A DIFFUSION DRIVEN INSTABILITY IN SYSTEMS THAT SEPARATE PARTICLES BY VELOCITY SEDIMENTATION

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ABSTRACT Velocity sedimentation has been used extensively to separate particles according to the magnitude of their sedimentation velocity in suitable media. This technique has been used over a wide range of particle size from protein molecules, viruses, subcellular particles to whole cells. Successful separation demands that collective particle motion should not occur. In practice it is observed that such systems may, under certain circumstances, suffer from a particular type of instability which destroys the normal dependence of sedimentation velocity on particle size and density. The aim of this paper is to identify the critical parameters that determine the development of this instability. Stability criteria are deduced and predictions of the theory compared with published observations. Satisfactory agreement between theory and observation is obtained. It is concluded that the simple stability criterion, namely that stable sedimentation will occur if the total density gradient is in the direction of the sedimenting force, grossly overestimates the particle load that can be separated in practice. Some specific recommendations for optimum particle loading are included. Earlier theoretical and experimental works are briefly reviewed.

INTRODUCTION

Attempts to study the biological functions of individual subpopulations of a heterogeneous mixture of cells are frequently dependent on the ability to separate the various subpopulations from one another. A commonly used method of achieving such a separation is that of velocity sedimentation (1–4) which isolates subpopulations on the basis of cell size. The same method has also been applied to the separation of subcellular particles (see for example refs. 5 and 6). The technique makes use of the fact that the velocity of a sphere sedimenting through a fluid under the influence of a gravitational or centrifugal force is given by Stokes' law

$$v_{sed} = \frac{2}{9} \cdot (\rho_{part} - \rho_s)(g/\eta) \cdot r_p^2, \quad (1)$$

where ρ_{part} and ρ_s are the density of the sphere and fluid respectively, η is the viscosity, g the sedimenting force per unit mass, and r_p is the radius of the sphere. In practice the particles to be separated are layered, as a thin band, onto a suitable medium and, provided Stokes' law is obeyed, the particles become distributed throughout the underlying medium in layers of constant $(\rho_{part} - \rho_s) \cdot r_p^2$. For whole cells,

using a medium whose density is close to that of water, fractionation is determined largely by cell radius since ρ_{part} does not vary widely between different cell types (1).

An essential prerequisite for particles to sediment in accordance with Eq. 1 is that no collective particle motion should occur. This constraint implies that the particle band must be stable throughout the sedimentation interval. The stability of such bands has received considerable theoretical (7–12) and experimental (1, 5, 6, 12–16) study. The Svensson criterion for stability states that the density gradient of the particle-fluid mixture should be in the same direction as the force producing the sedimentation. Violation of this condition results in interchange of fluid between regions of high and low density, i.e. the system suffers from convective instability.

Application of the technique of velocity sedimentation to the separation of bone marrow cells (2) or mouse spleen cells (1) has revealed that even when the Svensson criterion is satisfied an instability can develop above a certain critical cell load. A phenomenon termed "streaming" occurs in which the interface between the cell band and the underlying medium becomes distorted into thin streamers formed from the cell band layer. Miller and Phillips (1) observed that within a single streamer the cells moved collectively but without rouleaux formation in the direction of streamer growth. They also noted that the critical cell density at which streaming occurred could be modified by suitable choice of density gradient and was increased by increasing the sedimenting force.

Streamer or "droplet" formation has also been observed when suspensions of macromolecules have been prepared for sedimentation on sucrose or salt solution gradients (5, 15). Brakke (5) suggested that the droplet formation he observed when hemoglobin was layered over a sucrose gradient was produced by local diffusion of sucrose into the hemoglobin overlay. Such diffusion may invert the density gradient and lead to convective instability. This mechanism has been analyzed theoretically by Svensson et al. (7) but has failed to find experimental support (16). Sartory (11), by regarding the Svensson criterion as a necessary but not sufficient condition for stability, deduced that diffusion of the gradient-producing solute imposed a more severe limit on the macromolecular load than Svensson's diffusion analysis predicted. The experiments of Halsall and Schumaker (16) indicated however that, whereas Svensson's analysis overestimated the load that could be stably sedimented, that of Sartory underestimated it. Reasons for the latter discrepancy have been discussed (11).

In the present paper the effect of diffusion on stability is again examined. As in the work of Sartory the Svensson condition for stability is rejected and the presence of a diffusion-driven instability is deduced. The mechanism of this instability is precisely that described by Sartory (11) but different initial conditions are adopted in that continuous, rather than discontinuous gradients are considered.[1] It is of interest to note that formulated in this way the problem is closely analogous to that of mixing of the oceans due to the presence of thermal and salinity gradients discussed by Stern (17).

