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# PHASE RATIO DETERMINATION IN AN ION-EXCHANGE COLUMN HAVING PORES PARTIALLY ACCESSIBLE TO PROTEINS

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## SUMMARY

A method is suggested for determination of the hold-up volume and the phase ratio of a protein on a strong anion-exchange chromatographic column, which is based on mercury porosimetry and size-exclusion calibration with polymer samples.

## INTRODUCTION

Determination of thermodynamic parameters such as changes in enthalpy, entropy and Gibbs free energy associated with reversible binding of solute by the stationary phase in liquid chromatography is based on the measurement of the retention (capacity) factor, $k'$ (ref. 1), and is proportional to the solute distribution coefficient. The proportionality constant is the phase ratio, $\varphi$, which is determined by the relative magnitude of the stationary and mobile phases present in the column used.

Calculations of $k'$ values are very sensitive to the value used as the hold-up volume, $V_M$. Determination of $V_M$ presents both theoretical and practical problems. In the case of bonded stationary phases, such as reversed-phase[2] or polyethylenimine ion exchangers[3], a serious theoretical difficulty arises from the definition of the position of the interface between the mobile and stationary phases. The situation is further complicated by the fact that any bonded stationary phase will preferentially adsorb certain components from the mobile phase. The question is, are these adsorbed eluent components to be considered as part of the mobile phase or of the stationary phase?

Determination of the hold-up volume of a column containing bonded stationary phase has been studied mostly for reversed-phase chromatography of small solutes. $V_M$ has been determined several ways, including static methods[4,5], dynamic methods which involve injections of the mobile phase components[6–9] or isotopically labelled components of the mobile phase as well as radiolabelled compounds[10–13], injection of "non-retained" compounds[14–16], mathematical methods based on the linearization of retention data for homologous series[14,17,18], and measurement of retention values at different temperatures[19]. It was found that $V_M$ depends on the thermodynamic model chosen to describe the adsorption of the mobile phase components on the stationary phase[20]. Therefore, "the only good" hold-up volume value cannot be defined[14,18].

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Because they possess three-dimensional structure, there is a fundamental difference between the chromatography of proteins and that of small molecules[21]. Ion-exchange separation is generally performed using a salt concentration gradient from 0 to 0.5 *M* at constant pH[21], but proteins actually elute in a narrow salt concentration range. Under these mild conditions, the changes in the specific molar volumes of proteins and in the "thickness" of bonded stationary phases are negligible[22,23], particularly compared to the exclusion effect of the pores.

The concept of hold-up volume presented by Horváth and Lin[24] is especially relevant for the protein chromatographer. According to their analysis, the hold-up volume of a solute will vary between the interparticle volume, $V_0$, and the sum of the intraparticle and interparticle volume, $V_i + V_0$, of the column:

$$V_M = V_0 + K_{SEC}V_i \tag{1}$$

where the coefficient, $K_{SEC}$, represents the fraction of intraparticular space accessible to the solute ($0 < K_{SEC} < 1$). Usually $K_{SEC}$ decreases with increasing molecular mass of the solute and becomes zero for completely excluded solutes. This concept is essentially the same as the theory of retention in size-exclusion chromatography (SEC)[25]. If the retention factor of a protein in adsorption chromatography is

$$k' = \frac{V_R - V_M}{V_M} = K_d \frac{A_s}{V_M} \tag{2}$$

the combination of eqn. 1 and eqn. 2 leads to the general retention equation

$$V_R = V_0 + K_{SEC}V_i + K_dA_s \tag{3}$$

$V_R$ and $K_d$ are the retention volume and the "surface mediated" distribution coefficient of the solute, respectively. $A_s/V_M = \varphi$ is the phase ratio, where $A_s$ and $V_M$ are the stationary phase surface area and the mobile phase volume. This is a specific treatment for a more general problem (see Appendix 1).

