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Resolution and Peak Capacity in Equilibrium-Gradient Methods of Separation

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Summary

A theoretical analysis is made of the relative resolving power of equilibrium-gradient separation methods, such as isoelectric focusing and density-gradient sedimentation, and the corresponding kinetic methods, such as electrophoresis and kinetic centrifugation. Both general and specific equations are derived for resolution and peak capacity. It is concluded that peak capacity, the most general index of over-all resolving power, is of comparable magnitude for these two different approaches.

Two new equilibrium-gradient methods of separation are proposed, these employing dielectrical and thermal diffusion forces, respectively.

Parameters such as the "rate of generation of variance," or "plate height," $d\sigma^2/dZ$, "peak capacity," and "resolution" have been used extensively in the characterization of chromatographic systems. Although these parameters have found most use in gas and liquid chromatography as well as in gel permeation chromatography, their importance is more universal. Giddings (*1*) discussed their usefulness for characterizing and comparing the efficiency of separation methods as different as ultracentrifugal sedimentation and electrophoresis. In both cases separation is achieved through the introduction of an external field giving rise to forces acting on the particles and leading to a steady differential migration.

In this work we would like to extend the use of some of these characteristic separation parameters to encompass a general class of

equilibrium-gradient methods. These methods are exemplified by isoelectric focusing (*2*) and density-gradient (isopycnic) sedimentation (*3*). We will compare the intrinsic resolving power of such methods with their kinetic counterparts.

An equilibrium-gradient method, as the term is used here, denotes a method in which a gradient or combination of gradients causes each species to seek an equilibrium position along the separation path. At the equilibrium point the net force on a particle (molecule) is zero as shown in Fig. 1. Any deviation from this position caused by diffusion, etc., gives rise to a restoring force which thereby tends to keep the concentration pulse focused in a narrow region around the equilibrium point.

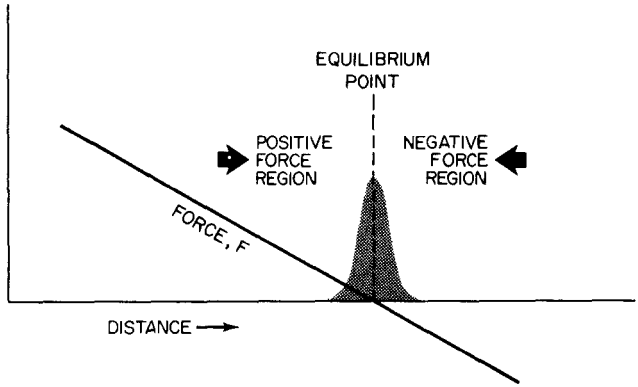

FIG. 1. Force vs. distance and the formation of a zone in an equilibrium-gradient separation system.

In practice equilibrium-gradient methods utilize a primary gradient (e.g., an electrical or "gravitational" potential), which is the same as in the corresponding kinetic method. Superimposed is a secondary gradient in some property (usually pH or density) which, in combination with the primary gradient, causes a reversal in the force at a given point. For example, the force per mole on a charged particle is $q\mathfrak{F}E$, where q is the effective charge, $\mathfrak{F}$ the faraday of electricity, and E the electric field strength. The latter is relatively constant and represents the primary gradient in electrical potential. Effective charge q is made to vary and, most importantly, to reverse sign at some point, by the secondary pH gradient.

Methods other than those employing electrical and centrifugal

forces are conceivable. We propose here two other possibilities. (a) In a strongly nonuniform electrical field, uncharged species with high dielectric constants migrate selectively to the high field regions. If a secondary gradient in the dielectric constant were imposed (such a gradient would occur naturally in an appropriate solvent mixture), each species would seek equilibrium at a point where the dielectric constant of the medium equaled its own. Components would thus separate according to differences in dielectric constant. This method would be applicable only to very large species. (b) The fractionating power of thermal diffusion could be used by employing a solvent mixture which, by its own partial separation, would reverse the sign of the thermal diffusion factor for each species at some point. The point of reversal would be the equilibrium point.