[1] The case of continuous gradients has apparently been considered by Halsall and Sartory (12). Lack of details in this publication unfortunately prevents comparison with the present work.

In addition to deducing a stability criterion an estimate is made of the amount by which particle sedimentation departs from a simple Stokes' law dependence when the instability is present. Such an estimate is of practical value since it appears that for particles, such as intact cells, for which the diffusion coefficient is negligible absolute stability can be achieved only at extremely low particle loads.

THEORETICAL PREDICTIONS

The theoretical analysis of the streaming instability is contained in the Appendix to this paper. It is shown in that section that an instability of a streaming type arises as a consequence of the difference between the diffusion coefficients of the sedimenting particles and the solute molecules of the suspending medium. In order that the total fluid density gradient should be parallel to the sedimenting force (Svensson criterion for stability) the solute and particle gradients must have opposite signs. A perturbation that leads to a downward displacement of the particle layer tends to grow as solute molecules diffuse into such a region faster than the sedimenting particles diffuse out. Thus the local density rises and the displaced element of fluid tends to sink, to be replaced by fluid from above which also has a lower solute concentration than the surrounding medium. The theoretical analysis shows that particle diffusion tends to stabilize the system but for large particles such as intact cells this stabilizing effect is negligible. However, even when the streaming instability is present it will assume practical importance only if the velocity of the individual streamers is comparable to the Stokes velocity of the sedimenting particles. The magnitude of the streamer velocity depends on a number of parameters including the initial particle number density so that the condition that the maximum streamer velocity should not exceed the particle sedimentation velocity can be used to define a critical initial particle number density below which sedimentation approximates to a Stokes' law dependence on particle size. In the Appendix it is shown that the critical initial particle number density is

$$\alpha(\text{the solute diffusion coefficient})^{-1/2}$$
$$\alpha(\text{the sedimenting force})^{1/2}$$
$$\alpha(\text{the viscosity of the suspending medium})^{-1/2}$$
$$\alpha(\text{the fluid density gradient})^{1/2}$$
$$\alpha(\text{particle radius})^{-1}$$

In Fig. 1 the theoretical dependence of the critical particle number density on particle radius is plotted for sedimenting forces of 1 g, 10 g, and 100 g. It is apparent that the critical number density increases with decreasing particle radius and increasing sedimenting force. These predictions accord with the observations of Miller and Phillips who found a higher critical particle number density for small cells (sheep erythrocytes) than for larger cells (mouse spleen cells) and that increasing the sedimenting force by operating in a zonal centrifuge did indeed raise the critical particle number density.

In Fig. 2 the dependence of the critical particle number density on density gradient is plotted for three values of sedimenting force. The density gradients used by Miller and Phillips have been marked on the diagram. The theory predicts a 4.3-fold increase

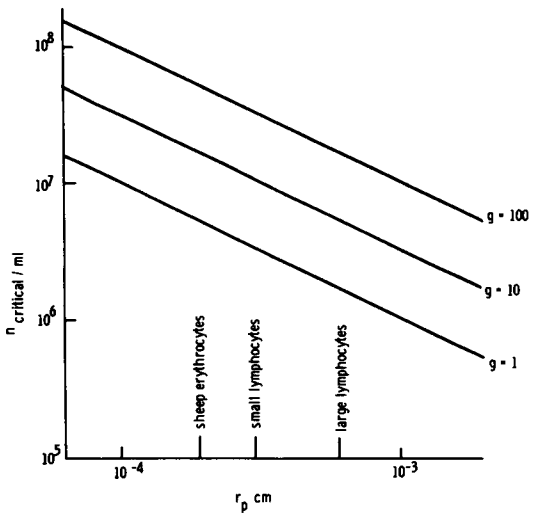

FIGURE 1

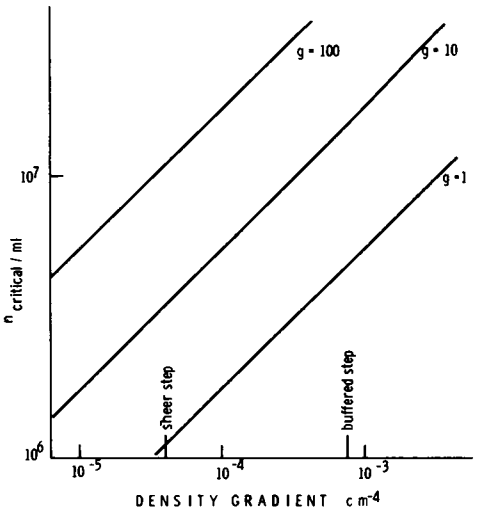

FIGURE 2

FIGURE 1 Variation of critical particle number density with particle radius for three values of accelerating force.

