DENSITY STABILITY

IN

BAND CENTRIFUGATION

by

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I. Introduction

In band centrifugation a thin layer of a solution containing macromolecules is applied to the top of a more dense solution in a rotating ultracentrifuge cell. The macromolecules then sediment as a band through the "bulk" solution.*

The general condition for the stability of a band[1] is that the total density gradient at any point must always be positive (or zero). The total density gradient is composed of two parts: 1) the density gradients in the bulk solution that would occur if there were no macromolecules present; 2) the density gradients due entirely to the band of macromolecules. The gradients in the trailing side of the band (closest to the top of the cell) are positive. However, the gradients in the leading side are negative; these negative gradients must be compensated by positive gradients of equal or greater magnitude in the bulk solution if the band is to be stable.

Figure 1 illustrates the combination of bulk solution and band gradients, and the stability requirement. If the solution gradient is zero, convection results in the complete loss of the band. If there is an inadequate positive solution gradient, the band is not lost, but only spreads enough to reduce the magnitudes of the negative band gradients to values smaller than that of the solution gradient.

The compensatory bulk solution gradients may be generated in two ways: 1) diffusion of small molecules (such as KCl, D_2O, or CH_3OH) which are present initially in different concentrations in the bulk solution and in the layer;

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* The general features of band centrifugation are described by Vinograd, Bruner, Kent, and Weigle, Proc. Natl. Acad. Sci., Vol. 49, No. 6, June, 1963, a copy of which is in Appendix I. The present report discusses an aspect of the topic with which this author was particularly concerned.

