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Resolution of Species Showing Microheterogeneity by Zone Electrophoresis and Chromatographic Systems: Theory

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Abstract

The resolution of a zone electrophoretic system or a chromatographic column is customarily defined in terms of the separation of two pure (homogeneous) species. For such separation the resolution increases indefinitely with time for analytic fractionation, or indefinitely with column length for elution separation. It is shown that microheterogeneity of either or both species imposes an upper limit on resolution. This may have a marked effect on conditions for optimal resolution, and on measurements of the apparent diffusion coefficient.

INTRODUCTION

Many authors have commented on the boundary spreading in electrophoresis that is due to microheterogeneity (i.e., a distribution of, rather than a single value of the mobility, for a given chemical species) (*1–7*). There are many causes of microheterogeneity; for example, proteins may show a distribution of net charge leading to a distribution of electrophoretic mobility because of deamidation, binding of ligands, partial

proteolysis or denaturation, and the existence of several stable conformations. The customary definition of resolution refers to the ability of the system to resolve two pure species, that is, species that do not exhibit microheterogeneity. These microheterogeneties can have an important effect on the resolution of a system. In particular, the resolving power of a column, defined in terms of two pure substances, can theoretically be increased indefinitely simply by increasing the column length. We demonstrate below that the effect of microheterogeneity is to set an upper limit to resolution viewed as a function of column length or duration of fractionation. In what follows we assume that the microheterogeneity properties remain constant in time, thus neglecting effects associated with denaturation, deamidation, or slow chemical reactions which might occur during the separation process.

THEORY

The column will be regarded as one dimensional, with x the spatial coordinate. We consider two cases, analytical fractionation in which the separation of two initially sharp peaks is observed after a fixed time, and elution (preparative) fractionation, in which the flux at the end of the column is measured. The reduced resolution of a column with respect to two proteins A and B may be defined to be

$$R = \frac{\mu_A - \mu_B}{\sqrt{\sigma_A^2 + \sigma_B^2}} \tag{1}$$

where μ denotes the mean peak position (centroid) at time t, and σ^2 is the second moment about the mean. [There are many variants of this definition. In order to bring it into line with the more commonly used form for the resolution, call it R_c, we would write $R_c = R/\sqrt{8}$ so that when $\sigma_1 = \sigma_2 = \sigma$, one has $R_c = \Delta\mu/(4\sigma)$ as used, for example, by Giddings (8).] When the column properties are homogeneous and diffusion or diffusion-like spreading is the only mechanism of bandspreading, then in analytical separation the μ's can be written

$$\mu = vt \tag{2}$$

in which t is the time, and the σ^2 depend on time as

$$\sigma^2 = 2Dt \tag{3}$$

where v (velocity) and D (apparent diffusion coefficient) are constants. Hence the resolution increases indefinitely as $\sqrt{t}$. In a similar way, if L

denoted the column length, the resolution is proportional to $\sqrt{L}$ for elution processes. We will show that the presence of microheterogeneity causes R to approach a limiting value as t or L become indefinitely large.

If the transport velocity of a specific molecule is v (for example, v in electrophoresis is ME, where M is mobility and E is the voltage gradient already defined), the concentration profile at time t of an initial pulse is

$$c(x,t;v) = \frac{c_0 f(v)}{\sqrt{4\pi Dt}} \exp\left[-\frac{(x - vt)^2}{4Dt}\right] \tag{4}$$

in which $c_0 f(v)$ is the initial amount of protein with velocity between v and $(v + dv)$. In what follows we characterize microheterogeneity solely as a distribution of values of v, while D is assumed to be constant.

This assumption is not necessary, but since the principal effects are due to the distribution of the transport coefficient, we consider only the simpler case.

