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J. Calvin Giddings[a]
[a] Department of Chemistry, University of Utah Salt, Lake City, Utah

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Generation of Variance, "Theoretical Plates," Resolution, and Peak Capacity in Electrophoresis and Sedimentation

J. CALVIN GIDDINGS

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF UTAH
SALT LAKE CITY, UTAH

Summary

The rate of generation of variance, $d\sigma^2/dX$, is a fundamental parameter which determines peak or boundary width and, thus, resolution in many differential migration systems. This parameter can be identified with the "plate height" of chromatography. By extending this nomenclature and the underlying concepts to electrophoresis and sedimentation, we arrive at parameters, particularly the "number of theoretical plates," which allow a comparison of the effectiveness of these diverse methods. Equations are derived for the plate number as well as for resolution and peak capacity. Numerical comparisons are shown. Optimization is discussed with reference to maximum resolution, peak capacity, and separation speed.

The variance, σ^2, of a solute peak (or front) in a uniform section of a chromatographic, electrophoretic, or sedimentation system increases in proportion to the distance migrated, X. The important ratio, $d\sigma^2/dX$, with the dimensions of a length, has often been used to define the "height equivalent to a theoretical plate" (or simply "plate height"), H, in chromatography (*1*). By this definition, the ties with unrealistic theoretical plate models are cut, and one is left with a simple length parameter, H, which describes the rate of generation of variance. The dimensionless "number of theoretical plates," N, is thus equal to the number of such H units in length X, and is X/H or $(X/\sigma)^2$ for a system uniform throughout. Parameter N has great utility in characterizing the efficiency of chromatographic operation. It can be shown to determine the degree of resolution of neighboring peaks and

to fix the "peak capacity," the maximum number of peaks separable. It is a valid and almost universally accepted index of chromatographic effectiveness.

The purpose of this work is to show that parameters H and N serve as equally valid indicators of electrophoretic and sedimentation efficiency, and provide a common basis for the comparison of the intrinsic capability of the various systems. Furthermore, we obtain simple expressions for the maximum number of theoretical plates, maximum resolution, and maximum peak capacity, thus making numerical comparisons possible. (Although the use of theoretical plate concepts is particularly inappropriate for continuous processes, the rate of variance generation, $d\sigma^2/dX$, is still of utmost significance. We call this "plate height" only to make a common connection with experimental chromatography.)

Any of the above techniques may be operated in a nonuniform mode, where gradients of some kind are used to achieve particular ends. Examples are gradient elution chromatography (*2*), chromothermography (*3*), isoelectric focusing (*4*), and density gradient centrifugation (*5*). Even centrifugation without gradients involves a force proportional to radial distance, but the variation over the migration path is not of significant proportions. In each case the maximum resolving power is related to the efficacy of the underlying processes, which may be characterized by H or some average of local H values. However, the present treatment is explicitly directed toward systems of a reasonally uniform nature.

Both electrophoresis and centrifugation may be operated either as zone or frontal (i.e., and or boundary) techniques. The spreading and resolution loss in either case can be described by plate height parameters.

The maximum possible resolution is that obtained in the absence of convection, stabilizing media, electrodiffusion, etc. Such nonidealities have been discussed in detail by Wieme for electrophoresis and are collectively responsible for what he calls "electrophoretic dispersion" (*6*). Addition of the latter to molecular diffusion yields an overall effective diffusion or dispersion coefficient, $\mathfrak{D}$. We will define a parameter, θ, as the dimensionless ratio

$$\theta = \mathfrak{D}/D \tag{1}$$

where D is the molecular diffusion coefficient. It follows that $\theta \geqq 1$; values of θ near unity are, of course, desired.

Let us assume that the external field exerts a force F on a mole of a given solute species. Its field-induced migration velocity through the medium will then be

$$U = F/\zeta \tag{2}$$

where ζ is the friction constant per mole of the species in the medium. For spherical particles the latter is given by Stokes law, $\zeta = 6\mathfrak{N}\pi\ \eta r$, but such an explicit form is not necessary.