Determination of $V_M$ is a more complicated problem for proteins than for small solutes. In the latter case a "very high eluent strength" is used to avoid sorption of the solute by the stationary phase[24]. In the case of ion-exchange chromatography of proteins, the retention as a function of mobile phase salt concentration is a "U"-shaped curve[26]. The minimum of this function occurs when the retention of a protein is determined by equal electrostatic and hydrophobic interactions. The minimum value of $K_d$ (in eqn. 3) is not necessarily equal to zero. Consequently, the hold-up volume of a protein cannot be determined using an "unretained compound". The method of "solvent disturbance" (injection of mobile phase component) requires that the mobile phase contain a certain amount of the protein of interest. The increase of the protein concentration in the mobile phase increases the viscosity of the mobile phase, decreases the diffusivity of the protein, increasingly excluding the sample components from the interior of the porous column material (see Appendix 2). The definition of $V_M$ as "the total volume of all the components of eluent present within the packed part of the column"[27] is not useful for proteins either.

The evaluation of the phase ratio with bonded phases involves the measure-

ment of mobile phase volume in the column and the determination of the surface area of the stationary phase that is accessible to the solute. The surface area of the stationary phase is usually estimated by the BET method[28], using small molecules such as nitrogen. As a consequence of the exclusion effect of the porous silica gel, BET data tend to overestimate the surface area accessible to even relatively low molecular mass sample components in liquid chromatography[1]. In the case of proteins, the error in the determination of surface area can be dramatically large. Furthermore, the BET measurement is a "dry" method, under which circumstances the hydrophilic stationary phase is flattened, resulting in a larger accessible surface area for small molecules than is available under chromatographic conditions, where hydration of the stationary phase reduces the pore diameter. Considering the difficulties mentioned above, the question may be asked: how should the phase ratio of a column having pores partially accessible to proteins be determined? In this paper we suggest a way to evaluate the hold-up volume and the phase ratio for proteins in porous ion-exchange columns.

## THEORETICAL

We use the following definition of phase ratio, $\varphi$ (ref. 24): the ratio of the fractions of the stationary phase area and mobile phase volume accessible to the protein (see eqn. 2):

$$\varphi = \frac{A_{s,acc}}{V_{M,acc}} \tag{4}$$

In the case of ion-exchange chromatography, a model was chosen in which the adsorbed "small" components of the eluent (water, salt ions) are considered as a part of the stationary phase. The volume of this layer is independent of mobile phase composition (salt concentration). This concept corresponds to the theory of SEC[25]. Consequently, each protein studied has its own distinct hold-up volume and phase ratio. The advantage of this model is that the phase ratio is independent of the mobile phase composition; therefore, application of this model to adsorption studies (for instance, to the determination of thermodynamic parameters) at different mobile phase compositions is very convenient.

The ideal method to determine $V_M$, would involve the use of an *alter ego* for each protein of interest, that would have the same size and shape as that protein, but would not interact with the stationary phase. Unfortunately, no such substitutes exist. Therefore, a series of non-retained polymers is used to evaluate the exclusion properties of the ion-exchange column. These polymers are calibrated along with proteins on a size-exclusion column having the same pore size as that of the ion exchanger. The calibration yields the apparent molecular mass of polymers relative to the proteins. Thus, the hold-up volume as a function of the logarithm of the molecular weight of the proteins ($V_{M,acc}$ *vs.* log $M_r$) can be determined for the ion-exchange column. Using data from mercury porosity measurements and size-exclusion calibration of the ion-exchange column, one can calculate the phase ratio as a function of the logarithm of the molecular weights of the proteins ($\varphi$ *vs.* log $M_r$). In this way, the hold-up volume and the phase ratio can be esetimated for a relatively wide range of molecular weight.

This method applies only to semi-rigid molecules such as proteins in their native state. It has been established by X-ray crystallography that conformational and vibrational displacement of amino acid residues in native protein seldom exceeds 1–2 Å in the most extreme case[29]. Exceptions are found in systems such as immunoglobulin G[30], and hexokinase[31] where there is a "hinged bending" or induced conformational change in the polypeptide at a specific location upon ligand binding. In contrast, segments of random-coiled polymers (*e.g.* polystyrene) diffuse randomly in both solution and on the surface of the sorbent. This makes it possible for segments of a random-coil polymer to enter pores of a porous chromatographic packing that are smaller than the radius of gyration of the polymer in solution.