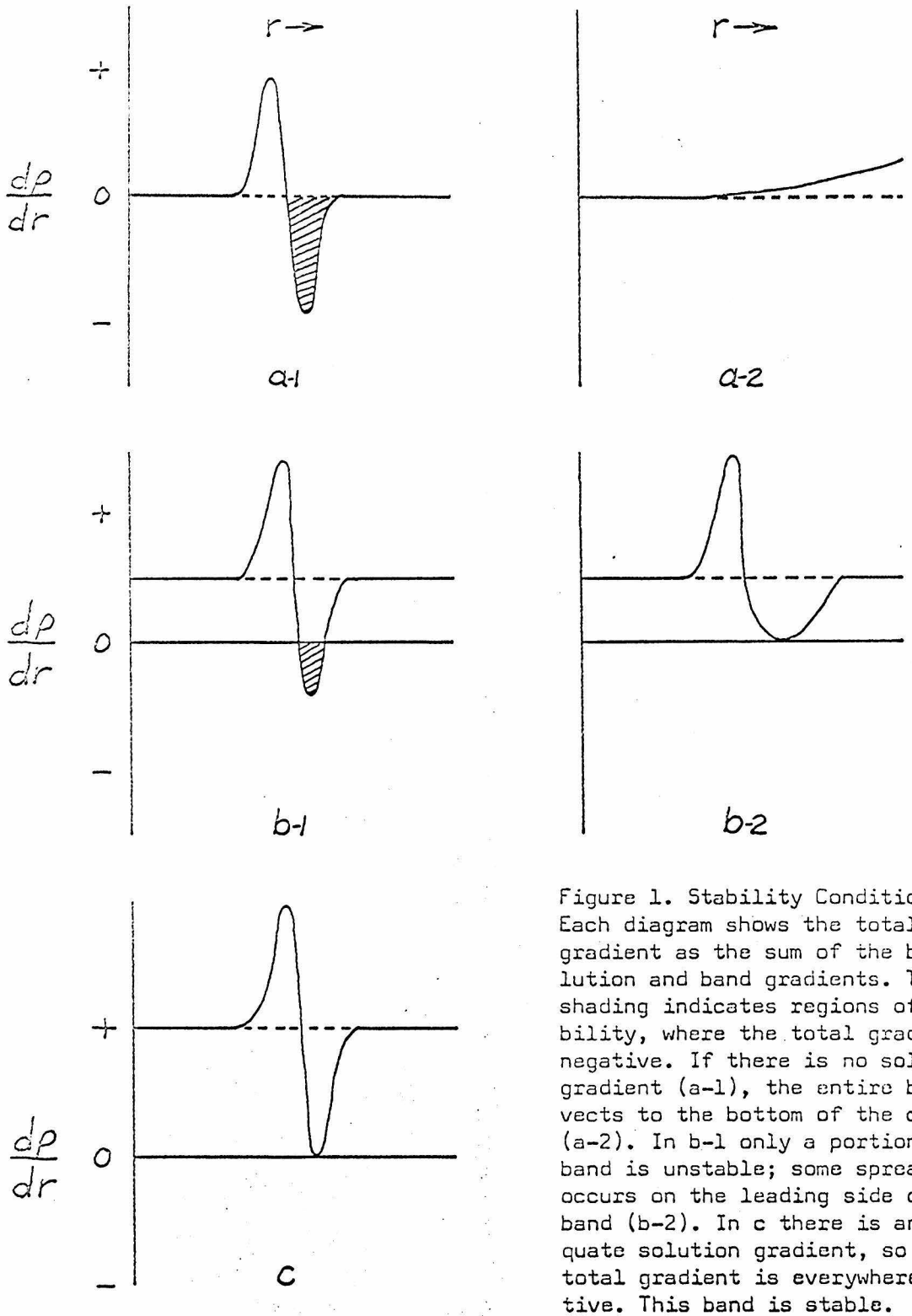

Figure 1. Stability Conditions. Each diagram shows the total density gradient as the sum of the bulk solution and band gradients. The shading indicates regions of instability, where the total gradient is negative. If there is no solution gradient (a-1), the entire band convects to the bottom of the cell (a-2). In b-1 only a portion of the band is unstable; some spreading occurs on the leading side of the band (b-2). In c there is an adequate solution gradient, so that the total gradient is everywhere positive. This band is stable.

2) combined sedimentation and diffusion of small molecules within the bulk solution, due to the centrifugal field.

It is of interest to predict whether or not a band will be stable in a given system. This involves estimation of both the band gradients and the compensatory gradients.

II. Density Gradients in a Band of Macromolecules

Density gradients in a band are estimated under the assumption of a Gaussian distribution of macromolecules in a rectangular cell. The concentration distribution in a Gaussian band[2],

$$c = \frac{M}{\sigma\sqrt{2\pi}} e^{-(r - r_o)^2/2\sigma^2} \tag{1}$$

can be differentiated with respect to distance to give the band concentration gradient at any point.

$$\frac{dc}{dr} = \frac{-(r - r_o)}{\sigma^2} c \tag{2}$$

In these equations, c is the concentration of macromolecules (weight solute/volume solution); M is the total mass of macromolecules in the band per unit cell area; $(r - r_o)$ is the distance from band center, r_o; σ is the standard deviation.

The maximum negative gradient which must be compensated is at the leading inflection of the band.

$$\left(\frac{dc}{dr}\right)_{\text{max. neg.}} = \left(\frac{dc}{dr}\right)_{\sigma} = \frac{-M}{\sigma^2\sqrt{2\pi e}} \tag{3}$$

In a band centrifuge experiment M is given by $c^o\delta$, where c^o is the concentration of macromolecules in the applied layer of thickness δ.

$$\left(\frac{dc}{dr}\right)_\sigma = \frac{c^o\delta}{\sigma^2\sqrt{2\pi e}} \tag{4}$$

A relation between the concentration gradient and the density gradient can be derived as follows. If v and v_1 are the specific volumes of the solution and solvent respectively, $\bar{v}_2$ the partial specific volume of the macromolecule, and g the weight fraction of macromolecules in the solution, then

$$v = g\bar{v}_2 + (1 - g)v_1$$

$$g = \frac{v - v_1}{\bar{v}_2 - v_1}.$$

$c = g\rho$, where ρ is the density of the solution, $1/v$.

$$c = \frac{v - v_1}{\bar{v}_2 - v_1}\rho$$

$$c = \frac{1 - \rho v_1}{\bar{v}_2 - v_1}$$

ρ_1 is the density of the solvent, $1/v_1$.

$$c = \frac{\rho_1 - \rho}{\rho_1\bar{v}_2 - 1}$$

Differentiation with respect to distance leads to

$$\frac{dc}{dr} = \frac{1}{1 - \bar{v}_2 \rho_1} \frac{d\rho}{dr} .$$

Since the amount of macromolecule used is very small ρ_1 may be replaced by ρ.

$$\frac{d\rho}{dr} = (1 - \bar{v}_2 \rho) \frac{dc}{dr} \tag{5}$$

Combining equations (4) and (5) gives the final result.

$$\left(\frac{d\rho}{dr}\right)_{\text{max. neg.}} = \left(\frac{d\rho}{dr}\right)_{\sigma} = \frac{-(1 - \bar{v}_2 \rho) c^{o} \delta}{\sigma^2 \sqrt{2\pi e}} \tag{6}$$

Table 1 gives some values of $(d\rho/dr)_{\sigma}$ in typical experiments. σ is estimated by the relation $\sigma^2 = \sigma^{o\,2} + 2D(t - t^{o})$, where D is the diffusion coefficient of the macromolecule and t is time in seconds. The superscript zero indicates a reference band. Initially gradients as large as about 200 $\times 10^{-4}$ g cm^{-4} may be present for materials such as proteins which have low light absorption coefficients and high diffusion coefficients. Halfway through the experiment the maximum negative gradient for most materials is about 4×10^{-4} g cm^{-4}. Positive solution gradients must be generated to compensate.