It follows from Eq. (4) that the first and second moments of the concentration of a single species are

$$\bar{x}(t) = \bar{v}t, \qquad \bar{x}^2(t) = \bar{v}^2 t^2 + 2Dt \tag{5}$$

where by definition

$$\overline{v^n} = \int_0^\infty v^n f(v)\, dv, \qquad n = \cdots, -2, -1, 0, 1, 2, \cdots \tag{6}$$

Hence we see that

$$\sigma^2 = (\overline{v^2} - \bar{v}^2)t^2 + 2Dt = \sigma_v^2 t^2 + 2Dt \tag{7}$$

as found by earlier authors (1–7). Thus the resolution is given by

$$R(t) = \frac{(\bar{v}_A - \bar{v}_B)\sqrt{t}}{\sqrt{(\sigma_{vA}^2 + \sigma_{vB}^2)t + 2(D_A + D_B)}} \tag{8}$$

which approaches an upper asymptote

$$R(\infty) = \frac{\bar{v}_A - \bar{v}_B}{\sqrt{\sigma_{vA}^2 + \sigma_{vB}^2}} \tag{9}$$

rather than increasing indefinitely with t, when σ_{vA}^2 and σ_{vB}^2 are not both zero. It is evident from Eq. (7) that the dimensionless quantity measuring the importance of microheterogeneity relative to that of diffusion as a cause of bandspreading for either species is

$$\alpha(t) = \sigma_v^2 t/(2D) \tag{10}$$

When $\alpha(t)$ is much less than 1, diffusion predominates in determining bandspreading, and when $\alpha(t) \gg 1$, the microheterogeneity predominates. As an example, some typical experimental parameters for a single protein in a gel electrophoresis column are $D = 4 \times 10^{-7}$ cm^2/sec, and a coefficient of variation (defined to be $\sigma_v/\bar{v}$) = 0.02. Assuming a mobility equal to 5×10^{-5} cm/(sec)(V) and a voltage gradient equal to 10 V/cm, we find that $\alpha(t) = 1.25 \times 10^{-4}\ t$ (sec). Thus for $t = 3$ hr $= 1.08 \times 10^4$ sec, $\alpha(t) = 1.35$, showing that microheterogeneity and diffusion contribute roughly equally to band spreading. A curve of $R(t)$ as a function of t is shown in Fig. 1 for the parameter values $\bar{v}_A - \bar{v}_B = 4 \times 10^{-5}$ cm/sec, $\sigma_{vA}^2 = \sigma_{vB}^2 = 1 \times 10^{-10}$ cm^2/sec^2, and $D_A = D_B = 4 \times 10^{-7}$ cm^2/sec. As can be seen from the solid curve, when microheterogeneity is present,

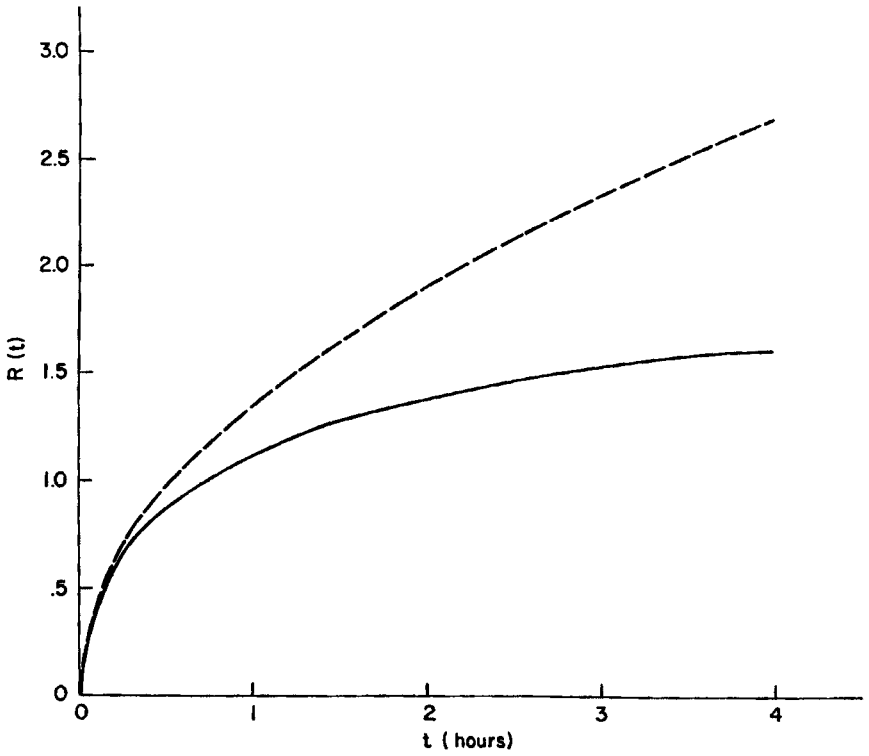

FIG. 1. A curve of resolution as a function of time for two species showing microheterogeneity. The parameter values are $\bar{v}_A - \bar{v}_B = 4 \times 10^{-5}$ cm/sec, $\sigma_{vA}^2 = \sigma_{vB}^2 = 1 \times 10^{-10}$ cm^2/sec^2, and $D_A = D_B = 4 \times 10^{-7}$ cm^2/sec.