## MATERIALS AND METHODS

Bovine serum albumin, ovalbumin, β-lactoglobulin, cytochrome *c*, insulin and dextran ($M_r$ 5 · $10^6$–40 · $10^6$) were purchased from Sigma (St. Louis, MO, U.S.A.). Thyroglobulin, Dextran T 10, T 20, T 40, T 70, and T 500 were obtained from Pharmacia (Piscataway, NJ, U.S.A.). A polyethylene glycol calibration standard kit was purchased from Polymer Labs. (Church Stretton, U.K.). Acetone, ethylene glycol, sodium chloride, and disodium hydrogen phosphate were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Carbon tetrachloride was purchased from EM Science (Cherry Hill, NJ, U.S.A.).

SEC of proteins and polymers was conducted using an LDC Constametric Model III pump and an LDC Refractomonitor refractive index detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.).

Synchropac Q 300 (lot. No. 1157-4), a strong anion-exchange support (Synchrom, Lafayette, IN, U.S.A.) was packed into a 150 × 4.1 mm column using a Shandon column packing pump (Shandon Southern Instruments, Sewickley, PA, U.S.A.). A Synchrom GPC 300 (250 × 4.6 mm) size-exclusion column was a generous loan from Synchrom. In the mobile phase was 100 m*M* sodium chloride in 20 m*M* disodium hydrogenphosphate, pH 7.0 at a flow-rate of 0.5 ml/min for both columns. The temperature was 23°C.

## RESULTS AND DISCUSSION

To control the retention properties of polymer samples in size-exclusion calibration of the ion-exchange column, the total volume of the mobile phase, $V_M$, and the mass of packing material, $m_s$, were determined gravimetrically[4] using water and carbon tetrachloride as the mobile phase: $m_s$ = 1.145 g, and $V_M$ = 1.56 ml. This $V_M$ datum is considered to be the maximum value for the mobile phase[6,14]. First, a molecular weight standard series of polyethylene glycols were used to calibrate a strong anion-exchange column. These standards have a narrow molecular weight distribution, but they were retained by the stationary phase, resulting in two diffuse bands characteristic of non-ideal SEC[32]. Therefore, another series of water-soluble polymers was chosen which had no interaction with the strong anion-exchange surface. Dextran samples similar to those produced for use as plasma protein substitutes, had no apparent retention on the stationary phase. These samples are heterodisperse systems with relatively broad molecular weight distributions. The hydrated dextran

TABLE I

PARAMETERS OF DEXTRAN SAMPLES USED FOR ANION-EXCHANGE COLUMN CALIBRATION

$[\eta]$, $\bar{M}_w$, and $\bar{M}_n$ data were obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A., Technical Service, by personal communication.

| *Dextran* | $[\eta]$* | $\bar{M}_w$** | $\bar{M}_n$*** | $\bar{M}_w/\bar{M}_n$§ |
|---|---|---|---|---|
| T 10 | 0.09 | 9900 | 5200 | 1.90 |
| T 20 | 0.14 | 20 000 | 13 300 | 1.50 |
| T 40 | 0.21 | 41 000 | 28 000 | 1.46 |
| T 70 | 0.26 | 70 000 | 42 500 | 1.65 |
| T 500 | 0.54 | 494 000 | 181 200 | 2.73 |

* Intrinsic viscosity, l/g.
** Weight-average molecular weight[36].
*** Number-average molecular weight.
§ Polydispersity.

molecules have a symmetrical shape in the low-molecular-weight range[33]. Increase in dextran molecular weight increases the asymmetry of the molecules, increasing their deviation from the "globular" shapes of serum proteins[34]. The type and extent of branching greatly affect the properties of many polymers[35]. Therefore, it is important to use the same type of polymers for calibration. The logarithmic form of the Mark-Houwink equation ($\log[\eta] = \log K + a \log M$, where $K$ and $a$ are the Mark-Houwink coefficients) was fitted to the intrinsic viscosity, $[\eta]$, and weight-average molecular weight, $\bar{M}_w$, data of dextran samples to determine whether these polymers are of the same type. The correlation coefficient was 0.990. Thus, the dextran samples are considered to be the same type. The values of the Mark-Houwink coefficients are $K = 1.57 \cdot 10^{-3}$ and $a = 0.451$, respectively. Table I shows the parameters of the dextran samples.

