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DETERMINATION OF STOICHIOMETRY AND EQUILIBRIUM CONSTANTS FOR REVERSIBLY ASSOCIATING SYSTEMS BY MOLECULAR SIEVE CHROMATOGRAPHY*

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The supratertiary architecture and self-assembly characteristics of enzyme complexes, viruses, membranes, immunochemical, and mechanochemical systems are central problems in molecular biology.^{1, 2} Among the simplest prototypes of such systems are association-dissociation phenomena of proteins and protein subunits. Recent investigations demonstrate that in a number of systems these phenomena occur only at very low concentrations.^{3, 4} The molecular sieve technique has special advantages for the study of such systems in the lower ranges of concentration for which only spectrophotometric or biological activity assay is available. Furthermore, elution behavior can often be related in a straightforward way to intrinsic molecular properties such as the diffusion coefficients,⁷ sedimentation coefficients,^{9, 10} and molecular weights.¹¹⁻¹³ It is the purpose of this paper to show that this technique may be used for quantitative analysis of the number of components and their equilibria in such dilute systems.

Recently, Winzor and Scheraga^{5, 6} demonstrated that molecular sieve chromatography possesses all of the qualitative features predicted by Gilbert¹⁵ for the sedimentation of a rapidly reversible, polymerizing system. However, no quantitative theory of the molecular sieve applied to such systems has been reported to date, although the Gilbert theory emphasized the analogy of sedimentation and chromatography. In this paper a quantitative theory for the molecular sieve is developed, and the formal relationship between the two transport methods is established. The theoretical predictions are tested against experimental data for the monomer-hexamer interaction of α -chymotrypsin and for the reversible dissociation of isoelectric human carboxyhemoglobin.

Theory.—In the phenomenological treatment to be developed no assumption is made as to the exact mechanism of operation of the column. The only necessary assumption is that the equilibrium of a reversibly aggregating system is not perturbed by the presence of the gel matrix. Evidence for the validity of this assumption for the systems investigated will be presented. This treatment is related to the theory for idealized chromatography of de Vault¹⁴ and to the theory of Gilbert for the sedimentation velocity of a rapidly equilibrating, monomer-*n*-mer system.¹⁵ In this idealized treatment, axial dispersion of the solute zone is ignored except as it arises as a result of the macromolecular interactions.

Behavior of a single homogeneous solute: The phenomenological parameter of molecular sieve chromatography is the molecular sieve coefficient, σ . This coefficient characterizes the interaction between the column gel phase and a given molecular species. It is related to the molecular Stokes radius, as well as to the gel porosity,^{7, 8} and represents the extent of solute equilibration with the gel phase of an infinitesimally thin section of the column. If dx is the height of such a section, αdx the void volume, and βdx the internal gel volume, the molecular sieve coefficient is defined by:

$$\sigma = \frac{Q}{\beta C} \quad (1)$$

Here 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

$$\left(\frac{\partial C}{\partial x}\right)_V + [\alpha + \beta\sigma] \left(\frac{\partial C}{\partial V}\right)_x = 0, \quad (2)$$

where x is distance from the top of the column. If a solute sample of volume S , for which both edges are sharp, is passed through the column, the respective solutions of equation (2) for the positions of the leading and trailing edges of the zone are

$$x_\sigma = \frac{V}{\alpha + \beta\sigma} \quad (3a)$$

$$x'_\sigma = \frac{V - S}{\alpha + \beta\sigma} \quad (3b)$$

Making the substitutions $V_0 = \alpha l$ and $V_i = \beta l$, where V_0 is the total column void volume, V_i , the internal gel volume, and $x_\sigma = x'_\sigma = l$, the column's length, gives

$$V_e = V_0 + \sigma V_i, \quad (4a)$$

$$V'_e = V_0 + \sigma V_i + S. \quad (4b)$$

V_e is the value of V at which the leading edge of the effluent solute will leave the column, and V'_e is the corresponding value for the trailing edge. Equations (4a) and (4b), in which axial dispersion is neglected, can be verified experimentally if the centroids of the gradients are taken to be the equivalent boundary positions.⁸