The irreducible minimum peak broadening is that due to molecular diffusion. The latter leads to the variance $\sigma^2 = 2Dt$, where D is the diffusion coefficient and t is time. Plate height H is then given by $d\sigma^2/dX = 2D\ dt/dX$ or, since $dX/dt = U$,

$$H = 2D/U \tag{3a}$$

If D is replaced by RT/ζ, where R is the gas constant and T is the absolute temperature, H becomes

$$H = 2RT/U\zeta \tag{3b}$$

This can be cast in terms of the force F by using Eq. (2),

$$H = 2RT/F \tag{3c}$$

The total number of theoretical plates along a uniform migration path of length X is, consequently,

$$N = XF/2RT = -\Delta\mu^0/2RT \tag{4}$$

where $-\Delta\mu^0$ is the chemical potential (or potential energy) drop of the species in migrating distance X. (The chemical potential "change," $\Delta\mu^0$, is negative; thus $-\Delta\mu^0$ has a positive value.) Quantity N is, therefore, one-half the ratio of two easily visualized energies: energy drop, $-\Delta\mu^0$, which structures the migration and separation, and thermal energy, RT, which makes it more diffuse.

Since N equals $(X/\sigma)^2$ (see the first paragraph), the above results show that the width of the peak (defined as 4σ) relative to the distance migrated through the medium, X, should ideally be

$$\frac{4\sigma}{X} = \left(\frac{32RT}{-\Delta\mu^0}\right)^{1/2} \tag{5}$$

The departure from this ideal measures the degree to which extraneous factors ("electrophoretic dispersion" and an analogous "sedimentation dispersion") are interfering with maximum performance or is a reflection of heterogeneity. The final result is

$$N = -\Delta\mu^0/2\theta RT \tag{6}$$

where θ is defined by Eq. (1). The ratio in Eq. (5) is enlarged by $\theta^{1/2}$ when the latter differs from unity.

For comparative purposes, we now calculate some order-of-magnitude N values from Eq. (4).

Electrophoresis: A potential drop of V volts for a species with effective charge number z (differing from the actual z because of double-layer effect), leads to $-\Delta\mu^0 = 96{,}500Vz$ J/mole. The product Vz often lies in the range of 10^2 to 10^4, giving $-\Delta\mu^0 \approx 10^7$–$10^9$ J/mole. Quantity $2RT$ is near 5000 J/mole at ordinary working temperatures. Thus, plate numbers are capable of reaching the vicinity of 10^3 to 10^5. The upper part of this range is equivalent to that of a very good gas chromatographic column and exceeds the capability of most liquid columns. However, these values may not necessarily be reached in electrophoresis because of electrophoretic dispersion; they represent merely a theoretical limit to performance.

Sedimentation: A simplified expression, adequate for present purposes, is $-\Delta\mu^0 = (1 - \rho_0/\rho)\ GML$, where ρ_0 and ρ are the densities of the medium and the solute, respectively, G is the centrifugal acceleration ($\omega^2 r$), M the molecular weight, and L the maximum value of X, i.e., the maximum length of migration path. For example, if $(1 - \rho_0/\rho)$ is 0.5, G is 10^6–10^8 cm/sec^2 ($\sim 10^3$–10^5 gravities) and L is 10 cm, then $-\Delta\mu^0$ is $5 \times (10^6$–$10^8)M$ ergs/mole and $-\Delta\mu^0/2RT$ is $(10^{-4}$–$10^{-2})M$. From Eq. (4) we see that this is the maximum number of attainable plates under the prescribed conditions. Of particular note is the proportionality to M. For $M = 10^5$, N is 10–10^3; for $M = 10^7$, N can reach from 10^3 to 10^5. These conditions, when compared to the electrophoretic results of the last paragraph, are more restrictive, although exceptions exist. One is more often interested in moderately charged species than in components of which the molecular weight exceeds 10^7. These result in about the same maximum plate number in electrophoresis and in centrifugation, respectively.

RESOLUTION

The *resolution* of two peaks is defined as the distance between their centers, ΔX, divided by $4\bar{\sigma}$ (see Fig. 1), where $\bar{\sigma}$ is the average of the two standard deviations. This measure is ordinarily applied to close-lying or overlapping peaks where $\Delta X \ll X$.

The incremental migration distance, ΔX, is proportional to the incremental velocity, ΔU. We have $(\Delta X/\bar{X}) = (\Delta U/\bar{U})$ or

$$\Delta X = \bar{X}\,\Delta U/\bar{U} \tag{7}$$

Since for a uniform column the plate height, $H = d\sigma^2/dX$, becomes $H = \sigma^2/X$, we have $\sigma = (\bar{H}\bar{X})^{1/2}$. Although σ resembles an rms average, it approaches the arithmetic average sufficiently well for similar, close-lying peaks. Using this $\bar{\sigma}$ and the ΔX from Eq. (7), along with the approximate expression $\bar{N} = \bar{X}/\bar{H}$, resolution becomes

$$Rs = \frac{1}{4}(\bar{N})^{1/2}\frac{\Delta U}{\bar{U}} \tag{8}$$

This expression shows that resolution is composed of two factors—the the efficiency of the system as measured by $(\bar{N})^{1/2}/4$ and the selectivity between solutes as measured by their relative velocity difference, $\Delta U/\bar{U}$. The same conclusion applies to chromatography (*1*).