There are two different ways of using broad molecular weight distribution polymer standards for size-exclusion calibration: the integral molecular weight distribution and the linear calibration methods[37]. Both methods are time-consuming and require that the complete molecular weight distribution of a broad polymer standard be known. In this case, the anion-exchange column was calibrated by measuring the retention volumes of the dextran peaks related to the average molecular weight of the sample, $\bar{M}_p$, assuming that $\bar{M}_p \approx \bar{M}_n$. The size-exclusion calibration curve of the anion-exchange column was constructed by plotting the number-average molecular weight, $\bar{M}_n$, of dextran samples against the size-exclusion distribution coefficient, $K_{SEC}$. Fig. 1 shows that the points of the dextran samples are on the linear part of the calibration curve. The point for dextran T 500 is at the very end of the linear range. The internal and external volumes of the ion-exchange column per unit mass of packing material are $V_i' = 0.48$ ml/g and $V_0' = 0.85$ ml/g, respectively. The difference between $V_M$ measured with a "non-retained solute", acetone, and by gravimetry is 0.04 ml.

Proteins were used as molecular weight standards for dextran samples on a size-exclusion column with the same pore size as that of the ion exchanger. To prove that the molecular weight, $\bar{M}_p$, represented by the eluted dextran peak is equal to the

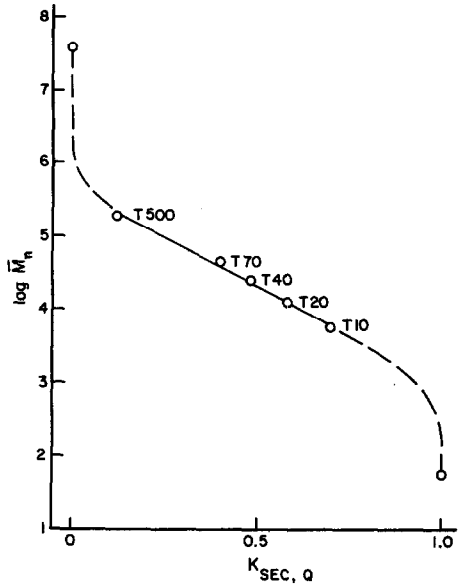


Fig. 1. Size-exclusion calibration of Synchrom Q 300 (150 × 4.1 mm) column using dextran samples. See conditions in Materials and methods.

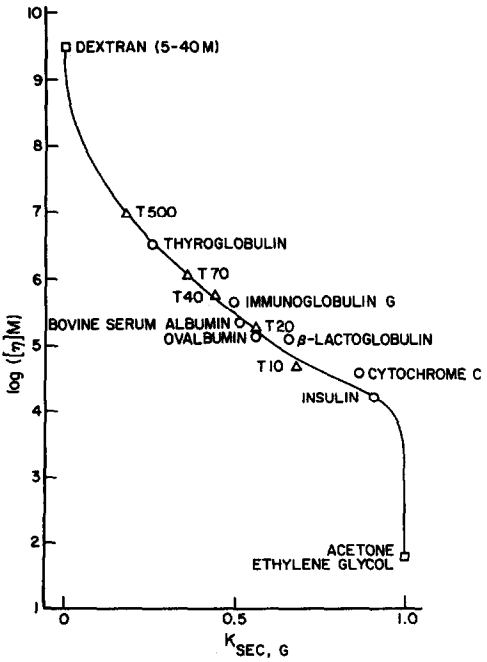


Fig. 2. Universal calibration curve of proteins and dextran samples on a Synchrom GPC 300 column. Intrinsic viscosity values of proteins are from refs. 41 and 43. See conditions in Materials and methods.

number-average molecular weight, $\bar{M}_n$, a universal calibration curve (log $[\eta]\bar{M}_n$ *vs.* $K_{SEC}$)[38] was determined for the proteins and the polymers (Fig. 2). Data points of dextrans calculated with $\bar{M}_n$ values lie on the curve. Thus, the assumption was correct that dextrans were suitable molecular weight standards for proteins. To avoid the problem of polydispersity of dextran samples, it would be desirable that the manufacturers produce samples having narrow molecular weight distribution.