FIGURE 2 Variation of critical particle number density with density gradient for three values of the accelerating force.

in critical particle number density in changing from the sheer step to the buffered step gradient. This result agrees well with the fourfold increase that Miller and Phillips observed in practice.

For small particles complete stability may be achieved provided the particle density gradient is not too steep. Thus if the ratio of particle density gradient to solute density gradient is less than the ratio of particle diffusion coefficient to solute diffusion coefficient the system is stable. Even in this case the limit set by the Svensson criterion cannot be reached in any practical case. In Fig. 3 the ratio "critical particle density gradient/solute density gradient" is plotted against the ratio "radius solute molecule/radius sedimenting particle." In deriving this figure from the theoretical analysis it has been assumed that diffusion coefficients are inversely proportional to particle radii. The figure shows that absolute stability at the Svensson limit (i.e. when particle and solute density gradients are of equal magnitude but opposite direction) occurs only when the particle and solute diffusion coefficients are equal. In general, the smaller the particles being separated the more nearly is the Svensson limit approached. Thus for hemoglobin on a sucrose gradient the particle density gradient is about 20% of the Svensson limit whereas for erythrocytes in an albumin gradient the stable gradient is less than 0.1% of that predicted by the Svensson criterion. In the Appendix the calculated particle density gradient for hemoglobin in a 0.02 $g \cdot cm^{-4}$ sucrose gradient is compared with the value obtained by Brakke (5). Predicted and observed values agree to within 25%.

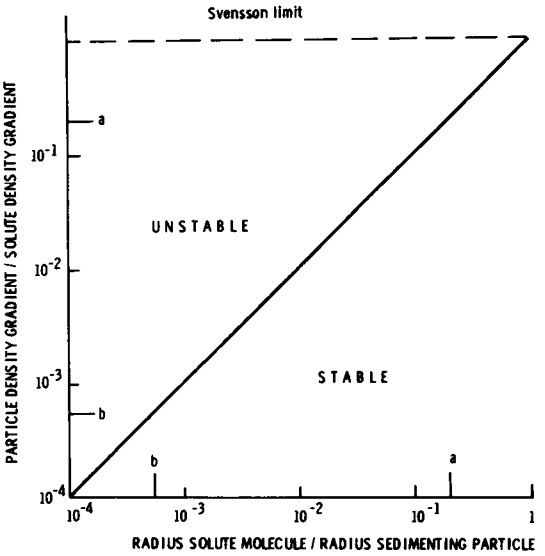

FIGURE 3 The limiting particle density gradient for complete stability. Points marked *a* correspond to hemoglobin sedimenting in a sucrose gradient. The system is theoretically stable up to 20% of the particle load predicted by the Svensson criterion. Points marked *b* correspond to sheep erythrocytes in an albumin gradient. Stability is achieved only well below the Svensson limit.

DISCUSSION

The underlying mechanism of the streaming instability outlined in the previous section appears to have been first discussed by Stern (17). It is common both to the present work and that of Sartory. The analysis differs from that of Sartory in considering continuous particle and solute gradients rather than a discontinuous boundary between two fluids. A discontinuous density change (in the mathematical sense) can hardly be achieved in practice so the continuous gradient analysis may be more appropriate even in those cases where an apparent discontinuity is set up (15, 16).

The theoretical analysis reveals that the phenomenon of streaming is influenced by a considerable number of variables. However, comparison of theory and experiment shows that the theory predicts to within a factor 3 the critical particle number density observed by Miller and Phillips and predicts Brakke's observation on hemoglobin sedimentation to within 25%. The theory also accounts for the fine-scale nature of the observed instability (i.e. the instability takes the form of thin streamers), predicts growth rates similar to those observed,[2] and indicates correctly the observed trend of the critical particle number density with particle size, sedimenting force and solute density gradient (1). Finally the theory satisfactorily accounts for the observation of Hilal et al. (13) that droplet size in a high viscosity gradient is larger than that in a

[2]Mason, D. W. Unpublished results.

low viscosity gradient but the droplets sediment faster in the latter. It may be concluded that the theory presented here appears to account adequately, in two widely different experimental regimes, for the streaming instability observed when particles are separated by velocity sedimentation.