While the dielectric and thermal fields proposed above are intrinsically weaker than electrical or centrifugal fields, the possibility of their use, perhaps under special circumstances, illustrates the broad generality of the equilibrium-gradient class of separations.

THEORY

We will first prove that the zonal shape for static equilibrium is approximately Gaussian. This has been shown earlier for the individual cases of density gradient centrifugation (*3*) and isoelectric focusing (*2*). Here we give a proof for the general case without introducing specific conditions. The desired general expression for the standard deviation, σ, will then be used to obtain values for resolution and peak capacity.

The force per mole acting on a species in a gradient field is, of course, a function of position z, $F(z)$. At the equilibrium point, $z = Z_\infty$, the force vanishes. $F(z)$ can be described by a Taylor expansion about this point

$$F(z) = F(Z_\infty) + \left[\frac{dF(z)}{dz}\right]_{z=Z_\infty} (z - Z_\infty) + \frac{1}{2}\left[\frac{d^2F(z)}{dz^2}\right]_{z=Z_\infty} (z - Z_\infty)^2 + \ldots \quad (1)$$

in which $F(Z_\infty) = 0$, as stated above. Terms of second and higher order may be assumed negligible for narrow zones. Therefore

$$F(z) \simeq \left[\frac{dF(z)}{dz}\right]_{z=Z_\infty} (z - Z_\infty) \quad (2)$$

If we let

$$k = -[dF(z)/dz]_{z=Z_\infty} \tag{3}$$

and transform to the coordinate system $z' = z - Z_\infty$, Eq. (2) becomes

$$F(z') = -kz' \tag{4}$$

which is simply a Hooke's law force with k the effective Hooke's law constant. The potential energy (equivalent to the chemical potential) in this Hookian well is $kz'^2/2$. Using this in the Boltzmann term gives the concentration relative to that at the equilibrium point

$$(C/C_\infty) = \exp(-kz'^2/RT) \tag{5}$$

This is a Gaussian distribution with standard deviation

$$\sigma = (RT/k)^{\frac{1}{2}} \tag{6}$$

This is the general expression needed for the study of resolution and peak capacity, below.

PLATE HEIGHT

Plate height is defined as $H = d\sigma^2/dZ$ for normal chromatography, sedimentation, and electrophoresis (*1*). However, with equilibrium-gradient methods, variance σ^2 is not generated in proportion to distance Z migrated, so the proportionality represented by H makes little sense in this case. With the former techniques plate height H and the number of plates N are useful stepping stones to resolution and peak capacity. Here we proceed directly to these parameters.

RESOLUTION

Resolution Rs for two peaks is defined as $\Delta Z/4\bar{\sigma}$, where ΔZ is the distance between peak centers and $\bar{\sigma}$ is the average standard deviation in width of the two peaks. For closely related substances, the two σ's will be approximately equal so that $\bar{\sigma}$ may be replaced by either individual σ. This step aids mathematical simplification.

The use of σ from Eq. (6) in the above expression for Rs yields

$$Rs = \frac{\Delta Z}{4(RT/k)^{\frac{1}{2}}} \tag{7}$$

This can be rearranged to give

$$Rs = \frac{1}{2}\left(\frac{\Delta\epsilon}{2RT}\right)^{\frac{1}{2}} \tag{8}$$

where $\Delta\epsilon$ is defined by

$$\Delta\epsilon = \tfrac{1}{2}k(\Delta Z)^2 \tag{9}$$

and, in view of the discussion following Eq. (4), is the energy needed to displace a species from its own equilibrium position to that of its neighbor. Thus Rs is determined by the ratio, $\Delta\epsilon/RT$, of two energies, a displacement energy $\Delta\epsilon$ and thermal energy RT. This type of ratio appears also in the kinetic methods, particularly in describing the number of theoretical plates.