The calculations have assumed ideal experimental conditions, such as perfect layering and vibration-free centrifugation. Experimental imperfections will result in larger density gradient requirements than calculated by equation (6).

Table 1

Maximum Negative Density Gradients in Bands of Macromolecules at Various Band Widths*

Material	$D \times 10^7$ $cm^2 sec^{-1}$	time hours	c^o %	σ cm	OD^{260} band center	$-(d\rho/dr)_\sigma \times 10^4$ $g\ cm^{-4}$
Protein	6.4	0	0.5	0.01	5.0	200
		1		0.07	0.6	4
Protein	3.2	0	0.3	0.01	3.0	120
		1		0.05	0.5	5
Virus (50% Nucleic Acid)	1.0	0	0.015	0.01	2.0	15
		0.5		0.02	0.5	4
Nucleic acid**	0.4	0	0.008	0.01	2.0	8
		0.5		0.015	1.2	3

* Calculated with equation (6). $\rho = 1.1\ g\ cm^{-3}$; $\delta = 0.02$ cm (10 μl layer in a 12 mm centerpiece).

** Concentration dependence of the sedimentation coefficient ignored.

III. Diffusion Compensatory Gradients

In ordinary band centrifugation experiments a light solution is layered onto a more dense solution containing small, rapidly diffusing, dense molecules (such as KCl or D_2O) which account for the density differences. The small molecules diffuse into the layer, producing positive density gradients in the cell. These "diffusion" gradients occur even if there is no centrifugal field, and are estimated here under the assumption that they are independent of any field.*

In typical experiments the volume of the applied layer ("lamellar solution") is 10 μl in a 12 mm centerpiece (or the equivalent 25 μl in a 30 mm centerpiece), resulting in a lamellar thickness, δ, about 0.2 mm. If the lamella is assumed to be infinitely thin, the system is equivalent to diffusion from a plane (the meniscus) in one direction, and is described in a rectangular cell by[2]

$$c = \frac{M}{\sqrt{\pi D t}} e^{-(r - r_a)^2/4Dt} . \qquad (7)$$

In this derivation c and M are the concentration and the mass per unit area of the small molecules responsible for the density differences. The presence of only one such species is assumed. r_a is the position of the meniscus.

Since $\rho = 1 + kc$, where k is an empirical constant,

$$\frac{dc}{dr} = \frac{1}{k} \frac{d\rho}{dr} \qquad (8)$$

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* In theory it is also possible to use light rapidly diffusing molecules (such as CH_3OH or $t\text{-}C_4H_9NH_2$) to account for the density difference. Such light molecules would be put in greater concentration in the lamellar solution than in the bulk solution so that positive gradients will result. The analysis of the diffusion gradients holds equally well in this case.

$M = \delta(c_L - c_B)$, where c_L and c_B are the initial concentrations of diffusing substance in the lamella and bulk solution respectively. Again using $\rho = 1 + kc$, and defining $\Delta\rho \equiv \rho_B - \rho_L$,

$$M = \frac{-\delta\Delta\rho}{k}. \tag{9}$$

The minus sign in equation (9) results from the fact that $\rho_B > \rho_L$. For dense diffusing molecules, k is positive and $c_L < c_B$.

Differentiation of equation (7) with respect to r, and substitution of equations (8) and (9) leads to

$$\frac{d\rho}{dr} = \frac{\delta\Delta\rho(r - r_a)}{2\sqrt{\pi D^3 t^3}} e^{-(r - r_a)^2/4Dt} \tag{10}$$

It is convenient to introduce into equation (10) the parameter β, defined by equation (11). Introduction of β gives equation (12)

$$\beta \equiv \frac{2\sqrt{\pi D^3}}{\delta\Delta\rho}\frac{d\rho}{dr} \tag{11}$$

$$\beta = \frac{(r - r_a)}{t^{3/2}} e^{-(r - r_a)^2/4Dt} \tag{12}$$

Graphs of $(r - r_a)$ against t can now be calculated for values of β for a system. For a given D, any combination of δ, $\Delta\rho$, and $d\rho/dr$ which give the same β result in the same graph. Graphs for systems with CsCl, D_2O, KCl, or NaCl are shown in Figures 2, 3, and 4.