The conventional calibration curve of proteins (log $M_r$ *vs.* $K_{SEC}$) measured on the size-exclusion column was used to determine the apparent molecular weight of dextrans relative to proteins (Fig. 3). $K_{SEC}$ values of the polymers lie in the linear range of the calibration curve. The point for dextran T 500 is at the very end of the linear part, as in Fig. 1. The equation of the linear part of the calibration curve ($0.35 < K_{SEC,G} < 0.91$) is:

$$\log M_{app} = aK_{SEC,G} + b \tag{5}$$

where $M_{app}$ is the apparent molecular weight of a solute relative to the protein standards, $K_{SEC,G}$ is the size-exclusion distribution coefficient measured on the size-exclusion column, $a = -2.92$, and $b = 6.46$. Coefficients of correlation and determination are 0.98 and 0.96, respectively, and the standard error of estimation is 0.163.

$K_{SEC,Q}$ values of polymers (measured on the ion-exchange column) as a function of $K_{SEC,G}$ values show a straight line for a wide range of molecular weights (Fig. 4). Deviation from this line at high molecular weight values is due to the difference between the total exclusion properties of the two columns. The value of the slope of the linear part deviates from one, probably because of the slight difference between the pore diameters of the columns. The equation of the linear part ($K_{SEC,G} > 0.35$) of the function $K_{SEC,Q}$ *vs.* $K_{SEG,G}$ is:

$$K_{SEC,Q} = AK_{SEC,G} + B \tag{6}$$

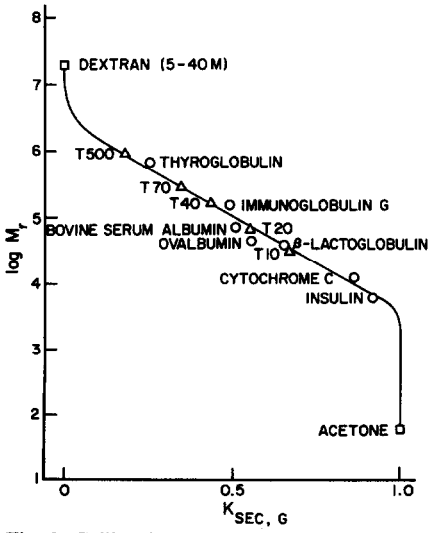


Fig. 3. Calibration curve of proteins on a Synchrom GPC 300 column used to determine the apparent molecular weight of dextran samples relative to proteins. See conditions in Materials and methods.

where $A = 0.922$ and $B = 0.072$. Both coefficients of correlation and determination are 0.999, and the standard error of estimation is $9.19 \cdot 10^{-3}$.

Combining eqns. 1, 5 and 6, and relating the volumes to the unit mass of packing material, the hold-up volume of a protein in a strong anion-exchange column having 1 g packing material, $V'_{MQ}$, can be expressed as the function of the protein molecular weight:

$$V'_{MQ} = V'_{oQ} + V'_{iQ}\left[\frac{A}{a}\log M_{app} + \left(B - \frac{Ab}{a}\right)\right] \tag{7a}$$

*i.e.*

$$V'_{MQ} = 1.86 - 0.152 \log M_{app} \tag{7b}$$

where $V'_{oQ}$ and $V'_{iQ}$ are the external and internal volumes of the ion-exchange column related to the unit mass of packing material. Considering that eqn. 6 is valid if $K_{SEC,G} > 0.35$, and that the last data point on the linear part of the function log $M_{app}$ *vs.* $K_{SEC,G}$ (Fig. 3) is that of insulin, eqn. 7b applies over the range of $6000 < M_{app} < 260\,000$. This function is plotted in Fig. 5 including values for the totally excluded dextran sample ($M_r$ $5 \cdot 10^6$–$40 \cdot 10^6$, $K_{SEC} = 0$) and for acetone ($K_{SEC} = 1$).

Mercury porosimetric measurements of Synchropak Q 300 strong anion-exchange material were performed by Dr. Richard Beaver. Cumulative pore area is plotted in Fig. 6 as a function of cumulative pore volume measured by porosimetry.

A comparison of $V'_i$ measured by SEC of acetone ($M_r = 58$) and the porosimetry data yields a minimum value of about 110 Å for the apparent ("dry") pore diameter accessible to acetone. Based on this value, the thickness of the hydrated polyethylenimine coating is estimated to be approximately 40–45 Å. This value agrees with data in the literature[3,39].

