A NEW TECHNIQUE FOR DETECTING DENSITY HETEROGENEITY IN THE ULTRACENTRIFUGE

A Ch 80 research report

submitted by

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(Computer programs will be found in pocket inside back cover)

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I. INTRODUCTION. The technique of density gradient centrifugation has proven to be a powerful tool in many areas of modern biochemistry. In the analytical ultracentrifuge it is possible to obtain a high degree of resolution of molecular components of a material according to their density in a buoyant salt solution. Furthermore, in the preparative ultracentrifuge components can be quantitatively separated and collected. A method to increase the resolution of density heterogeneity in the ultracentrifuge could become a useful technique for biochemists. The subject of this paper is a new design for an ultracentrifuge cell whose effectiveness in increasing such resolution will be analyzed in detail.

II. CELL DESIGN.

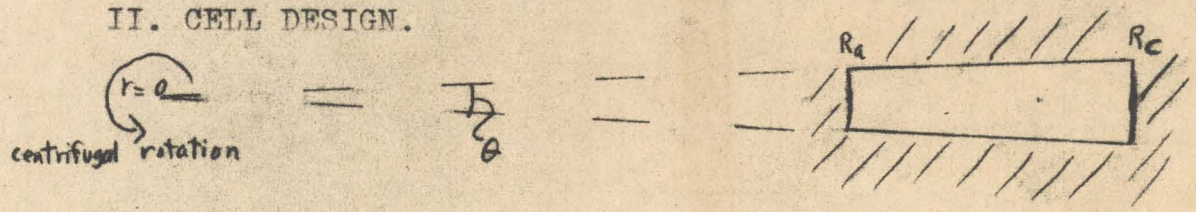

Fig. 1 a
"USA" cell

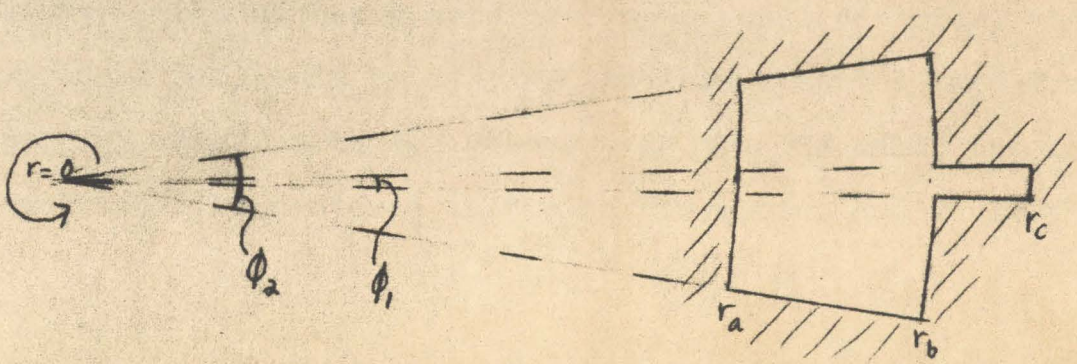

Fig. 1 b
"DSA" cell

The normal shape of the analytical ultracentrifuge cell is shown in Fig. 1a. It is termed the "uniform sector angle cell", or the USA cell. The design of the new cell is shown in Fig. 1b. It is called the "dual sector angle cell", or DSA cell. The centrifuge cell is filled with a salt solution such as CsCl, and at high angular velocity a density gradient is formed which is assumed to be linear at equilibrium. A high molecular weight material, such as DNA or protein, if placed in the cell with a solution of appropriate density, will form a band in the central region of the cell. Assuming a constant centrifugal field in the region of the band, and ignoring effects at the two ends of the cell, a material homogeneous with respect to both density and molecular weight will have a perfectly gaussian concentration distribution. It is important to keep in mind that the band will be gaussian regardless of the particular shape of the cell. Thus a homogeneous material in the new DSA cell will always form a gaussian band, but the magnitude of the peak concentration will vary greatly depending on the location of the band in the cell. Therein lies the key to the increased resolution possible with the DSA cell. To illustrate by the simplest example, suppose a sample were composed of two materials of equal amount and molecular weight but of slightly different densities. In the normal USA cell the observed band would be the sum of two gaussian bands separated by a small distance, and it would be difficult to distinguish this observed band from a perfect gaussian band. However, in the DSA cell, the heavier band would be centered in the narrow sector and would thus have