From each graph can be read the two times at which a gradient (the value of which is in β) reaches a given $(r - r_a)$. In the interval the density

Figures 2, 3, and 4. Diffusion Compensatory Gradients and Centrifugal Field Compensatory Gradients. The diffusion gradients are calculated from equation (12). Each diffusion gradient curve is characterized by a value of D and a value of β. Any combination of δ, $\Delta\rho$, and $d\rho/dr$ which give the same β will give the same curve. The field gradients are shown only in Figure 4; they are calculated from equations (13) and (14).

Figure 2. $D = 1.27 \times 10^{-5}$ $cm^2 sec^{-1}$ (NaCl in water)
β = (from the inside curve outward) 2×10^{-6}, 2×10^{-7}, and 2×10^{-8}

The values of $d\rho/dr$ on the curves are for the system: $\delta = 0.02$ cm, $\Delta\rho = 0.04$ g cm^{-3}.

Figure 3. $D = 1.6 \times 10^{-5}$ $cm^2 sec^{-1}$ (KCl in water)
$\beta = 4.3 \times 10^{-3}$, 4.3×10^{-4}

The values of $d\rho/dr$ on the curves are for the system: $\delta = 0.02$ cm, $\Delta\rho = 0.022$ g cm^{-3}.

Figure 4. Diffusion gradients.
$D = 2.2 \times 10^{-5}$ $cm^2 sec^{-1}$ (CsCl or D_2O in water)
$\beta = 3.66 \times 10^{-6}$, 3.66×10^{-7}, 3.66×10^{-8}

The values of $d\rho/dr$ on the curves are for the system: $\delta = 0.02$ cm, $\Delta\rho = 0.1$ g cm^{-3}

Field gradients. (dashed line)
CsCl, $\rho = 1.5$ g cm^{-3}, $\omega^2 = 13.93 \times 10^6$ sec^{-2} (35,600 rpm), $d\rho/dr = 10^{-4}$ g cm^{-4}.