Equilibrium of monomer with a higher polymeric species: Consider the case of an associating system subjected to a transport process in which displacement from equilibrium by differential migration of the participating species is slow compared

FIG. 1.—Characteristic elution patterns for carboxyhemoglobin undergoing simultaneous molecular sieve transport and reversible dissociation. The diagram (a) is a tracing from the pattern obtained with $C_0 = 5.0 \times 10^{-6}$ moles/liter and (b) is the concentration gradient across the zone.

concentrations C_m of monomeric and C_p of polymeric species:

$$K = \frac{C_m^n}{C_p} \quad (5)$$

Within either boundary the conservation equation (1) is written for each species:

$$\left(\frac{\partial C_m}{\partial x}\right)_V + \xi_m \left(\frac{\partial C_m}{\partial V}\right)_x = 0 \quad (6a)$$

$$\left(\frac{\partial C_p}{\partial x}\right)_V + \xi_p \left(\frac{\partial C_p}{\partial V}\right)_x = 0 \quad (6b)$$

where $\xi_m = \alpha + \beta\sigma_m$, $\xi_p = \alpha + \beta\sigma_p$; σ_m and σ_p are the respective individual coefficients of monomer and polymer. From equations (5) and (6a, 6b) the conservation equation for total mass transport may be written:

$$\left(\frac{\partial C_m}{\partial x}\right)_V + \left[\frac{K\xi_m + n\xi_p C_m^{n-1}}{K + nC_m^{n-1}}\right] \left(\frac{\partial C_m}{\partial V}\right)_x = 0. \quad (7)$$

Two types of experiment may be performed. (a) The solute zone is so small that a plateau region having the initial solute concentration is not present in the effluent. If this is the case, the apparent molecular sieve coefficient will vary inversely with column length, since the equilibrium is shifted by dilution to favor dissociation. Whereas this type of experiment is a useful qualitative means of detecting dissociation (cf. Andrews, 1964), the resulting elution patterns cannot be quantitatively related through equation (7) to the degree of dissociation. (b) The initial solute zone is sufficiently large that a plateau region is present in the effluent zone. If the chromatography is carried out in this manner so that the leading and trailing boundaries continually "feed" from a constant plateau concentration as they spread, the centroid positions will still move with constant velocity, independently of column length, in spite of the fact that the shapes of the edges are changing appreciably.⁸ It is possible, under these circumstances, to relate the configurations of the bound-

to the rate of attainment of equilibrium. The primary effects observed are a sharp leading edge and diffuse trailing boundary^{5, 8} (see Fig. 1). These elution characteristics are caused by the extreme concentration dependence of the apparent molecular sieve coefficient arising as a result of the monomer-n-mer reaction. Neglecting the boundary dispersion which is normally present for a single solute, it is possible to relate these boundary characteristics directly to the molecular sieve coefficients of the individual species, to the stoichiometry, and to the equilibrium constant.

For the instantaneous equilibrium $nM \rightleftharpoons M_n$ the equilibrium constant, K , is related to the

aries to the molecular sieve coefficients of the participating species, to their respective concentrations, and to the plateau concentration.

