[h]		
Gel (%)	Values of r Calculated by Equation (9)					Mean r	Mean Deviation (%)
2	112.1	115.0	115.0	92.4	106.2	108.1	6.5
3	77.5	83.2		99.9	66.0	81.6	12.1
5		60.5	55.0	50.0	69.7	58.8	10.7
6		53.7	51.9	57.2		54.3	3.6
7		33.1	22.0	31.7	32.5	29.8	13.2
8				27.8	29.8	28.8	3.5

[a] Polson (1937). [b] Nichol and Deutsch (1948). [c] Thompson (1956). [d] Williams (1953) by electron microscopy. [e] Miller and Price (1946). [f] Loeb and Zinder (1961) estimated from electron micrographs. [g] Fischbach and Anderegg (1963). [h] Mol wt determined by the empirical calibration method of Andrews gives a value of 30,000–35,000, consistent with the 32,000 obtained on Andrews' columns. Here the Stokes radius of a half-mer (mw: 34,000) is used.

column data. Comparison of values of r calculated for a column with effluent peak volume data for a series of different particles of known a provides a test of equation (9).

Conformity of G-200 and Agar Columns to the Theory. —Values of r calculated from data extant in the literature by means of equation (9) and the known Stokes radii are given in Table I for two sets of data obtained with Sephadex G-200 columns and in Table II for data from granulated agar columns. Validity of equation (9) to a first approximation is indicated by the generally good agreement of effective pore radii calculated from Stokes radii of different particles for a given column. The agreement obtained with the G-200 columns is seen to be excellent. Variations in actual r values for different columns may be due to differences in nature and preparation of bed materials or of buffer conditions employed. The Sephadex G-200 columns appear to conform somewhat more closely to the theory than do the agar columns. The agar columns are not as closely correlated with any of the molecular size parameters as are the Sephadex gels by either the empirical methods (Andrews, 1962; Steere and Ackers, 1962) or by the present method. It is known that ion-exchange interactions of proteins with the sulfate groups may occur in the agar gels even though the ion-exchange capacity is very low. Systematic deviations in calculated values of r may result from what appear to be even slight interaction effects. A case in point is the behavior of cytochrome c on the agar columns as shown from Andrews' data in Figure 4. As pointed out by Andrews (1962) an inverse variation in elution volume with ionic strength may be indicative of specific interaction.

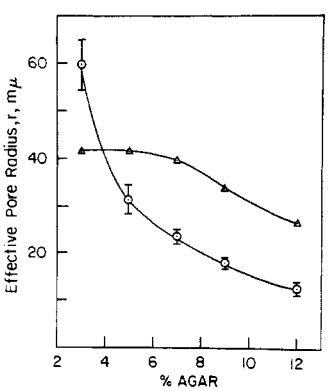

FIG. 4.—Effective gel-pore radii from Andrews' data. ⊙, mean values calculated from molecular species as given in Table II; △, values calculated from elution volumes of cytochrome c. Specific interaction effects between an effluent particle and the column bed may be detected by such deviations, provided a series of different particles is used for calibration.

Once r is known for a given column it should be possible to predict the column distribution coefficient (or elution position) of a molecular species from the Stokes radius by means of equation (9). Such theoretical distribution coefficients calculated for the proteins listed in Table IB are shown in Figure 5 as the solid line. The experimental K_D values are also plotted and it is seen that the agreement between the theoretical curve and the experimental distribution coefficients is excellent. For comparison, a plot of distribution coefficients against the logarithms of molecular weights is

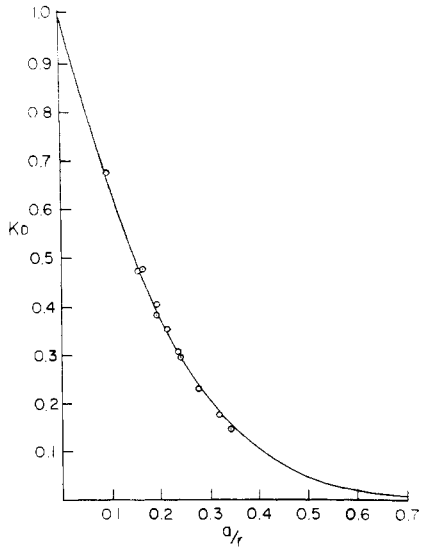

FIG. 5.—Test of equation (3). Theoretical column distribution coefficients predicted from the literature diffusion coefficients by equation (9) with r = 18.76 mμ are given by the solid line. Experimentally obtained values for the proteins shown in Table IB are plotted as points.