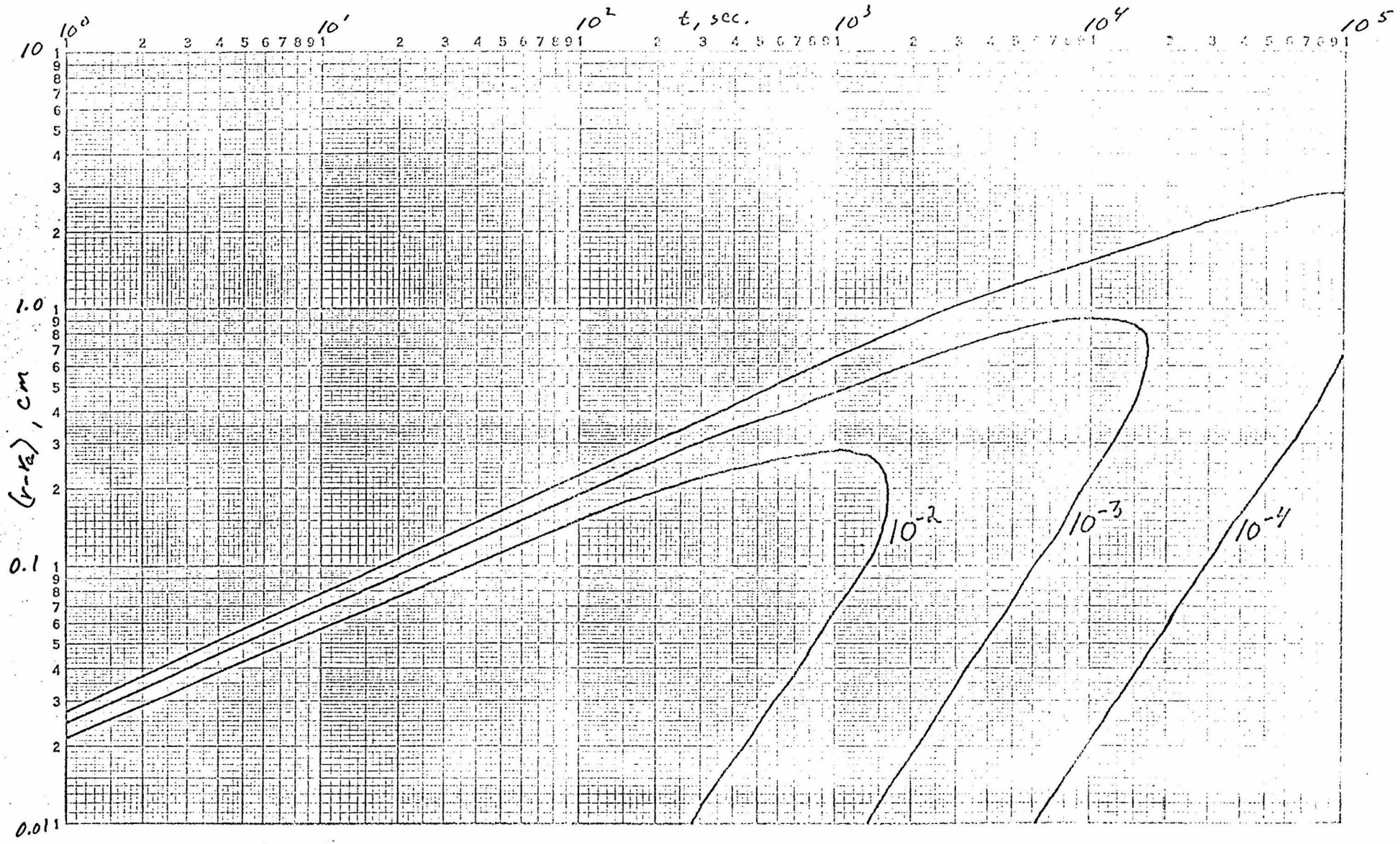

Figure 2.

NaCl

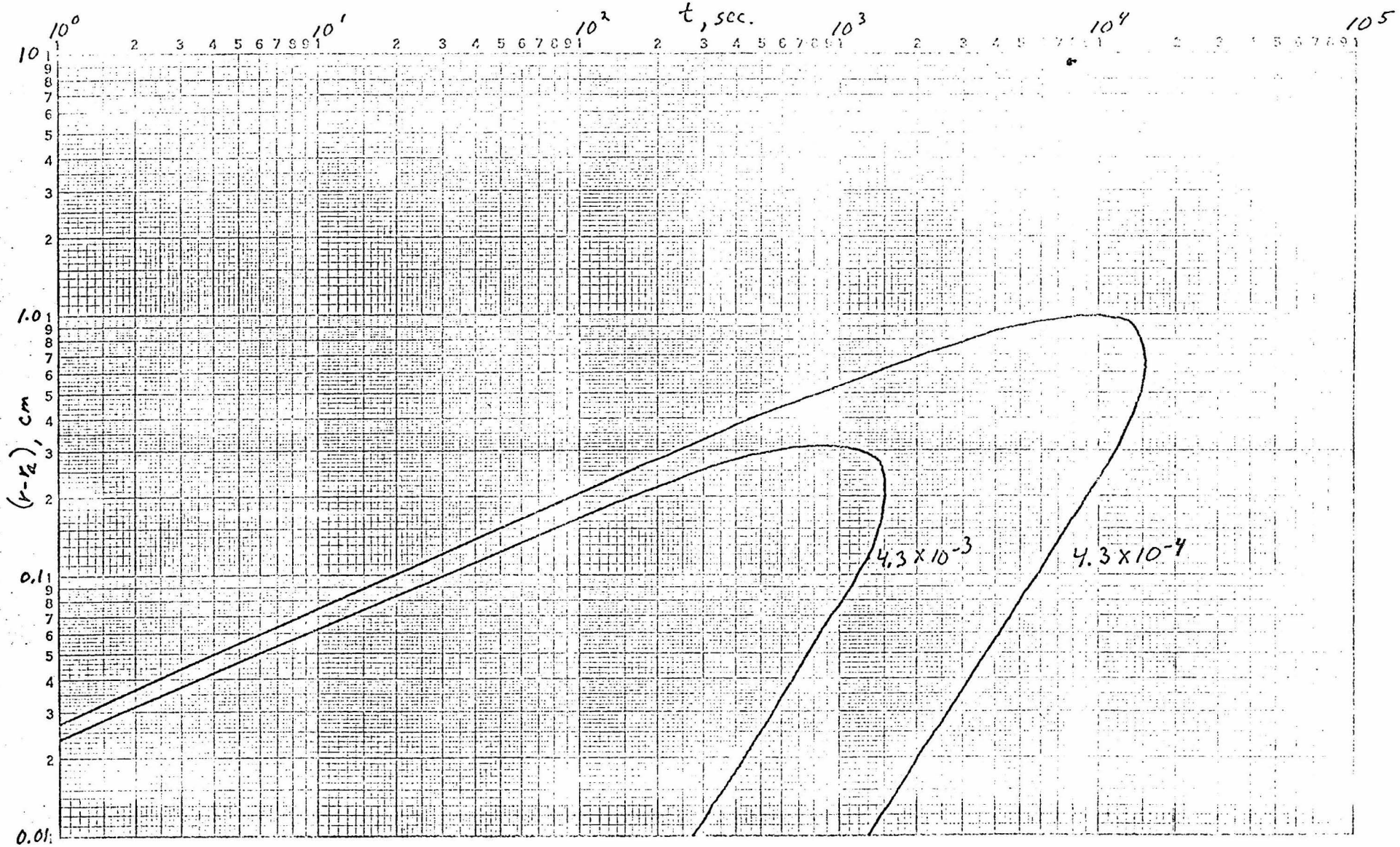
t, sec.
10^0
10^1
10^2
10^3
10^4
10^5
10
1.0
0.1
0.01
$(r-r_a)$, cm
4.3×10^{-3}
4.3×10^{-4}