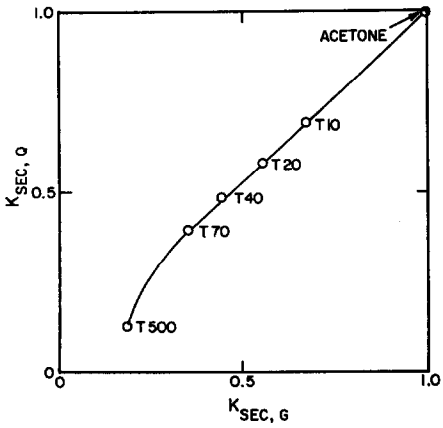


Fig. 4. Size-exclusion distribution coefficient of dextran samples on a strong anion-exchange column ($K_{SEC,Q}$) *versus* a size-exclusion column ($K_{SEC,G}$). See conditions in Materials and methods.

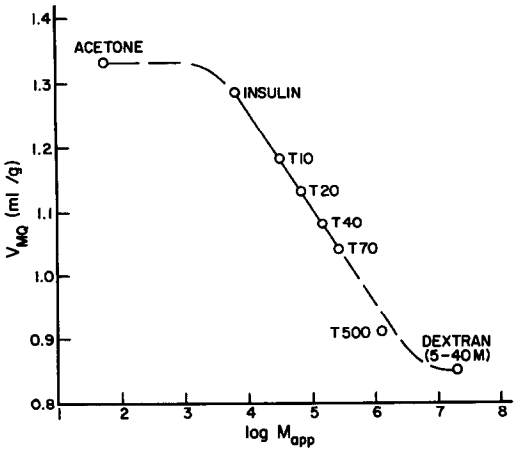


Fig. 5. Hold-up volume of proteins as a function of the logarithm of their molecular weight on a Synchrom Q 300 strong anion-exchange column having 1 g packing material. The solid line represents the effective part of the curve.

The external surface area of the stationary phase in the ion-exchange column was estimated using a simple model, calculating the total surface area of the spherical particles in the column. Assuming that the diameter of a particle is 6.5 $\mu$m[40], the external surface area of 1 g of support is $A_0'$:

$$A_0' = \frac{V_{empty} - V_0}{V_p} \frac{A_p}{m_s} = 0.203 \text{ m}^2/\text{g} \quad (8)$$

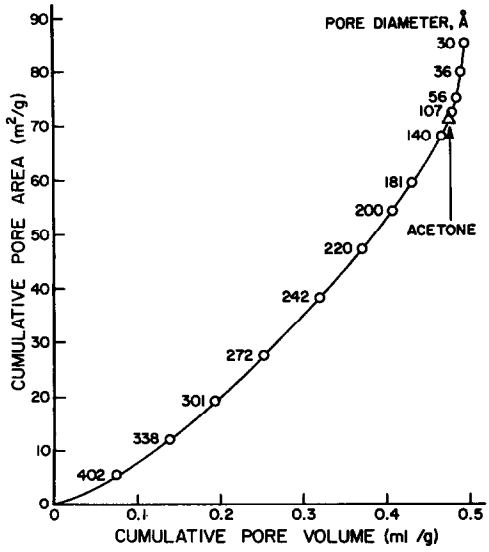


Fig. 6. Cumulative pore area as a function of cumulative pore volume for Synchrom Q 300 strong anion-exchange packing material determined by mercury porosimetry. "Dry" pore diameter values are displayed as well.