If two solutes in a particular medium have a specified selectivity, $\Delta U/\bar{U}$, the number of plates required to separate them is, from Eq. (8),

$$\bar{N} = 16/(\Delta U/\bar{U})^2 \tag{9}$$

If the fractional velocity difference is 1 part in 10, the required $\bar{N}$ is 1600; if 1 part is 100, $\bar{N} = 160{,}000$, the latter requiring more than ordinary effort as the earlier calculations showed.

By using Eq. (4) with Eq. (9), we can also show the minimum chemical potential drop needed to achieve unit resolution. This is

$$-\Delta\mu^0 = 32RT/(\Delta U/\bar{U})^2 \tag{10}$$

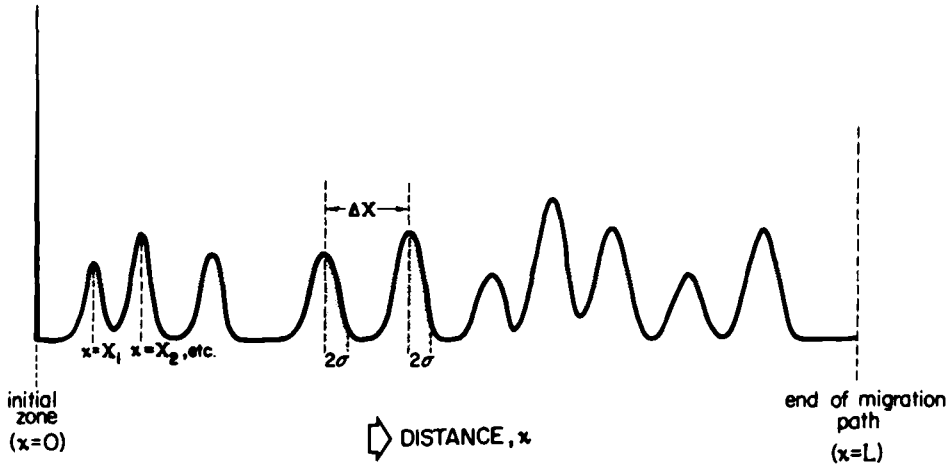

FIG. 1. Schematic illustration of separated peaks. *Peak capacity* is the maximum number of peaks the system is capable of separating (here ~ 12). Resolution is $\Delta X/4\bar{\sigma}$. Maximum length of migration is L.

If θ departs significantly from unity, it must be included in the numerator to get the actual (rather than minimum) value.

PEAK CAPACITY

The *peak capacity* is the upper limit of resolvable components for a given technique under prescribed conditions (7) (see Fig. 1 for an illustration). It is obtained by allowing each hypothetical peak to occupy a distance equal to its own width, 4σ, and, then, by calculating how many of these peaks can be accommodated in the allowable migration range L. The calculations are by nature approximate, but the results provide useful estimates of maximum performance.

We may distinguish three limiting cases, which depend on the properties responsible for separation. If we consider the final solute spectrum, the $\Delta\mu^0$ value can depend on migration path length X as follows:

a. $-\Delta\mu^0 = \text{const. } X^2$. This limit is approached for species which have comparable sizes (and thus ζ), and the separation of which depends on differences in charge (electrophoresis) or density (sedimentation).

b. $-\Delta\mu^0 = \text{const. } X$. This limit is opposite to (a) and corresponds to a constant force, F, for each species. The separation occurs by virtue of differences in friction coefficient ζ (i.e., by virtue of size) rather than in applied forces.

c. $-\Delta\mu^0 = \text{const.}$ Here the force is inversely proportional to the distance migrated. This may approximate a series of increasing size for which the charge or density increases only gradually with radius, i.e., with $(\text{radius})^{1/2}$.

From Eq. (5) the peak width is

$$4\sigma = \left(\frac{32RT}{-\Delta\mu^0/X^2}\right)^{1/2} \tag{11}$$

In (a), $-\Delta\mu^0/X^2$ is constant, thus yielding a constant peak width. Peak capacity is simply the maximum migration length over the peak width, $L/4\sigma$ or

$$n = (-\Delta\mu^0_{\max}/32RT)^{1/2} \qquad \text{case (a)} \tag{12}$$

where $-\Delta\mu^0_{\max}$ is the highest value of $\Delta\mu^0$ among the species.