The solution of equation (7) for the trailing boundary, under the conditions just described, is

$$C_m = \left[\frac{K}{n} \cdot \frac{\theta \xi_m - 1}{1 - \theta \xi_p} \right]^{1/(n)-1} \quad (8)$$

where $\theta = x/V$. From equations (5) and (8), the total solute concentration C_T within the boundary can be expressed as a function of the equilibrium constant, the degree of polymerization, and the coordinates x and V :

$$C_T = \left[1 + \frac{1}{n} \cdot \frac{\theta \xi_m - 1}{1 - \theta \xi_p} \right] \left[\frac{K}{n} \cdot \frac{\theta \xi_m - 1}{1 - \theta \xi_p} \right]^{1/(n-1)} \quad (9)$$

In order to express the measurable solute concentration C_T across the boundary in terms of the molecular sieve coefficients of the individual species and the effluent volume V that has passed through the column, consider the behavior of the system at $x = l$. First, the following coordinate transformations are defined in order to obtain a system of reduced volume coordinates by which all column elution diagrams may be normalized.

$$\nu = \frac{V - V_0}{V_t}; \quad V > V_0 \quad (10a)$$

$$\nu' = \frac{V - V_0 - S}{V_t}; \quad V > V_0 + S. \quad (10b)$$

Expressed in reduced coordinates, the elution curves $C_T(\nu)$ and $C_T(\nu')$ for the leading and trailing boundaries, respectively, are superimposable for all column experiments. Setting $x = l$ and making the substitution (10b), equation (9) becomes:

$$C_T(\nu') = \left[1 + \frac{1}{n} \cdot \frac{\sigma_m - \nu'}{\nu' - \sigma_p} \right] \left[\frac{K}{n} \cdot \frac{\sigma_m - \nu'}{\nu' - \sigma_p} \right]^{1/(n-1)} \quad (11a)$$

Equation (9) is the analogue of Gilbert's equation (6) for the velocity ultracentrifugation of a polymerizing system.¹⁵ The formal relationship between the sedimentation and molecular sieve methods is more clearly established with the further substitution: $\phi = (\sigma_m - \nu')/(\sigma_m - \sigma_p)$. Then equation (11a) becomes:

$$C_T = \left[1 + \frac{1}{n} \cdot \frac{\phi}{1-\phi} \right] \left[\frac{K}{n} \cdot \frac{\phi}{1-\phi} \right]^{1/(n-1)} \quad (11b)$$

which is identical in form to Gilbert's equation (6).¹⁵ The gradient of total solute concentration may be given as

$$\frac{\partial C_T}{\partial \nu'} = - \frac{\phi^{(2-n)/(n-1)}}{(n-1)(\sigma_m - \sigma_p)} \left[\frac{K}{n} \right]^{1/(n-1)} \left[\frac{1}{1-\phi} \right]^{(2n-1)/(n-1)} \quad (12)$$

Equation (12) has a single minimum for $n > 2$ at the position

$$\nu'_{\min} = \frac{2n-1}{3(n-1)} \sigma_m + \frac{n-2}{3(n-1)} \sigma_p \quad (13)$$

From equation (10b) the effluent volume at which the minimum in the gradient of total solute concentration will occur is

$$V'_{\min} = V_0 + \left[\frac{2n-1}{3(n-1)} \sigma_m + \frac{n-2}{3(n-1)} \sigma_p \right] V_i + S. \quad (14)$$

As in the case of sedimentation and electrophoresis, the reduced coordinate position of the minimum is independent of K and C , and depends only on the stoichiometry of the reaction for the two given species. The degree of polymerization can be computed directly from the coefficients of the individual species and the minimum in the gradient by solving equation (13) for n . Then

$$n = \frac{3\nu'_{\min} - \sigma_m - 2\sigma_p}{3\nu'_{\min} - 2\sigma_m - \sigma_p}. \quad (15)$$

A fundamental limitation is imposed on the effective resolution of the column, since

$$\lim_{n \rightarrow \infty} \nu'_{\min} = \frac{2}{3} \sigma_m + \frac{1}{3} \sigma_p. \quad (16)$$

The practical upper limit lies in the region $n \geq 10$, as is also the case for the sedimentation velocity method.

For dimerization ($n = 2$), there is a single maximum but no minimum in the gradient curve, equation (12). It is thus possible to distinguish qualitatively between this case and the more general one when $n > 2$. The ideal gradient patterns for the two cases are shown in Figure 2.