In practical terms a number of specific recommendations may be made. Thus in order to maximize the number of particles that can be separated

(*1*) the medium for separation should have a low viscosity and a small diffusion coefficient;

(*2*) the density gradient of the medium should be as steep as is practicable;

(*3*) separation should be carried out, where possible, at a high value of sedimenting force.

In addition, for small particles such as proteins, viruses, and subcellular fractions, where the particle diffusion coefficient is not negligible one may obtain completely stable sedimentation by ensuring that the ratio of particle density gradient to solute density gradient is less than the ratio of the corresponding diffusion coefficients.

APPENDIX

In order to examine the stability of the sedimenting band an infinitesimal perturbation of the equilibrium condition is considered. If any such perturbation can be shown to grow then the system is unstable since thermal motion will always ensure the presence of the initial departure from equilibrium.

In the following analysis the z coordinate is taken parallel to a constant gravitational acceleration. Thus (xy) planes are surfaces of constant gravitational potential. Density gradients are taken to be positive in the direction of increasing z. $n(z)$ is the particle density in the unperturbed initial state; v is the volume of one particle; $\rho(z)$, is the total fluid density taking into account the presence of the suspended particles; D_s is the diffusion coefficient of the solute of the suspending medium; and D_p is the diffusion coefficient of the particles.

Consider a perturbation in particle density of the form

$$n = n(z_o) + n' \cdot \exp(-[(z - z_o)/b]^2) \cdot \sin kx \sin ky,$$

where z_o is some arbitrary depth in the fluid. Then the continuity equations for particles and solute, respectively, are

$$2D_p k^2 n' + (\mathrm{d}n'/\mathrm{d}t) + [\mathrm{d}n(z_o)/\mathrm{d}z] \cdot v_Z = 0, \tag{2}$$

$$2D_s k^2 \rho_s' + (\mathrm{d}\rho_s'/\mathrm{d}t) + (\mathrm{d}\rho_s/\mathrm{d}z) \cdot v_Z = 0, \tag{3}$$

where V_z = fluid velocity arising as a result of the perturbation and ρ_s' is the perturbed density of the suspending medium. For deriving Eqs. 2 and 3 the following inequalities have been assumed:

$$[1/n(z)][\mathrm{d}^2 n(z)/\mathrm{d}z^2] \ll k^2,$$

$$[1/n(z)][\mathrm{d}n(z)/\mathrm{d}z] \ll k^2 b,$$

$$1 \ll k^2 b^2.$$

Equating gravitational and viscous forces, taking $nv \ll 1$ and ignoring the acceleration term

which can be shown to be small, the kinetic equation for the fluid is

$$\rho_s' + \rho_p' = (2\eta/g) \cdot k^2 V_z, \tag{4}$$

where $\rho_p' = (\rho_{\text{part}} - \rho(z)) \cdot n' \cdot v$.

Eqs. 2, 3, and 4 yield

$$2(\eta/g) \cdot k^2(\mathrm{d}^2\rho_p'/\mathrm{d}t^2) + [4(\eta/g) \cdot k^4(D_p + D_s) + (\mathrm{d}\rho(z)/\mathrm{d}z)](\mathrm{d}\rho_p'/\mathrm{d}t) + (8D_pD_s \cdot k^6(\eta/g) + 2k^2[D_p(\mathrm{d}\rho_s/\mathrm{d}z) + D_s(\mathrm{d}\rho_p/\mathrm{d}z)])\rho_p' = 0. \tag{5}$$

In order to satisfy the Svensson criterion the coefficient of $\mathrm{d}\rho_p'/\mathrm{d}t$ in Eq. 5 must be positive. Hence the auxiliary equation will have one positive root (corresponding to an unstable perturbation) if the coefficient of ρ_p' is negative. Thus the stability criterion may be written

$$8D_pD_s \cdot k^6(\eta/g) + 2k^2[D_p(\mathrm{d}\rho_s/\mathrm{d}z) + D_s(\mathrm{d}\rho_p/\mathrm{d}z)] > 0 \text{ stable.}$$

If $D_p(\mathrm{d}\rho_s/\mathrm{d}z) + D_s(\mathrm{d}\rho_p/\mathrm{d}z)$ is positive then the system is stable for all values of k. Otherwise there is a critical value of k given by

$$k_{\text{crit}}^2 = \tfrac{1}{2}(g/\eta)^{1/2} \cdot D_p^{-1/2} \cdot D_s^{-1/2} \cdot (-[D_p(\mathrm{d}\rho_s/\mathrm{d}z) + D_s(\mathrm{d}\rho_p/\mathrm{d}z)])^{1/2}.$$

For $k^2 > k_{\text{crit}}^2$ the system is stable.