a peak concentration relatively higher than the lighter band which is centered in the wide sector. The observed band, as the sum of these two, would be distorted to some degree by this asymmetry, and it might be possible to resolve the bands more easily.

III. CASES TO CONSIDER. Out of the wide variety of problems that could be analyzed in the DSA cell, there are three general cases that stand out for two reasons. First, they are similar to practical problems that have been encountered, and so analysis could provide very useful information. Second, they are amenable to treatment with relatively straightforward mathematical and computer techniques.

1. The sample contains two bands of equal amount and molecular weight but of different densities. For instance, a sample of homogeneous DNA that is denatured into complementary strands will satisfy approximately the above conditions.

2. The sample contains predominantly one component but has a minor component of greater density whose presence is obscured by the dominant band.

3. The sample contains an essentially infinite range of components whose densities are symmetrically distributed around a central density; the amount of each component is a gaussian distribution around the center with respect to density. This case is referred to as a gaussian distribution of gaussian bands, and it will be shown that the composite band is actually a true gaussian curve in the USA cell, but not in the DSA cell.

IV. MATHEMATICAL ANALYSIS. For a single homogeneous component in the DSA cell, the concentration C(r) at equilibrium is

$$C(r) = C^{o} e^{-\frac{(r-r_m)^2}{2\sigma^2}}$$

where r = distance from center of rotation

r_m = position of band peak

σ = band width, or standard deviation of band (a length, dependent on the density gradient)

C^o is determined by conserving mass within the system:

$$M = \int_{r_a}^{r_c} h r \phi(r) \cdot r \cdot C(r)\, dr$$

where r_a, r_b, r_c are as indicated in Fig. 1b

$\phi(r)$ is as in Fig. 1b: $\phi(r) = \phi_2$ between r_a and r_b

$\phi(r) = \phi_1$ between r_b and r_c

h = thickness of the cell (the third dimension)

M = amount of component

Ignoring end effects (i.e. $(r_a - r_m)/\sigma = -\infty$ and $(r_c - r_m)/\sigma = \infty$), integration yields the result

$$C^{o} = \frac{M}{h\phi_1\sigma\left[(1-\alpha)\sigma e^{-\frac{(r_m-r_b)^2}{2\sigma^2}} + \sqrt{\frac{\pi}{2}}\, r_m \left((\alpha+1)+(1-\alpha)\,\mathrm{erf}\left(\frac{r_m-r_b}{\sqrt{2}\,\sigma}\right)\right)\right]}$$

where $\alpha = \phi_2/\phi_1$ = ratio of large to small angle in DSA cell

$\mathrm{erf}(x)$ = error function = $\frac{2}{\sqrt{\pi}} \int_0^x e^{-u^2} du$

Note that $\mathrm{erf}(-x) = -\mathrm{erf}(x)$

$\mathrm{erf}(0) = 0$ and $\mathrm{erf}(\infty) = 1$

For simplification and convenience in manipulation it is

worthwhile to shift the scale of distance from "r" to a new dimensionless variable $s = (r-r_b)/\sqrt{2}\sigma$. Also we define $s_m = (r_m-r_b)/\sqrt{2}\sigma$. The concentration C(s) as a function of s becomes $C(s) = C^o e^{-(s-s_m)^2}$

$$C^o = \frac{M}{h\phi_1\sigma\left[(1-\alpha)\sigma e^{-s_m^2} + \sqrt{\frac{\pi}{2}} r_m\left((1+\alpha)+(1-\alpha)\operatorname{erf} s_m\right)\right]}$$