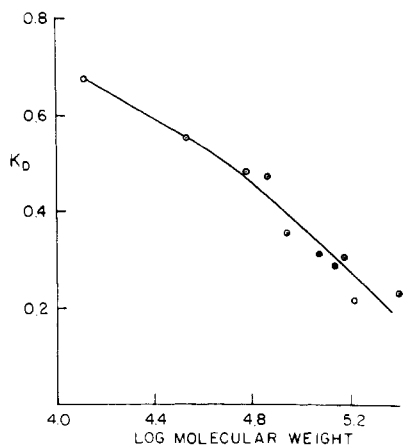

FIG. 6.—Correlation of column distribution coefficients with molecular weights. The data are for the proteins of Table IB. Molecular weights are taken from Edsall (1958).

given in Figure 6 and is seen to provide a much poorer correlation.

For systems that conform to equation (9) a fundamental limitation is placed on the maximum possible separation obtainable for two given macromolecules on a column of any specified size. From equation (1) the volume of separation between effluent peak positions of two macromolecules of radii $a > a'$ is:

$$V_s = V_e^{a'} - V_e^{a} = (K_D' - K_D)V_i \qquad (10)$$
$$= K_s V_i$$

where K_s is defined as the separation coefficient for the two molecular species. For given ratios of Stokes radii the separation coefficients calculated as a function of a/r are shown in Figure 7. Making use of these curves, a rational approach to the separation of two known particles on gels for which the effective pore size can be varied would be: (a) ascertain the maximum theoretical K_s for the two particles, (b) employ a bed material with the corresponding pore size r. Then if α is the ratio of total bed volume to internal volume for a column of the composition selected, the bed volume V_b required is given by:

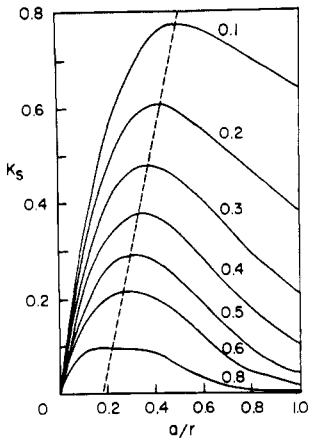

FIG. 7.—Separation coefficients for different ratios a'/a of molecular radii as a function of the particle to pore-radius ratio a/r.

$$V_b = \alpha \frac{V_s}{K_s} \qquad (11)$$

where V_s is the desired volume of separation between peaks of the two molecular species. In practice, the optimal pore size for a desired separation may not be available. In that case the porosity corresponding to the largest available separation coefficient can be selected as a best choice and the feasibility of a given separation can be evaluated. Thus the practical limitations and possibilities of specific separations can be assessed. In cases where interaction effects within the column do occur the separation coefficients will be altered correspondingly.

DISCUSSION

Although it is generally recognized that elution positions on molecular-sieve columns are correlated with molecular "size," there is not general agreement in the literature as to which size parameter is appropriate. Part of this uncertainty arises from the fact that most of the "well-behaved" proteins commonly available to serve as calibrating standards possess closely similar partial specific volumes and frictional ratios. For such proteins a correlation of molecular weights with elution position is essentially the same as the correlation with molecular radii. It is desirable, however, that variations in elution position due to effects of molecular asymmetry and hydration, if present, be taken into account by any column-calibration procedure used in the study of an unknown or impure molecular species. Polson (1961) has concluded that elution positions were better correlated with diffusion coefficients than with molecular weight, on the basis of the behavior of hemoglobin and serum albumin. Andrews (1962) has suggested that hemoglobin may have been dissociated under the conditions employed, and found a better correlation for his data with molecular weights. Whitaker (1963) has obtained an excellent linear correlation between elution position and logarithm of molecular weight for a series of globular proteins of low frictional ratio on Sephadex G-100 columns. However there was the same discrepancy for the case of hemoglobin versus serum albumin, and uncertainty as to the molecular form of the hemoglobin was again present. Current investigations of the reversible dissociation phenomena of hemoglobin on molecular-sieve columns being carried out in this laboratory will be reported later. It is clear from these studies, however, that under conditions where the hemoglobin exists as a 67,000 mw