The solution of equation (7) for the leading boundary is of the "discontinuous" or hypersharp kind first described by

FIG. 2.—Ideal concentration gradient patterns for the trailing edge of a solute zone where monomer is in reversible equilibrium with a single polymeric species. (a) When $n > 2$, the gradient exhibits two maxima and a single minimum. (b) For the dimerization reaction, $n = 2$, a single maximum only is present.

Wilson.¹⁶ It can be shown that the centroid positions of both the hypersharp leading and diffuse trailing boundaries will occur, respectively, at $\nu = \bar{\sigma}_\omega$ and $\nu' = \bar{\sigma}_\omega$, where $\bar{\sigma}_\omega$ is a weight average of the coefficients of the individual species.⁸ For the leading boundary the centroid volume $\bar{V}$ is

$$\bar{V} = V_0 + [\sigma_p + \alpha(\sigma_m - \sigma_p)]V_i, \quad (17)$$

where α is the weight fraction of monomeric species in the plateau region. It follows from equations (17) and (4a) that $\bar{V}$ is also a weight average of the V_e values of the reactant species:

$$\bar{V} = \alpha V_m + (1 - \alpha)V_p. \quad (18)$$

Also the weight fraction of monomer is given by

$$\alpha = \frac{\bar{V} - V_p}{V_m - V_p} = \frac{\bar{\sigma}_\omega - \sigma_p}{\sigma_m - \sigma_p} = \frac{\bar{\nu} - \sigma_p}{\sigma_m - \sigma_p}, \quad (19)$$

where $\bar{\nu}$ is the centroid position of the leading edge.

Determination of the equilibrium constant: Once n is known, the equilibrium constant can be calculated from the leading edge by equation (19) and the relation

$$K = \frac{\alpha^n C_0^{n-1}}{1 - \alpha}, \quad (20)$$

where C_0 is the total solute concentration in the plateau region. For the trailing edge, equation (11a) may be solved for K :

$$K = \frac{n C_{eq}^{n-1}}{\lambda \left(1 + \frac{\lambda}{n}\right)^{n-1}}, \quad (21)$$

where C_{eq} may be taken as the total solute concentration at the centroid of the trailing boundary, in which case $\lambda = (\sigma_m - \bar{v}')/(\bar{v}' - \sigma_p)$. In addition to this method, valid for any n , the value of K may also be determined by using positions of the maximum and minimum. If $n = 2$, C_{eq} may be taken as the concentration at the maximum of the gradient curve and $\lambda = (\sigma_m - \nu'_{\max})/(\nu'_{\max} - \sigma_p)$, where $\nu'_{\max}$ is its coordinate position. If $n > 2$, C_{eq} may be taken as the concentration corresponding to the minimum in the gradient curve, in which case

$$\lambda = \frac{\sigma_m - \nu'_{\min}}{\nu'_{\min} - \sigma_p}.$$

Determination of individual molecular sieve coefficients: For these calculations it is necessary to have reasonably accurate values for σ_m and σ_p . It may be possible to obtain this information either by modifying the reaction conditions so as to prevent either association or dissociation, or to utilize extrapolation procedures. The latter may in some cases be used effectively with the molecular sieve technique for determination of σ_m . The determination of σ_p presents a more difficult problem, since only partial boundary resolution will often exist at the highest practical concentrations. In this case, although it is not possible to obtain σ_p from a single elution diagram, all the necessary information for its determination can be obtained by combining data from two columns of identical bed material, but of different bed volume.⁸