Figure 3.

KCl

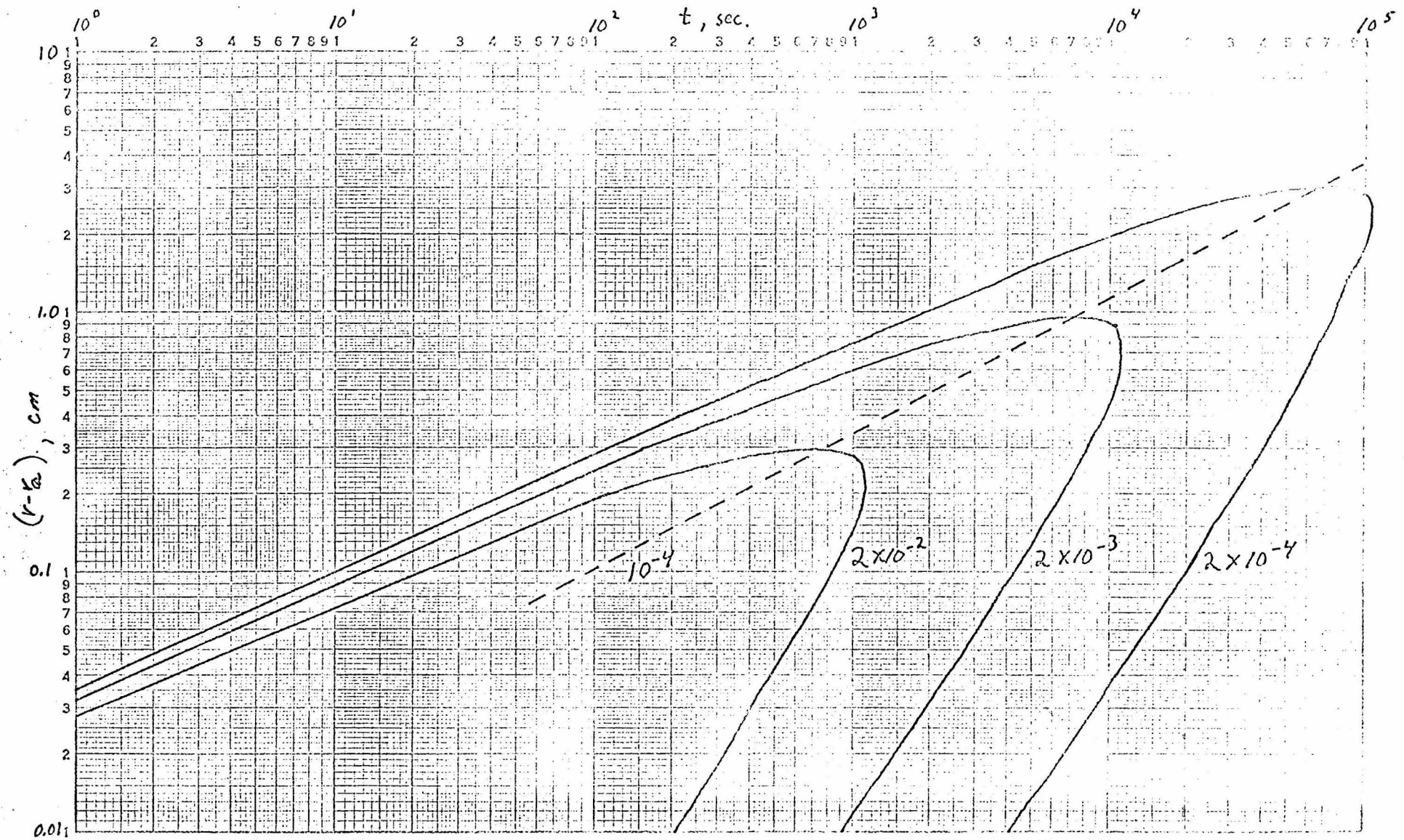

Figure 4.