TABLE III
PARAMETERS FOR COLUMN CALCULATIONS[a]

$\frac{V_e - V_o}{V_i}$	$\frac{a}{r}$	$\frac{V_e - V_o}{V_i}$	$\frac{a}{r}$	$\frac{V_e - V_o}{V_i}$	$\frac{a}{r}$	$\frac{V_e - V_o}{V_i}$	$\frac{a}{r}$	$\frac{V_e - V_o}{V_i}$	$\frac{a}{r}$
0.9595	0.0100	0.9200	0.0200	0.8816	0.0300	0.8442	0.0400	0.8078	0.0500
0.7725	0.0600	0.7381	0.0700	0.7048	0.0800	0.6725	0.0900	0.6413	0.1000
0.6110	0.1100	0.5817	0.1200	0.5533	0.1300	0.5259	0.1400	0.4995	0.1500
0.4740	0.1600	0.4495	0.1700	0.4258	0.1800	0.4031	0.1900	0.3812	0.2000
0.3602	0.2100	0.3400	0.2200	0.3207	0.2300	0.3022	0.2400	0.2845	0.2500
0.2675	0.2600	0.2514	0.2700	0.2359	0.2800	0.2212	0.2900	0.2072	0.3000
0.1939	0.3100	0.1813	0.3200	0.1693	0.3300	0.1579	0.3400	0.1471	0.3500
0.1369	0.3600	0.1273	0.3700	0.1183	0.3800	0.1097	0.3900	0.1017	0.4000
0.0941	0.4100	0.0870	0.4200	0.0804	0.4300	0.0742	0.4400	0.0684	0.4500
0.0630	0.4600	0.0580	0.4700	0.0533	0.4800	0.0489	0.4900	0.0449	0.5000
0.0412	0.5100	0.0377	0.5200	0.0345	0.5300	0.0316	0.5400	0.0289	0.5500
0.0264	0.5600	0.0242	0.5700	0.0221	0.5800	0.0202	0.5900	0.0184	0.6000
0.0168	0.6100	0.0154	0.6200	0.0141	0.6300	0.0129	0.6400	0.0118	0.6500
0.0108	0.6600	0.0099	0.6700	0.0090	0.6800	0.0083	0.6900	0.0076	0.7000
0.0070	0.7100	0.0064	0.7200	0.0058	0.7300	0.0054	0.7400	0.0049	0.7500
0.0045	0.7600	0.0041	0.7700	0.0037	0.7800	0.0034	0.7900	0.0030	0.8000
0.0027	0.8100	0.0024	0.8200	0.0022	0.8300	0.0019	0.8400	0.0017	0.8500
0.0014	0.8600	0.0012	0.8700	0.0010	0.8800	0.0009	0.8900	0.0007	0.9000
0.0005	0.9100	0.0004	0.9200	0.0003	0.9300	0.0002	0.9400	0.0001	0.9500
0.0001	0.9600	0.0000	0.9700	0.0000	0.9800	0.0000	0.9900	0.0000	1.0000

[a] Values of a/r are obtained with this table from the experimental values of $V_e - V_o/V_i$. For column calibration a molecular species of known a is used and the effective pore radius r is thereby determined. Once the column is calibrated, unknown values of a for different macromolecules can be similarly obtained.

species, such as reported here for the G-200 experiments, the discrepancy in elution volumes between hemoglobin (f/fo = 1.16) and bovine serum albumin (f/fo = 1.30) on G-75 and G-100 columns is largely compensated when elution volume is correlated with molecular Stokes radius instead of molecular weight and is very accurately corrected for the G-200 columns. It is clear from Figures 5 and 6 that the Sephadex G-200 column behavior correlates with Stokes radius more accurately than with molecular weight. Whereas this fact may be of some disadvantage for practical situations in which a simple rapid estimate of molecular weight is desired, it actually makes the column a more valuable tool when used in conjunction with a second physical method. Thus an accurate determination of molecular weight can be obtained by the usual combination of the diffusion coefficient (obtained from the column Stokes radius) with the sedimentation coefficient or intrinsic viscosity.