Experimental.—Calculations for α -chymotrypsin: From the molecular sieve data of Winzor and Scheraga⁵ on the α -chymotrypsin system in 0.02 M sodium phosphate buffer at pH 7.9, it is possible to determine the degree of polymerization and the equilibrium constant. From their Figure 3, $V'_m - S = 25.0$ ml, $V'_{\min} - S = 21.5$ ml. Assuming, in accord with common experience for G-100 Sephadex columns, that the void volume is $29 \pm 2\%$ of the total column volume and that $V'_p - S = V'_0$, so that $V'_p - S = 11.8$ ml, we obtain by equation (22) a value of 5.9 ± 0.9 for n . Whereas the above calculation shows the molecular sieve data to be most consistent with a value of $n = 6$, it could only be concluded with certainty, without a measured value at V'_0 , that n lies between 5 and 7. From Figure 3 of the data of Winzor and Scheraga,⁵ it is also seen that the gradient peaks have almost equal area for a concentration of 3.4 mg/ml. This result is in accord with the corresponding concentration of 3.5 mg/ml, at which the monomer and polymer peaks have equal area in the sedimentation velocity experiments of Massey, Harrington, and Hartley.^{17, 18} This agreement strongly indicates that the equilibrium is not appreciably perturbed by the presence of the gel under these conditions.

Dissociation of human carboxyhemoglobin: Applicability of the molecular sieve technique to the quantitative study of interacting protein systems at very low concentrations was tested by a study of human carboxyhemoglobin dissociation in the concentration range 5–900 μ g/ml. Recent stud-

ies^{4, 18, 19} have established that the hemoglobin molecule dissociates into half molecules in this concentration range under isoelectric conditions and moderate ionic strength. This dissociation was chosen for study as a critical test of the method since (a) it occurs in a concentration range where adsorptive interaction effects with the column, if present, might strongly influence the elution behavior of the zones, and (b) it is a monomer-dimer equilibrium and, consequently, $\sigma_m - \sigma_p$ is minimal.

Experimental procedures: Hemoglobin was prepared from outdated human blood and crystallized according to the procedure of Drabkin.²⁰ Crystals were dissolved in 0.2 M sodium phosphate buffer, pH 6.8, in an approximate concentration of 50 mg/ml, and the solution was dialyzed against the same buffer, subsequently saturated with carbon monoxide, and stored at 4°C. Samples for chromatography were prepared by passing a 1.0-ml sample of the above stock solution through a Sephadex G-25 column of 25.0-ml bed volume, which was previously equilibrated with CO-saturated buffer; the effluent protein was diluted to the desired concentration in the same buffer. The concentrations of stock solutions and column samples were determined by absorbance measurements at 542 m μ and 220 m μ .

Dry Sephadex G-200 that had been sifted through a 200-mesh screen was allowed to soak for 4 days in 0.2 M sodium phosphate buffer pH 6.8. Glass columns of 1.0 cm inside diameter were

fitted with a disk of porous polyethylene at the bottom above the rubber stopper and were packed by gravity flow. The bottom of the column was attached by means of a short Teflon tube into the sample flow cuvette of a Vanguard ultraviolet analyzer (model 1056-O.D.) set to record absorbance at 220 m μ . The reference cell was filled with buffer and closed. A sample consisting of 25.0 ml of protein solution of the desired concentration, saturated with carbon monoxide, was applied to the column, followed by buffer. The resulting elution curve was recorded. Effluent volumes were measured by passing the output from the analyzer flow cell into a burette by which volumes were measured at any desired chart position. This method provides a precision in volume measurement of about 1-2% which is approximately the same precision as the spectrophotometric assay. The data presented here are from a single column of volume 56.1 ml, void volume, 15.6 ml (determined from the effluent volume of tobacco mosaic virus), and an internal volume V_i of 38.5 ml. The column had a flow rate of 8-10 ml/hr.