In (b) the width is

$$4\sigma = \left(\frac{32RT}{-\Delta\mu^0/X}\right)^{1/2} (X)^{1/2} = \beta X^{1/2} \tag{13}$$

where β is equal for all species. We treat the present case by noting that the number of peaks, dn, which can fit in a small interval dX is $dX/4\sigma = dX/\beta X^{1/2}$. Upon integration

$$n = 2\beta^{-1}(L^{1/2} - L_0^{1/2}) \approx 2L^{1/2}/\beta \tag{14}$$

When β is replaced by its equivalent from Eq. (13), we have

$$n = \left(\frac{-\Delta\mu^0_{\max}}{8RT}\right)^{1/2} \qquad \text{case (b)} \tag{15}$$

Case (c) is equivalent to a constant plate number, N, and is a reasonably good approximation in chromatography. By means of the previous arguments, $4\sigma = 4XN^{-1/2}$ and

$$n = \frac{N^{1/2}}{4} \ln \frac{L}{L_0} = \left(\frac{\Delta\mu^0}{32RT}\right)^{1/2} \ln \frac{L}{L_0} \qquad \text{case (c)} \tag{16}$$

which is essentially equivalent to an earlier derived chromatographic equation. Here L_0 is the distance (usually 0.1–0.01 $\times$ L) below which resolution is seriously damaged by initial peak size. Such a limit is needed in this case because the prediction that the peaks get sharper as they approach the origin must fail at some point L_0. (Note that none of the equations relating 4σ and X contradicts the assumed proportionality of σ^2 to X for a single peak because the former describes a sequence of peaks involving different species.)

It is noteworthy that case (a), (b), and (c) all result in similar peak capacities despite different assumptions. Undoubtedly little would be changed if we had a mixture of the three cases. Consequently, a rather general expression can be obtained. Taking the intermediate case (b) as typical and combining this with Eq. (4), we get

$$n \approx 0.5(N_{\max})^{1/2} \tag{17}$$

where $N_{\max}$ is the highest number of theoretical plates among the species. This is comparable to the equation for liquid chromatographic columns.

If θ is significantly different from unity, Eq. (17) is unchanged, but Eqs. (12), (14), (15), and (16) must all be multiplied by $\theta^{-1/2}$.

Equation (17) shows that the separation of ten distinct peaks requires at least 400 theoretical plates; one hundred peaks (comparable to a number of experimental examples from gas chromatography) requires a minimum of 40,000 plates. The latter result is near the upper limit of performance in most electrophoresis and sedimentation devices, as the previous calculations have shown.

OPTIMIZATION

Optimization is a complex topic, of which only some rudimentary but central aspects will be covered here. Criteria for optimization arise rather naturally from the foregoing equations.

Maximum Resolution and Peak Capacity

Both resolution and peak capacity increase with plate number N. Thus an optimization of parameters to maximize both of the former involves the maximizing of N. Equation (6) shows the necessary steps. The chemical potential drop should be as large as possible and θ should be minimal. In electrophoresis the former involves increasing the effective charge or ζ potential and increasing the total voltage drop. The study of θ is much more involved and cannot be adequately summarized in this space (*6*). However it is dependent on $-\Delta\mu^0$, an interaction that must be allowed for. Temperature, T, should also be a minimum, but this parameter ordinarily provides little leeway.

Maximum Speed

Macromolecular transport processes are inherently sluggish, leading to separation times far in excess of those of, e.g., gas chromatography. The slowness hinders data collection and the opportunity to experiment freely over a wide range of parameters.

Since N measures the efficiency of separation, N/t measures the time rate of generation of efficiency. From Eq. (4), $N/t = UF/2RT$, where U has replaced X/t. With the help of Eq. (2) and the inclusion of θ, this becomes

$$\frac{N}{t} = \frac{F^2}{2RT\zeta\theta} \tag{18}$$

A maximum N/t is achieved by employing the maximum possible force, F, and by reducing θ and ζ, which is equivalent to decreasing viscosity. Temperature is more complex because it strongly affects ζ; altogether T should be as high as is consistent with the system so that viscosity and ζ are minimal.

This brief treatment does not allow for the many subtleties of optimization theory. The analogous problem for chromatography, which illustrates the complexities, has been more extensively discussed (1).

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