$R(t)$ approaches a limiting value very slowly with increasing time. It is possible, from Eq. (8), to specify the time, t_θ, at which the resolution becomes any arbitrary fraction θ of its limiting value. Then from the equation

$$R(t_\theta)/R(\infty) = \theta \tag{11}$$

we calculate t_θ as

$$t_\theta = \frac{\theta^2}{1-\theta^2}\left(\frac{D_A + D_B}{\sigma_{vA}^2 + \sigma_{vB}^2}\right) \tag{12}$$

which depends only on the relative importance of the two mechanisms of bandspreading and the parameter θ.

The calculations related to resolution can be made without reference to a specific model of microheterogeneity because they involve only two moments. Let us examine the effect of a specific model of microheterogeneity on the concentration profile. We suppose that velocity has a uniform distribution over the range $V - V_1 < v < V + V_1$:

$$\begin{aligned} f(v) &= 0, && v < V - V_1 \\ &1/2V_1, && V - V_1 \leqslant v \leqslant V + V_1 \\ &0, && v > V + V_1 \end{aligned} \tag{13}$$

where V and V_1 are constants, and

$$\bar{v} = V, \qquad \sigma_v = V_1/\sqrt{3} \tag{14}$$

The integrated concentration profile can be expressed in terms of the error function

$$\phi(x) = (2\pi)^{-1/2}\int_{-\infty}^{x} \exp(-u^2/2)\, du \tag{15}$$

as

$$\begin{aligned} \frac{C(x,t)}{c_0} &= \int_0^\infty \frac{c(x,t;v)}{c_0}\, dv \\ &= \frac{1}{2V_1 t}\left\{\phi\left(\frac{(V+V_1)t - x}{\sqrt{2Dt}}\right) - \phi\left(\frac{(V-V_1)t - x}{\sqrt{2Dt}}\right)\right\} \end{aligned} \tag{16}$$

In the limit $V_1 = 0$, $C(x,t)$ is given by Eq. (4) with $v = V$. In order to assess the effects of microheterogeneity on the concentration profile, we have plotted in Fig. 2 several normalized concentration profiles for the parameters $D = 4 \times 10^{-7}$ cm^2/sec, $V = 4 \times 10^{-4}$ cm/sec, and different

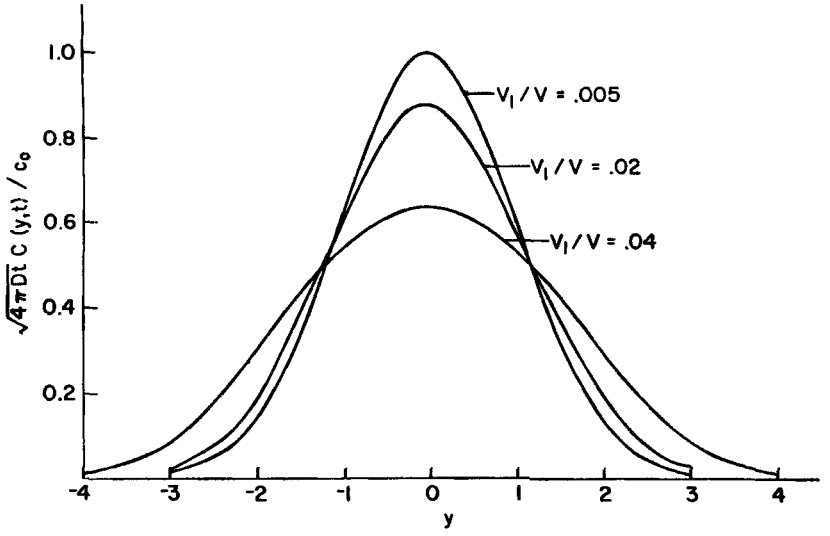

FIG. 2. Normalized concentration profiles when the velocity has a rectangular distribution around the mean. The parameters are $D = 4 \times 10^{-7}$ cm^2/sec and $V = 4 \times 10^{-4}$ cm/sec.