The possibility of chromatographic separation on the basis of molecular friction within a gel was suggested long before the development of molecular-sieve chromatography (Tiselius, 1949). However, diffusional restriction mechanisms have been generally discounted on the basis of the insensitivity of elution position to flow rate of the mobile phase (Tiselius *et al.*, 1963). It should be recognized, however, that there are several inherent features of the column that would tend to produce such extreme flow-rate insensitivity under a diffusion-controlled mechanism. The stationary gel particles must be surrounded by stagnant layers and regions of liquid. Solute from the advancing liquid stream then must be delivered to the bead across the stagnant layers by diffusion before penetration into the stationary-gel phase can occur, and must be delivered again from the bead across the surrounding film to enter the mobile stream. For simple systems in which exact calculations can be made it is known that the thickness of the surrounding layer varies inversely with the flow rate in such a way as to make the solute flux across the layer highly insensitive to velocity of flow of surrounding liquid. For example, the flux of solute onto the surface of a solid bead falling in a liquid solution varies as the cube root of the rate of descent (Levich, 1962). Such an effect must certainly come into play in the molecular-sieve column. Since the individual gel particles are in intimate contact with each other as well as with the column-void liquid, there must be a great deal of interparticle diffusion as well as liquid-particle exchange and this would further reduce the sensitivity to flow rate.

Although there have been few studies of the role played by structural features of the stationary phase on chromatographic processes, it has been shown that, for partition chromatography in cellulose fibers, a molecule of radius greater than 40 A will encounter large diffusional restriction within the cellulose fiber (Stewart and Shin, 1959). Evidence of such an effect was found for migration of steroids in impregnated paper (Starka and Prusikova, 1959). The R_F was found to increase to a maximum with an increasing stationary phase.

It is likely that both the molecular-exclusion effect and diffusional restriction effect are operative to some extent in all types of molecular-sieve columns. The latter should be more prominent in gels of narrow pore-size distribution. When a minor exclusion effect is present in addition to the diffusional restriction mechanism, this effect may be masked by incorporation into the adjustable parameter r. On the other hand, it is possible that the restricted diffusion effect may play an appreciable role in the G-75 and G-100 gels and yet be masked by specific interaction effects. These effects, known to be more prominent in the more highly cross-linked gels and with the smaller protein species, would tend to increase the negative slope of an equilibrium K_D versus molecular radius profile for a given gel (Figs. 1 and 2).

A calibrated molecular-sieve column may be used for estimation of the diffusion coefficient and Stokes radius of a macromolecule present in impure form or unknown concentration provided an activity assay is available. An upper limit for the hydrated molecular weight can then be determined by the usual calculation:

$$M = \frac{4\pi Na^3}{3\bar{V}} \qquad (12)$$

provided the partial specific volume $\bar{V}$ is known or

can be reasonably estimated as in the case of most proteins. In cases where the preparation is not sufficiently pure to be studied successfully by other physical methods, it is frequently of great interest to have even a rough estimate of the molecular weight. As with other relative methods, such as the porous-disk-diffusion technique, the necessity for a standard of calibration is an inherent disadvantage.

For many experimental purposes equation (9) may be found a valid approximation for column calibration and for determination of Stokes radii or corresponding diffusion coefficients and for subsequent molecular weight calculations incorporating these parameters. To facilitate use of this method a brief table of values of the function (eq. 9) has been calculated by means of an IBM 1401 computer. Values of a/r are given in Table III for corresponding $(V_e - V_o)/V_i$ ratios which can be obtained from experimental measurements.

The theory goes further than providing an empirical correlation for measurements in that (a) it explains in terms of a fundamental process the discrepancy between static-batch-equilibrium experiments and corresponding nonequilibrium K_D values for the G-200 columns, (b) it provides an explanation for the fact that diffusion coefficients are better correlated with elution volumes than are molecular weights, (c) it predicts the exact shape of the curve relating molecular size to elution positions on the column, (d) it provides a rationale for the commonly observed limitations in obtainable separations, and (e) it provides a basis for the prediction of numerical separation coefficients. By comparison with other physical methods the molecular-sieve column is noted for ease of operation, simplicity of equipment required, and rapidity of results obtainable. Although this technique may continue to be of greatest value in preparative procedures, it should find increasing utility as an analytical tool.

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