We may now inquire into the general effect of changing the steepness of the secondary gradient. If there is an M-fold increase in gradient, there will be correspondingly an M-fold increase in the force at any point and thus an M-fold increase in the Hookian force constant k. The distance between peak centers ΔZ will, on the other hand, change with M^{-1}, since increasing steepness will bring the peaks together. The net effect on $\Delta\epsilon$ of this M-fold gradient increase is therefore a change by a factor of $M(M^{-1})^2 = M^{-1}$. Therefore, Rs, which Eq. (8) shows to depend on $(\Delta\epsilon)^{1/2}$, will change by $M^{-1/2}$. In summary, resolution is inversely proportional to the square root of the gradient. While narrow peaks are obtained in steep gradients, they become crowded together to more than an offsetting degree.

Density-Gradient Sedimentation

In the particular case of density-gradient sedimentation, the force is given by

$$F(z) = (\rho - \rho_0)VG \tag{10}$$

where G is the centrifugal acceleration, $\omega^2 z$, V the molar volume, and ρ and ρ_0 the densities of the entrained species and of the solvent, respectively. A gradient exists in the latter. The force constant, defined by Eq. (3), becomes

$$k = VG\, d\rho_0/dz \tag{11}$$

The distance between peak centers is

$$\Delta Z = \Delta\rho/(d\rho_0/dz) \tag{12}$$

i.e., it is the distance in which the density increment due to the gradient equals $\Delta\rho$, the density difference of the two components. The substitution of these two expressions into Eq. (7) yields

$$Rs = \frac{\Delta\rho}{4}\left(\frac{VG}{RT\, d\rho_0/dx}\right)^{\frac{1}{2}} \tag{13}$$

an equation which gives the explicit conditions necessary for unit resolution. Such an equation has not heretofore been available for density-gradient sedimentation.

Equation (13) can be used to predict the resolvability of biological species under a given set of experimental conditions, provided their densities and molar volumes are known. As an illustration of this, we can apply the resolution expression to the classical separation of isotopically labeled and unlabeled *E. coli* DNA performed by Meselson and Stahl (*4*). The density difference between the two types of DNA is $\Delta\rho = 0.014$ g/cm^3, the density of unlabeled DNA being 1.710 g/cm^3. The molecular weight in CsCl solution was determined as 9.4×10^6 for N^{14}DNA, which gives $V = 9.4 \times 10^6/1.71$ cm^3. Quantity G in the experiment was 140,000g and $RT = 2500$ Joule/mole or in CGS units 25×10^9 ergs/mole at the ambient temperature. Assuming a gradient $d\rho_0/dz = 0.08$ g/cm^4, we can predict from Eq. (13) a resolution, $Rs = 2.2$. The measured value, from Fig. 2b of their paper, is 1.5. The accord is good in view of the fact that any imperfection in the system will detract from the theoretical limit, 2.2.

Isoelectric Focusing

In the case of isoelectric focusing the basic force equation, as mentioned earlier, is

$$F = q\mathfrak{F}E \tag{14}$$

There are equivalent forms involving the zeta potential. Force constant k from this and Eq. (3) becomes

$$k = -\mathfrak{F}E\frac{dq}{dz} = -\mathfrak{F}E\frac{dq}{d\mathrm{pH}}\frac{d\mathrm{pH}}{dz} \tag{15}$$

Peak separation ΔZ is obtained as

$$\Delta Z = \Delta\mathrm{pH}/(d\mathrm{pH}/dz) \tag{16}$$

where ΔpH is the isoelectric pH increment between the two components. The substitution of these two expressions in Eq. (7) gives

$$Rs = \frac{\Delta\text{pH}}{4}\left(\frac{-\mathfrak{F}E\ dq/d\text{pH}}{RT\ d\text{pH}/dz}\right)^{\frac{1}{2}} \tag{17}$$

which again gives the explicit dependence of Rs on basic system parameters. A similar equation has been obtained by Vesterberg and Svensson (*5*).