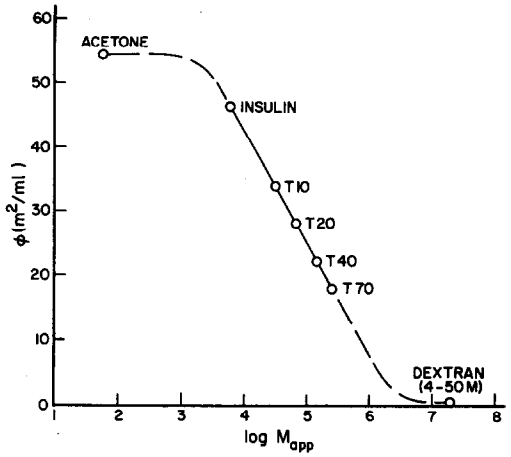


Fig. 7. Phase ratio of proteins as a function of the logarithm of their molecular weight on a Synchrom Q 300 strong anion-exchange column having 1 g packing material. The solid line represents the effective part of the curve.

where $V_{empty}$ is the empty column volume, $V_p$ and $A_p$ are the volume and the surface area of a particle, respectively. Error arising from the particle size distribution and irregularity of the particle surface is neglected. The contribution of the external surface area to the accessible surface area is generally small, and the effect of this error on the phase ratio decreases with decreasing molecular weights of the proteins.

Knowing the functions of $V'_M$ *vs.* log $M_{app}$ (eqn. 7b), the pore surface area *versus* pore volume (Fig. 6), and the values of external volume and external surface area per unit mass of packing material ($V'_0$ = 0.85 ml/g and $A'_0$ = 0.203 m²/g), the phase ratio can be calculated as a function of protein molecular weight (Fig. 7). The effective part of the curve of phase ratio *vs.* log $M_{app}$ is also linear, and its equation is:

$$\varphi = 112 - 17.4 \log M_{app} \tag{9}$$

where 6000 < $M_{app}$ < 260 000. Coefficients of correlation and determination are both 0.999, and the standard error of estimation is 0.235.

The Achilles' heel of the method is the protein calibration curve. The shape and density of the proteins are slightly different, as verified by the different intrinsic viscosity values[41]. This results in deviations from the calibration curve. Therefore, when the curves are used to determine $V_M$ and $\varphi$ values for a rod-shaped solute, the calculated values will be greater than the actual ones[42]. However, if the shape of the proteins used for calibration is similar to that of the proteins studied, this method can result in very good estimations. Hold-up volumes and phase ratios of proteins, calculated using eqn. 7 and eqn. 9, are shown in Table II. The change in the phase ratio as a function of the molecular weight of the proteins is much greater than the change in the hold-up volume. This is due to the smaller contribution of the external surface area to the total accessible surface area than that of the external volume to the accessible hold-up volume.

TABLE II

HOLD-UP VOLUME AND PHASE RATIO OF PROTEINS ON A SYNCHROM Q 300 STRONG ANION-EXCHANGE COLUMN HAVING 1 g PACKING MATERIAL

| *Protein* | $M_r$* | $V'_M$ *(ml/g)* | $\varphi$ *($m^2$/ml)* |
|---|---|---|---|
| Immunoglobulin G | *ca.* 150 000 | 1.07 | 22.1 |
| Conalbumin | 77 000 | 1.12 | 27.1 |
| Bovine serum albumin | 69 000 | 1.12 | 27.9 |
| β-Glucosidase | 65 150 | 1.13 | 28.4 |
| α-Amylase | 55 000 | 1.14 | 29.6 |
| Ovalbumin | 43 500 | 1.15 | 31.4 |
| β-Lactoglobulin | 35 000 | 1.17 | 32.8 |
| Carbonic anhydrase | 30 400 | 1.19 | 34.1 |
| Soybean trypsin inhibitor | 20 100 | 1.21 | 37.2 |
| Myoglobin | 17 500 | 1.22 | 38.3 |
| Insulin | 5700 | 1.29 | 46.7 |
| Acetone | 58 | 1.33 | 54.4 |

* Ref. 44.

## CONCLUSIONS

We suggest a method to calculate hold-up volumes and phase ratios of proteins on a strong anion-exchange column, which is based on mercury porosimetric measurements and size-exclusion calibration using non-retained dextran samples. This method can be used for a very wide range of protein molecular weights, from 6000 to 260 000 in the case of Synchrom Q300 column.