TABLE 1
EQUILIBRIUM CONSTANTS FROM SEPHADEX
G-200 COLUMN EXPERIMENTS FOR HUMAN
CARBOXYHEMOGLOBIN

$C_0 \times 10^6$ moles/liter	$\bar{V}$	ν	α	K
0.01	38.0	0.581	1.0	—
0.30	37.8	0.577	0.95	5.4
1.00	37.4	0.567	0.825	3.9
2.00	37.1	0.559	0.725	3.8
2.00	36.8	0.550	0.612	1.9
5.00	36.5	0.544	0.537	3.1
8.00	36.4	0.542	0.513	4.1
8.00	36.5	0.544	0.550	5.4
10.00	36.2	0.536	0.438	3.4
12.00	36.0	0.530	0.362	2.5
12.00	36.1	0.534	0.412	3.5
12.00	36.4	0.542	0.513	6.2
14.00	36.2	0.536	0.438	4.8
1000	34.9	0.501	0	—

Average $K = 4.0 \pm 1.7 \times 10^{-6}$ moles/liter.

To provide an independent determination of the equilibrium constant for the dissociation of carboxyhemoglobin, porous disk diffusion measurements were performed using a magnetically stirred cell of the type described by Stokes.²¹ The cell had upper and lower volumes of 31.43 ml and 33.26 ml, respectively, a medium grade porous disk 0.08 cm $\times$ 3.0 cm diameter, and a cell constant, A/liter, of 6.42 cm. A preliminary diffusion of 5 hr was followed by a final diffusion of 48-150 hr, carried out in a thermostat at 20°C $\pm$ 0.02.

Results and Discussion.—From elution volumes of the leading boundary the degree of dissociation, α , was calculated for each experiment by equation (19), and the equilibrium constant by equation (20). Values of $\sigma_m = 0.581$ and $\sigma_p = 0.501$ were obtained from experiments at 10^{-8} moles/liter and 10^{-3} moles/liter, respectively. From these results, shown in Table 1, the average value obtained for K was 4.0×10^{-6} moles monomer/liter.

Equilibrium constants were calculated from the integral diffusion data for each experiment according to a method of Ackers.⁸ Values of K and α calculated from

the porous disk measurements are given in Table 2 at concentrations, $\bar{C}$, for which the measured integral diffusion coefficients, D_i , are equal to differential coefficients. For the calculation of α , values of D_m and D_d , the diffusion coefficients of monomer and dimer, were obtained from differential measurements at concentrations of 10^{-8} and 10^{-5} moles/liter, respectively (see Table 2). The average value obtained for the

equilibrium constant was 3.6×10^{-6} moles/liter. This value is in good agreement with the equilibrium constant 4.0×10^{-6} moles/liter obtained from the column experiments.

It is a basic assumption of this treatment that the macromolecular dissociation reaction is not perturbed by the solute-gel matrix interactions. Although there is no *a priori* reason to expect such a perturbation, at the present time

the validity of this assumption can only be assessed experimentally for each individual system. The agreement between theoretical predictions and experimental results obtained for the monomer-hexamer equilibrium of α -chymotrypsin and for the monomer-dimer equilibrium of carboxyhemoglobin indicates that these systems are not appreciably perturbed by the gel matrix. It does not follow, however, that this conclusion would be valid for all protein systems under all column conditions. The general reliability and range of applicability of the method must await detailed investigation of a number of systems covering a range of molecular size, shape, and multiplicity of interaction.

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TABLE 2

EQUILIBRIUM CONSTANTS FROM POROUS DISK
DIFFUSION MEASUREMENTS FOR HUMAN
CARBOXYHEMOGLOBIN

$D_i \times 10^7$ $\frac{\text{cm}^2}{\text{sec}}$	$K \times 10^6$ moles/liter	$\bar{C} \times 10^6$ moles/liter	$\alpha = \frac{D_i - D_d}{D_m - D_d}$
8.51 ± 0.1	—	0.100	1.0
8.21 ± 0.1	3.59	0.998	0.815
7.79 ± 0.1	3.55	3.97	0.558
7.62 ± 0.1	3.78	10.3	0.454
6.88 ± 0.1	—	100.0	0

Average $K = 3.6 \pm 0.2$ moles/liter.