The above resolution expressions all hinge on the linear force approximation given in Eq. (2). In the above case the validity of the approximation is less obvious because $dq/d\text{pH}$ is generally not constant over a pH range extending more than about 1.5 pH units on each side of the isoelectric point. This is illustrated for ovalbumin in Fig. 2 where electrophoretic charge z is plotted against pH (*6*).

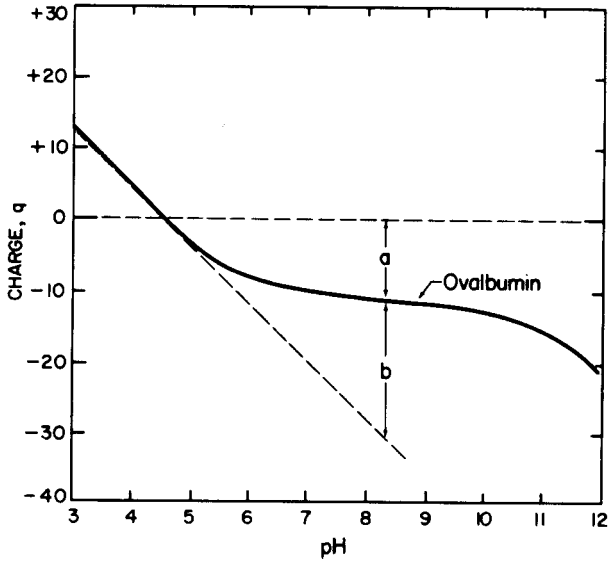

FIG. 2. Variation of electrophoretic charge q with pH for ovalbumin [Overbeek (*6*)].

In order to examine the effect of the nonlinearity, we calculate σ in pH units, σ_{pH}, for a typical system involving ovalbumin. Using $\sigma_{\text{pH}} = \sigma\ d\text{pH}/dz$ along with $\sigma = [RT/-\mathfrak{F}E(dq/d\text{pH})(d\text{pH}/dz)]^{1/2}$, the latter obtained by combining Eqs. (6) and (15), we obtain

$$\sigma_{\text{pH}} = \left(\frac{RT\ d\text{pH}/dz}{-\mathfrak{F}E\ dq/d\text{pH}}\right)^{\frac{1}{2}} \tag{17b}$$

The tangent line in Fig. 2 shows $dq/d\text{pH} = -9$ at the isoelectric

point. If we assume a pH gradient of 0.05 pH/cm and a field of 25 V/cm, Eq. (17b) yields $\sigma_{pH} = 2.4 \times 10^{-3}$. The total peak would be spread over about $4\sigma_{pH}$ or about 10^{-2} pH units. Since observable deviations from linearity do not occur in less than one or two pH units, the effect of nonlinearity is negligible.

Since peak width in pH units is typically 10^{-2}, neighboring peaks can be resolved if their isoelectric points differ by only 0.01. This can be confirmed directly from Eq. (17) by noting that a unit resolution is obtained using the above parameters.

PEAK CAPACITY

Peak capacity, n, is the maximum number of components resolvable by a given technique under specified conditions. If component peaks of average width $4\bar{\sigma}$ are distributed over path length L, the peak capacity is clearly

$$n = L/4\bar{\sigma} \tag{18}$$

From Eq. (6), $\bar{\sigma} = (RT)^{1/2}\overline{(1/k)^{1/2}}$, a term that can be replaced by $(RT)^{1/2}(1/k)^{1/2}$, where k is the average denoted by $1/[(1/k)^{1/2}]^2$. The peak capacity thus becomes

$$n = \left(\frac{kL^2}{16RT}\right)^{\frac{1}{2}} \tag{19}$$

This equation shows that peak capacity increases in proportion to total path length L and with the square root of the secondary gradient as reflected in k.