## ACKNOWLEDGEMENTS

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## APPENDIX 1

The change of free energy of the chromatographic process can be calculated according to the following equation:

$$\Delta G^\circ = -RT \ln K_d \quad \text{(A1)}$$

where $R$ and $T$ are the universal gas constant, and the absolute temperature, respectively. $K_d$ is the distribution coefficient of a solute. Since $K_d$ can not be measured directly, it is calculated, generally, from the retention factor and the phase ratio:

$$K_d = k'/\varphi \quad \text{(A2)}$$

Thus, eqn. A1 can be written as:

$$\Delta G^{\circ} = -RT(\ln k' - \ln \varphi) \tag{A3}$$

In the case of liquid–liquid partition chromatography,

$$K_d = [P]_S/[P]_M \tag{A4}$$

where $[P]_S$ and $[P]_M$ are the volumetric concentrations (mol/m$^3$) of solute P in the stationary and the mobile phase, respectively. The phase ratio can be expressed as the ratio of the volumes of the stationary and mobile phases:

$$\varphi = V_S/V_M \tag{A5}$$

Thus, $K_d$ and $\varphi$ are both dimensionless.

In the case of adsorption chromatography,

$$K_{da} = C_{P,S}/[P]_M, \tag{A6}$$

where $C_{P,S}$ is the surface concentration of solute P (mol/m$^2$) on the stationary phase surface. The phase ratio is the following:

$$\varphi_a = A_S/V_M \text{ (m}^2/\text{m}^3) \tag{A7}$$

where $A_S$ is the stationary phase surface area. Both $K_{da}$ and $\varphi_a$ have dimensions. But, the change of free energy of a chromatographic process in adsorption chromatography should be calculated with dimensionless parameters, otherwise the magnitude of the calculated value will be a function of the chosen dimension of $K_{da}$ or $\varphi_a$ (in eqn. A1 or eqn. A3). To avoid this problem, we introduce a factor, $f$, called "solute factor", expressing the ratio of the fractions of the mobile phase volume and the stationary phase area occupied by a solute P molecule:

$$f = V_P/A_P = 4R_P/3, \tag{A8}$$

where $V_P$, $A_P$ and $R_P$ express the volume, the largest cross sectional area, and the radius of a spherical solute. Consequently, the dimensionless distribution coefficient, $K_{dDL}$:

$$K_{dDL} = K_d/f, \tag{A9}$$

and the dimensionless phase ratio, $\varphi_{DL}$ in adsorption chromatography:

$$\varphi_{DL} = \varphi_a f = \frac{A_S}{V_M}\frac{V_P}{A_P} = \frac{A_S/A_P}{V_M/V_P} = \frac{n_{P,S}}{n_{P,M}} \tag{A10}$$

where $n_{P,S}$ and $n_{P,M}$ are the capacities of the stationary and the mobile phases in amount of substance (in moles).

The introduction of the solute factor in the dimensionless phase ratio (in eqn. A10) makes it possible to define another thermodynamic system, where the adsorbed solute molecules are considered as a part of the stationary phase, where the volume

of the stationary phase would be represented by the volume of the adsorbed solute molecules. Therefore, the increase of the amount of solute molecules adsorbed would increase the volume of the stationary phase and decrease the volume of the mobile phase. Consequently, the phase ratio would increase. This model would make it very difficult to use the data of adsorption isotherms previously measured based upon a model of fixed phase ratio.

## APPENDIX 2

The phenomenon mentioned in the case of "solvent disturbance" method for proteins may be used to determine the hold-up volume of a protein. An increase in concentration of protein injected results in a decrease in diffusivity, *i.e.*, in retention volume. Furthermore, an increase in sample concentration increases the mass of protein bound to the stationary phase, consequently, the hold-up volume decreases. The results of these two phenomena have an effect on the shape of the retention volume *versus* concentration of protein sample plot. The intercept of this plot would give the hold-up volume of the protein studied.

## NOTE ADDED IN PROOF

During the review of this paper the question arose as to whether adsorption of a protein on the surface of an ion-exchange column could change its structure, allowing it to penetrate into small pores. Although this is possible, it should be noted that electrostatic adsorption has been widely used in the industrial immobilization of enzymes. Adsorption has also been widely used as a technique for the immobilization of antibodies in clinical assays. If structural changes do occur they are probably on the exterior of the protein and do not produce a global alteration of protein structure. Otherwise, biological activity would be lost. We conclude that electrostatic interactions of proteins with the surface of ion-exchange media generally will not change protein structure sufficiently to alter their migration into porous sorbent matrices. The exception to this might be the "hinged" proteins noted above.

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