CsCl or D_2O

gradient at $(r - r_a)$ is larger. Similarly, the two positions of a gradient at a given time may be read. Between these positions the gradient is larger. With a family of curves for various β, hence various $d\rho/dr$, the magnitude of the diffusion gradient at any t and $(r - r_a)$ may be estimated.

Inspection of Figures 2 to 4 shows that immediately upon layering (t = 0) large gradients are formed. In all three figures gradients on the order of 10^{-2} g cm^{-4} reach 0.1 cm (roughly 1/10 of the length of the cell) in less than one minute. Table 1 indicates that 10^{-2} g cm^{-4} is about the largest band gradient encountered. Since it is not practical to use experimental conditions under which the band reaches 0.1 cm in a minute, it is apparent that the diffusion gradients alone are more than sufficient to provide stability in the early moments of an experiment.

Table 1 indicates that gradients on the order of 4×10^{-4} g cm^{-4} will be required halfway through the experiment, $(r - r_a) = 0.5$ cm. For the systems in the accompanying figures, the requirement will be satisfied between about ten minutes and several hours, with one exception. For 0.5 M KCl bulk solution the requirement will be satisfied only between about one and four hours. Thus it is possible in this system to operate at too high a speed and reach insufficient diffusion gradients during the experiment. Especially if higher initial concentrations of macromolecules are used, it is also possible to operate too slowly and reach a position where the gradient is insufficient for stability and is dying out. (It should be obvious that the diffusion gradients approach the condition $d\rho/dr = 0$ throughout the cell.)

Even in 0.5 M KCl, the least stabilizing system discussed, it is generally possible to adjust operating speed and initial concentration of macromolecule so that a satisfactory experiment can be made. Stability can also be increased by increasing $\Delta\rho$ or δ.

As the cell bottom is approached, the validity of equations (10) or (12) decreases, because the cell can no longer be consider infinitely long. The effect of the cell bottom is to decrease the magnitudes of the gradients, but the relative error introduced should be quite small except very near the bottom.

IV. Centrifugal Field Compensatory Gradients

Application of a centrifugal field to a binary bulk solution causes combined sedimentation and diffusion according to the Lamm differential equation of the centrifuge. Concentration gradients, hence density gradients result from this action. Fujita and MacCosham[3] have obtained an approximate solution to the differential equation for the sedimentation of a low molecular weight solute with sedimentation coefficient s and diffusion coefficient D both independent of concentration. Their solution is dependent on the semi-infinite cell condition that there is a region in the cell where no significant change has occurred in the concentration distribution.

If the macromolecular component and the lamellar solution are ignored, the Fujita-MacCosham results may be applied to the bulk solution.

The solution for the concentration gradient is given by their equation (23) with a multiplicative factor of $e^{-z/2}$.

$$\frac{\partial(c/c^{\circ})}{\partial(r/r_a)} = \frac{e^{-\tau}e^{-z/2}}{\epsilon}\left\{\left[1-\phi\left(\frac{z+\tau}{2\sqrt{\epsilon\tau}}\right)\right]\left(2+\frac{z+\tau}{\epsilon}\right)e^{z/\epsilon} - \frac{2}{\sqrt{\pi}}\sqrt{\frac{\tau}{\epsilon}}\,e^{-(\tau-z)^2/4\epsilon\tau}\right\} \qquad (13)$$

$\tau = 2\omega^2 st$, $\epsilon = 2D/r_a^2\omega^2 s$, $y = (r/r_a)^2$, $z = \ln y$, and $\phi(x)$ denotes the error function of x. ω is the angular velocity. r_a is the distance of the meniscus from the axis of rotation. c^o is the concentration of the sedimenting component initially in the bulk solution.