If we define the term ΔE by analogy to the definition of ΔE in Eq. (9), we have

$$\Delta E = \tfrac{1}{2}kL^2 \tag{20}$$

In terms of this energy parameter, the peak capacity from Eq. (19) takes the form

$$n = \left(\frac{\Delta E}{8RT}\right)^{\frac{1}{2}} \tag{21}$$

which, like Eq. (8) for resolution, involves a ratio of energy terms. The ratio of n to Rs can be shown, using Eqs. (8), (9), (20), and (21), to have the simple form, $L/\Delta Z$.

More importantly, for it encourages the comparison of equilibrium-gradient and kinetic methods, Eq. (21) resembles closely the equation for peak capacity in the kinetic case

$$n_{kin} = \left(\frac{-\Delta\mu^{max}}{32RT}\right)^{\frac{1}{2}} \tag{22}$$

where in this case the energy term, $-\Delta\mu^{max}$, is the chemical potential or energy change of a species migrating the full path length L under the influence of the primary field. Below we seek to compare $-\Delta\mu^{max}$ and ΔE since this permits the direct comparison of peak capacities by the two basic methods.

We postulate a model system with a uniform gradient throughout. Thus the force is a linear function of distance for each component. The force curve for the component whose equilibrium location is at position L is shown in Fig. 3 as the diagonal line which, of course, reaches zero at equilibrium point L. The slope of the line is $-k$, as shown by Eq. (3). The shaded triangular area, which represents the energy drop of the species in moving from the origin to L, is seen by the figure geometry to be $\frac{1}{2}kL^2$. This, of course, is ΔE.

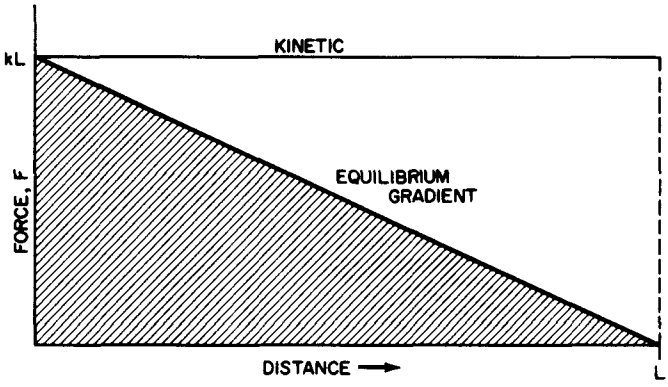

FIG. 3. Comparison of force vs. distance plots for kinetic and equilibrium-gradient methods.

The same component separated by a kinetic method would have a uniform force throughout, represented by the upper horizontal line. (The two lines under optimum conditions will begin at the same point since the highest point available will provide in one case the maximum gradient and in the other case the maximum force.) The energy (or chemical potential) drop of the component moving from the origin to L under this circumstance would be simply the rectangular area on the plot, $-\Delta\mu^{max} = kL^2$. Thus we have the approximation

$$-\Delta\mu^{\max} = 2\Delta E \tag{23}$$

The comparison of Eqs. (21) and (22) now yields

$$n = \sqrt{2}n_{\text{kin}} \approx n_{\text{kin}} \tag{24}$$

which shows the peak capacity to be the same order of magnitude whether equilibrium-gradient or kinetic methods are used.

The foregoing comparison hinges on the linear model, the validity of which we will discuss shortly, and on a particular chemical potential model, Case a of Ref. *1*, which leads to Eq. (22). However, it was shown in the last-named reference that the final result is not strongly dependent on the model chosen.