Under all conditions to be considered, $r_m/\sigma \gg 1$ and since $e^{-s_m^2} \leq 1$ the first term of the denominator, $(1-\alpha)\sigma e^{-s_m^2}$, can be ignored without significantly affecting results. The expression can be further simplified by defining

$$K = \frac{1-\alpha}{1+\alpha} \quad \text{and} \quad D = \frac{M}{\sqrt{\frac{\pi}{2}}\, h\phi_1\sigma(1+\alpha)} = \frac{M}{\sqrt{\frac{\pi}{2}}\, h\sigma(\phi_1+\phi_2)}$$

Thus

$$C^o = \frac{D}{r_m(1+K\operatorname{erf} s_m)}$$

$$C(s) = C^o e^{-(s-s_m)^2}$$

We are now ready to proceed with analyses of the specific cases mentioned on page 3.

1. Two components of equal amount and molecular weight but banding at slightly different densities. The total concentraion C(s) is

$$C(s) = C_1(s) + C_2(s) = C_1^o e^{-(s-s_m(1))^2} + C_2^o e^{-(s-s_m(2))^2}$$

$$C_1^o = \frac{D_1}{r_m(1)\left(1+K\operatorname{erf}(s_m(1))\right)}$$

$$C_2^o = \frac{D_2}{r_m(2)\left(1+K\operatorname{erf}(s_m(2))\right)}$$

Since the quantity in each band is the same, $D_1 = D_2 = 1$ for simplicity. At this point it is appropriate to insert reasonable values for the parameters $r_m(1)$, $r_m(2)$, $s_m(1)$, $s_m(2)$, and K. Then the result can be plotted with the aid of the computer. The particular case studied was one with the two bands both equidistant from r_b, the sector discontinuity, but on opposite sides of it. The separation between bands, $\Delta r = r_m(2) - r_m(1) = \sqrt{2}\sigma[s_m(2) - s_m(1)]$ was varied between 0 and 2.0σ. $K = \frac{1-\alpha}{1+\alpha}$, $\alpha = \frac{\phi_2}{\phi_1}$ was tried at several values. The design of the analytical cell is such that the maximum value of α for a cell that functions acceptably is probably around 10. Note that for $\alpha = \phi_2/\phi_1 = 1$, $K = 0$, the cell is a normal USA cell, and the equations behave accordingly. Values of α between 1 and 10 were tried. The values of $r_m(1)$ and $r_m(2)$ were determined by setting $r_b = 5.0$ cm (the exact value depends on the design of the cell, but is not critically important here), letting $\sigma = 0.02$ cm (a reasonable value for a DNA sample), and noting

$$r_m(1) = r_b - \frac{\Delta r}{2} \quad \text{and} \quad r_m(2) = r_b + \frac{\Delta r}{2}$$

The effect of putting a sample as described above into the DSA cell is shown in Program I. For each value of Δr between 0 and 1.6σ, 5 values of α from 1 (the USA cell) to 16 were tried, and the resultant curves were plotted. The amplitudes were adjusted so that regardless of the value of α, a single band centered at r_b would have a peak height of 0.2 in arbitrary units. The results show that the DSA cell has a very significant effect in cases where $\Delta r \gtrsim \sigma$.

A method of analysis which shows the precise effect of the DSA cell is the following. Assume that a sample contains

either a) two components as described above, or b) a homogeneous material. If the sample is placed in the USA cell, the band will be described by curve A if it contains two components, or curve B if it is homogeneous.