For aqueous CsCl solutions the left hand side of equation (13) may be written in terms of density gradients using the relation for CsCl[4] $g = 137.48 - 138.11/\rho$, along with the general relation $c = g\rho$.

$$\frac{\partial(c/c^o)}{\partial(r/r_a)} = 137.48 \frac{r_a}{c^o} \frac{d\rho}{dr} \qquad (14)$$

Using equations (13) and (14)* the progress of a small density gradient, 10^{-4} g cm^{-4}, was evaluated as a function of time for the following system: aqueous CsCl, $\rho = 1.5$ g cm^{-3}, $\omega^2 = 13.93 \times 10^6$ sec^{-2} (35,600 rpm), $r_a = 6.0$ cm, $s = 0.5 \times 10^{-13}$ sec [5], $D = 2.2 \times 10^{-5}$ $cm^2 sec^{-1}$. These values result in $\epsilon = 1.75$ and $\tau = 14 \times 10^{-7} t$. The results of this calculation are indicated by the dashed line in Figure 4.

It is apparent from Figure 4 that early in the experiment field gradients are small compared to diffusion gradients. The field gradient in CsCl, $\rho = 1.5$ g cm^{-3}, is much slower than comparable diffusion gradients in more dilute systems. The initial field gradients are even smaller than calculated here, since the calculations assume full speed from the beginning.

Later in the experiment the field gradient may become significant. Unlike the diffusion gradients, the field gradients increase regularly with

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* For this example the approximation $\frac{\partial(c/c^o)}{\partial(r/r_a)} = \frac{2}{\epsilon}\left[1 - \phi\left(\frac{z+\tau}{2\sqrt{\epsilon\tau}}\right)\right]$ was found to yield results within 1% of those from the full equation (13). The general validity of the approximate equation was not investigated, and is not obvious since the neglected terms are not individually negligible, but cancel in this case.

time approaching an equilibrium value. Therefore the importance of the field gradients relative to the diffusion gradients increases late in the experiment. At all times the two gradients act in the same direction, and thus reinforce each other.

The field gradient calculations are valid only as long as the semi-infinite cell assumption holds. Since gradients similar to those calculated are formed from the cell bottom upward, the small gradient 10^{-4} g cm^{-4} can be followed for slightly less than half the cell length. Thus in the analytical ultracentrifuge, the field gradient results in Figure 4 are meaningful only to about 0.5 cm. In contrast to the diffusion gradient case, the effect here of the cell bottom is to produce gradients larger than those calculated.

In bulk solutions such as 95% D_2O or dilute salt, the field gradients are much smaller than in the example.

V. Experimental Considerations

It is extremely difficult to verify in detail the conclusions of the previous three sections. The clue to band instability in an experiment is front spreading (Fig. 1-b-2), but front spreading may have other causes such as inhomogeneity of the sedimenting component. In this section will be presented two examples of the use of the foregoing calculations. These examples will show that in general the diffusion gradients provide very ample stability under normal experimental conditions.

If the bulk solution is aqueous CsCl, $\rho = 1.01$, the diffusion gradients may be found from Figure 4, and are 1/10 the magnitudes indicated on the curves. It is seen that gradients on the order of 10^{-3} will take at least 15 minutes to reach 0.5 cm (if such gradients ever progress that far). Experiments in

which T-4 viruses were sedimented 0.5 cm in 8 minutes in such a system resulted in extensive forward spreading. From Table 1 it is seen that the band gradient is about 10^{-3}. Table 1 and Figure 4 have therefore predicted the instability in this system.

If the bulk solution is aqueous CsCl, $\rho = 1.25$, the diffusion gradients are 2½ times those indicated on the curves in Figure 4. The 10^{-3} gradient reaches 0.5 cm in 9 minutes. Experiments with T-4 viruses were performed in this system. If the band was accelerated at different rates so that it reached 0.5 cm in 10, 8, and 7 minutes in three different runs, the spreading was found to be very significantly greater at that position in the faster runs. In the first run the band at 0.5 cm was nearly comparable to bands under much mor stable conditions.

It appears therefore that Table 1 and Figures 2 to 4 can serve as useful guides in the prediction of band stability and the determination of desirable experimental conditions.

References

1. Svensson, H. , L. Hagdahl, and K. D. Lerner, Science Tools, 4:1 (1957).

2. Crank, J., The Mathematics of Diffusion, Oxford, Clarendon Press, 1957, pp. 10-11.

3. Fujita, H., and V. J. MacCosham., J. Chem. Phys., 30:291 (1959).

4. Vinograd, J., and J. E. Hearst, Prog. in Chem. Org. Nat. Prod.,XX:372 (1962).

5. Pedersen, K. O., J. Phys. Chem., 62:1282 (1958).