values of the ratio V_1/V corresponding to crudely estimated experimental values in gel electrophoresis. The curves display $\sqrt{4\pi Dt}C(x,t)/c_0$ as a function of the dimensionless parameter $y = (Vt - x)/\sqrt{2Dt}$ for $t = 3$ hr. As can be observed, appreciable broadening occurs only for the value of $V_1/V = 0.04$ which is equivalent to $\sigma_v/\bar{v} = (1/\sqrt{3})(V_1/V) = 0.023$.

Similar calculations can be made for elution chromatography or preparative scale electrophoresis. If the column length is denoted by L, then the elution profile is given, to a good approximation, by

$$\frac{E(t)}{c_0} = \frac{L}{\sqrt{4\pi Dt^3}} \int_0^\infty f(v) \exp\left[-\frac{(L - vt)^2}{4Dt}\right] dv \tag{17}$$

The expected elution time and the associated variance can be written

$$\bar{t} = L\overline{v^{-1}}, \qquad \sigma_T^2 = L^2\rho^2 + \frac{LD}{2}\overline{v^{-3}} \tag{18}$$

where $\rho^2 = \overline{v^{-2}} - (\overline{v^{-1}})^2$. Hence the contribution to the bandspreading of heterogeneity relative to diffusion is

$$\beta(L) = 2L\rho^2/D\overline{v^{-3}} \tag{19}$$

If we choose, as a model of heterogeneity the uniform distribution given in Eq. (13), then it follows that

$$\rho^2 = \frac{1}{V^2 - V_1^2} - \frac{1}{4V_1^2} \ln^2\left(\frac{V + V_1}{V - V_1}\right) \qquad (20)$$
$$\overline{v^{-3}} = V/(V^2 - V_1^2)^2.$$

Assuming the parameters $D = 4 \times 10^{-7}$ cm^2/sec, $V = 5 \times 10^{-4}$ cm/sec, and $V_1/V = 0.04$, we find that $\beta(t) = 1.33\ L$ (cm) so that microheterogeneity would have an important effect for any L greater than approximately 1/2 cm. The analog of Eq. (8) gives the resolution as a function of column length L as

$$R(L) = \frac{(v_B^{-1} - v_A^{-1})\sqrt{L}}{\sqrt{(\rho_A^2 + \rho_B^2)L + D_A v_B^{-3} + D_B v_A^{-3}}} \qquad (21)$$

with a limiting value

$$R(\infty) = \frac{\overline{v_B^{-1}} - \overline{v_A^{-1}}}{\sqrt{\rho_A^2 + \rho_B^2}} \qquad (22)$$

In practice, when optimizing a preparative (elution) system, it is necessary to optimize resolution for some finite time, or alternatively, to optimize resolving efficiency, i.e., resolution per unit time (9). These calculations can be extended to the case where properties of the column are inhomogeneous, by using the theory of Weiss and Dishon (*10*, *11*). The effects of microheterogeneity on the separation of proteins of greatly differing amounts is not covered by the present theory because the expression in Eq. (1) is inappropriate, but we feel that no matter what criteria are used to characterize resolution, the qualitative result of the present paper will hold.

Finally, we note that the parameter σ_v^2 in Eq. (7) can be found either by curve fitting $\sigma^2(t)$ to a quadratic form in t, or in electrophoresis by using the fact that bandspreading due to microheterogeneity is reversible when the field is reversed (*4*). If the field is reversed at time T, then $\sigma^2(t)$ is given by

$$\sigma^2(t) = \sigma_v^2(2T - t)^2 + 2Dt \qquad (23)$$

valid for $t \geqslant T$. Hence if we choose the two times at which peak positions coincide, t and $2T - t$, then we have

$$\sigma^2(2T - t) - \sigma^2(t) = 4D(T - t) \qquad (24)$$

from which the pure diffusion contribution is easily found.

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