A) $$C_A(s) = \frac{1}{r_{m(1)}} e^{-(s+\frac{\Delta r}{2\sigma})^2} + \frac{1}{r_{m(2)}} e^{-(s-\frac{\Delta r}{2\sigma})^2}$$

B) $$C_B(s) = \left(\frac{1}{r_{m(1)}} + \frac{1}{r_{m(2)}}\right) e^{-\gamma s^2}$$

where $$\gamma = 1 - \log\left[\cosh\left(\frac{\Delta r}{2\sigma}\right)\right]$$

The gaussian curve B has been adjusted so that it represents a "best fit" to curve A, the sum of two gaussians. The computer plots (see Program II) show that for $\Delta r \lesssim 1.5\sigma$, there is essentially very little difference in the shape of curve A and curve B. This means in the USA cell one cannot determine whether the sample contains one component or two. However, if the sample is then placed in a DSA cell having $\alpha = 10$ (the practical maximum), there is a significant difference in the observed band depending on whether one component or two is present. If it is a two component sample, the apparent density (i.e. the density at the peak) and the peak height are both increased. It appears that for cases with $\Delta r \gtrsim \sigma/2$, the two component nature of the sample should be measurable.

The suggested method of analyzing an unknown sample for the presence of two equal components is as follows. While the sample is in the ultracentrifuge the temperature of the cell can be varied over a wide range. Changing the temperature will change the bulk solution density, the sample density,

and the density gradient, so the band position will thereby change. The precise effect of temperature on the first three parameters is known[1], so by appropriate control of temperature the band can be caused to move from one side of the sector discontinuity to the other. Both the peak height and peak position can be determined as a function of temperature. From this the effect of the sector angle discontinuity alone on band height and position can be deduced. Finally, this observed effect can be compared to the theoretical effect as a function of Δr as calculated on the computer, thus allowing an estimation of the band separation Δr to be made.

2. Components of equal molecular weight, but in different quantities and of different densities. If the band separation is Δr and both peaks are equidistant from r_b, then

$$C(s) = C_1^\circ e^{-\left(s+\frac{\Delta r}{2\sqrt{2}\sigma}\right)^2} + C_2^\circ e^{-\left(s-\frac{\Delta r}{2\sqrt{2}\sigma}\right)^2}$$

$$C_1^\circ = \frac{D_1}{\left(r_b-\frac{\Delta r}{2}\right)\left(1+k\,\mathrm{erf}\frac{\Delta r}{2\sqrt{2}\sigma}\right)} \qquad C_2^\circ = \frac{D_2}{\left(r_b+\frac{\Delta r}{2}\right)\left(1+k\,\mathrm{erf}\frac{\Delta r}{2\sqrt{2}\sigma}\right)}$$

Two cases were analyzed, and for each case the curves were plotted for $\alpha = 1$ (USA cell) and $\alpha = 10$ (DSA cell).

Case 1. A denser component with 5% of the amount of the major component, located 2σ from the major band.

Thus $\Delta r = 2\sigma$ and $D_2/D_1 = 0.05$

Case 2. A 1% impurity located 4σ from the main peak.

Thus $\Delta r = 4\sigma$ and $D_2/D_1 = 0.01$

The results are plotted in Program III. In the USA cell the minor component is not noticeable in either case. In the DSA cell the minor component can be seen as a definite

skewing of the large band in case 1 and as a separate peak with height 7% of the larger one in case 2. Thus it is clear that the DSA cell can be very useful in solving problems of this nature.

3. A gaussian distribution of gaussian bands. Ignoring end effects and radial dilution effects, the concentration distribution C(r) of a gaussian distribution of gaussian bands is

$$C(r) = \int_{-\infty}^{\infty} g(x)\, C(r,x)\, dx$$

where $g(x)dx = \frac{1}{\beta} e^{\frac{-(x-x_m)^2}{2\beta^2}} dx$ = fraction of sample with equilibrium position between x and x+dx

$$C(r,x) = C^{\circ}(x)\, e^{-\frac{(r-x)^2}{2\sigma^2}}$$

x_m = peak position of the composite band

σ = band width of each fraction, assumed to be constant

β = standard deviation of the density distribution

a) For a USA cell,

$$C^{\circ}(x) = \text{constant}/\sigma = F/\sigma$$

$$C(r) = \frac{F}{\beta\sigma} \int_{-\infty}^{\infty} e^{-\frac{(x-x_m)^2}{2\beta^2} - \frac{(r-x)^2}{2\sigma^2}} dx$$