Density-Gradient Sedimentation

The combination of Eqs. (11) and (19) yields

$$n = \left(\frac{VGL^2\, d\rho_0/dz}{16RT}\right)^{\frac{1}{2}} \tag{25}$$

where, of course, V, and to a lesser extent G and $d\rho_0/dz$, are appropriate averages. An alternate form is obtained by replacing $L\, d\rho_0/dz$ by $[\rho_{0L} - \rho_{00}]$, the total density increment of the solvent over the separation path. This substitution gives

$$n = \left(\frac{VGL\,[\rho_{0L} - \rho_{00}]}{16RT}\right)^{\frac{1}{2}} \tag{26}$$

The ratio of n to Rs, as shown by combining this equation with Eq. (13), is the simple density ratio, $[\rho_{0L} - \rho_{00}]/\Delta\rho$.

From Eq. (25) we can estimate approximately the number of resolvable components in a certain cell with a certain steepness of the density gradient. Assuming $V = 6 \times 10^6$ cm^3, $G = 105{,}000g$, $d\rho_0/dz = 0.05$ g/cm^4, $RT = 25 \times 10^9$ ergs/mole and a cell length, L, of 1.5 cm, Eq. (25) predicts a peak capacity of $n = 13$. Note, however, that this rapidly becomes smaller as molecular size decreases.

Isoelectric Focusing

Here one combines Eqs. (15) and (19) to get

$$n = \left(\frac{-\mathfrak{F}E(dq/d\text{pH})(d\text{pH}/dz)L^2}{16RT}\right)^{\frac{1}{2}} \tag{27}$$

where $dq/d\text{pH}$ is the appropriate average. The alternate form is obtained by replacing $L\ d\text{pH}/dz$ by $[\text{pH}_L - \text{pH}_O]$, the total pH increment. In this case we have

$$n = \left(\frac{-\mathfrak{F}E(dq/d\text{pH})L[\text{pH}_L - \text{pH}_O]}{16RT}\right)^{\frac{1}{2}} \tag{28}$$

The uniform-gradient model is approximate for isoelectric focusing because $dq/d\text{pH}$ is not constant, as seen in Fig. 2. This does not affect the above two equations, but does alter the comparison of kinetic and equilibrium-gradient methods. The peak capacity of the former differs from our earlier estimate since $-\Delta\mu^{\max}$ becomes $kL^2a/(a+b)$ in place of kL^2 (a and b are defined by Fig. 2). Thus the equation

$$-\Delta\mu^{\max} = 2a\Delta E/(a+b) \tag{29}$$

replaces Eq. (23). However, since a and b will be of similar magnitude, the conclusion stated in Eq. (24), that peak capacities are comparable in value whether a kinetic (in this case electrophoresis) or equilibrium-gradient (isoelectric focusing) method is used, is still valid.

DISCUSSION

The explicit formulas obtained here for resolution and peak capacity provide guidelines for achieving separations. Of equal importance, a meaningful comparison of the potential of kinetic and equilibrium-gradient methods has been made. The latter comparison merits additional amplification.

Although kinetic and equilibrium-gradient methods utilize the same primary fields (electrical or gravitational), they will not necessarily fractionate the same sample systems. The density gradient method, for example, will not fractionate solutes that have different sizes but equal density, whereas the kinetic sedimentation method will fail for systems where the net force and frictional coefficient are proportional to one another. This basic difference in function must provide the initial criterion between the two methods. However, for solutes with a reasonably broad spectrum in properties, the total of resolvable peaks (the peak capacity n) provides a general criterion of over-all resolving power. Resolution does not provide such a criterion because it is specific for a particular pair and the answer depends on the detailed properties assumed for each component, the choice of which may arbitrarily favor one method over the other. Therefore the peak capacity

provides a general, and at the same time an experimentally meaningful, criterion of fractionation efficacy.

It has been stated that the resolving power, at least in sedimentation, is inherently better in kinetic than in equilibrium-gradient methods (*7*). Conversely higher resolving power has been claimed for isoelectric focusing than for electrophoresis (*5*). Our own conclusion is that the two approaches are generally comparable in resolving effectiveness for any of the basic primary fields. However, the important matter of resolution time, not considered in detail here, perhaps favors the kinetic methods.

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