Integration of this yields

$$C(r) = \sqrt{2\pi}\frac{F}{\phi}\, e^{-\frac{(r-x_m)^2}{2\phi^2}}$$

where $\phi^2 = \sigma^2 + \beta^2$

Thus in the USA cell the sample behaves exactly as a homogeneous material with standard deviation ϕ.

b) In the [illegible] cell,

b) In the DSA cell,

$$C'(x) = \frac{F}{\sigma\left(1+K\,\mathrm{erf}\left(\frac{x-r_b}{\sqrt{2}\sigma}\right)\right)}$$

where $K = \frac{1-r}{1+r}$ as before

Then

$$C(r) = \frac{F}{\phi\sigma}\int_{-\infty}^{\infty} \frac{e^{-\frac{(x-x_m)^2}{2\phi^2} - \frac{(r-x)^2}{2\sigma^2}}}{1+K\,\mathrm{erf}\left(\frac{x-r_b}{\sqrt{2}\sigma}\right)}\,dx$$

Direct manipulation of this yields

$$C(r) = \frac{\sqrt{2}F}{\phi}\, e^{-\frac{(r-x_m)^2}{2\phi^2}} \int_{-\infty}^{\infty} \frac{e^{-v^2}\,dv}{1+K\,\mathrm{erf}(A(r)-Bv)}$$

$$B = \sqrt{2}\beta/\phi \qquad A(r) = \frac{\beta^2(r_b-r)}{\sqrt{2}\phi^2\sigma}$$

Again invoking the assistance of the computer, the effect of the DSA cell can be determined in this situation. The analysis is similar to that made for the two component problem on page 7. Suppose the sample is either a) homogeneous, with band width ϕ, or b) a gaussian distribution of gaussian bands with $\phi^2 = \sigma^2 + \beta^2$. Two cases were analyzed:

case 1. $\beta = \sigma$ and $\phi = \sqrt{2}\,\sigma$

case 2. $\beta = 2\sigma$ and $\phi = \sqrt{5}\,\sigma$

In the USA cell, the band is gaussian, and the nature of the sample cannot be determined. The results for the DSA cell with $\alpha = 10$ are shown in Program IV. In both cases it can readily be seen whether the sample is homogeneous or a gaussian distribution of gaussians. For an unknown sample, analysis in the manner described on pages 7-8 will allow estimation of β. It is interesting to note that Sueoka[2] has studied the density heterogeneity of several types of DNA and has found that the variance in density, which corresponds to β as described above, is of the same order of magnitude as σ, the standard deviation of the homogeneous components. Thus there in fact exist materials which would definitely

show distributional effects in the DSA cell. Although such materials do not necessarily have density distributions even approximating gaussian form, the analysis described above can be modified and extended to handle any type of density heterogeneity.

V. PRESENT STATUS OF THE RESEARCH. It is unfortunate that a DSA cell has not yet been used for experiments, nor has one even been built. This is because of a misjudgment I made at a critical point in the project. When I first tackled the problem in the summer of 1965, I was not trained in computer programming, nor did I realize that the computer would be so useful that I should have learned to program at that time. I did succeed in the fundamental analysis of the problem and attempted to try test cases by manual computation. I chose the case of two equal bands with very little separation; for the specific values I picked, the DSA cell had little effect, so I decided (too hastily, obviously) that the DSA cell was not practical. When I began to write this report I decided to use the computer to analyze the problem and thereby discovered that the DSA cell promises to become a very effective tool for biochemists. I regret that I will not have a chance to carry out the numerous experiments which should be done with the DSA cell; having laid the groundwork, I will leave the experimentation and further development of analytical methods to someone else.

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1. Vinograd, Greenwald, and Hearst, Biopolymers, 3: 109 (1965)

2. Sueoka, Noboru, Proc. Natl. Acad. Sci., 45: 1480 (1959)