Estimation of the Growth Rate of the Instability. If $D_p \ll D_s$ and $-(\mathrm{d}\rho_p/\mathrm{d}z) \ll (\mathrm{d}\rho_s/\mathrm{d}z)$ it is readily shown that Eq. 5 has the solution

$$(1/\rho_p')(\mathrm{d}\rho_p'/\mathrm{d}t) = -2k^2 \cdot D_s(\mathrm{d}\rho_p/\mathrm{d}z)[4(\eta/g) \cdot k^4 \cdot D_s + (\mathrm{d}\rho(z)/\mathrm{d}z)]^{-1}. \tag{6}$$

Thus the initial perturbation grows with a growth rate $1/\tau$ equal to $(1/\rho_p') \cdot (\mathrm{d}\rho_p'/\mathrm{d}t)$. Eq. 6 yields a maximum growth rate

$$(1/\tau)_{\max} = -D_s^{1/2} \cdot (\mathrm{d}\rho_p/\mathrm{d}z)[4(\eta/g)(\mathrm{d}\rho(z)/\mathrm{d}z)]^{-1/2} \tag{7}$$

and this occurs at a value of k given by

$$k(1/\tau)_{\max} = [(g/4\eta D_s)(\mathrm{d}\rho(z)/\mathrm{d}z)]^{1/4}. \tag{8}$$

Finally, writing $n'(t) = n'(0) \cdot \exp(t/\tau)$ where $n'(0)$ is the amplitude of the initial perturbation, Eqs. 2 and 7 give

$$V_z = \tfrac{1}{2} \cdot D_s^{1/2}(\rho_{\text{part}} - \rho(z)) \cdot v(g/\eta)^{1/2} \cdot (\mathrm{d}\rho(z)/\mathrm{d}z)^{-1/2} \cdot n'(0) \cdot \exp(t/\tau).$$

This equation indicates that the velocity of growth of the perturbation increases exponentially with time. However the perturbed density $n'(0) \cdot \exp(t/\tau)$ has a limiting value equal to the maximum particle density in the sedimenting band. Thus the fastest growing mode will grow to a limiting velocity V_{lim} given approximately by

$$V_{\text{lim}} \approx \tfrac{1}{2} \cdot D_s^{1/2} \cdot (\rho_{\text{part}} - \rho(z)) \cdot v(g/\eta)^{1/2} \cdot (\mathrm{d}\rho(z)/\mathrm{d}z)^{-1/2} \cdot n(z)_{\max}.$$

The instability will have a significant effect on particle separation by velocity sedimentation if V_{lim} is comparable in magnitude to the sedimentation velocity V_{sed} for a single particle, i.e. if

$$V_{\text{lim}}/V_{\text{sed}} = \tfrac{9}{4} \cdot D_s^{1/2}(\eta/g)^{1/2} \cdot (\mathrm{d}\rho(z)/\mathrm{d}z)^{-1/2} \cdot n(z)_{\max} v r_p^{-2} \gtrsim 1. \tag{9}$$

The theoretical analysis shows that viscosity and particle diffusion both tend to stabilize the system. At large values of k viscosity is important. Eq. 5 yields two stable roots for $1/\tau$, namely

$$1/\tau = -k^2 D_p \text{ and } -k^2 D_s.$$

Thus short wavelength modes are stabilized by viscosity and the solute and particle perturbations are dissipated by diffusion.

If D_p, the diffusion coefficient of the particle, is sufficiently large so that $D_p \cdot (d\rho_s/dz) + D_s \cdot (d\rho_p/dz)$ is positive then the system becomes stabilized by particle diffusion for perturbations of any wavelength. In practice this condition is satisfied for particles of small size only since there is an inverse relationship between diffusion coefficient and particle radius. For larger particles, such as intact cells particle diffusion is negligible. In this case an initial perturbation grows exponentially. A maximum growth rate $(1/\tau)_{max}$ may be identified (Eq. 7) and this occurs at a particular value of k given by Eq. 8.

As an example of the application of the theory it may be applied to the case of sheep erythrocytes sedimenting under unit gravity in an albumin gradient. The following parameters are assumed

$$\begin{aligned} D_s &= 6 \times 10^{-7}\,\text{cm}^2 \cdot \text{s}^{-1} \\ d\rho(z)/dz &= 1 \times 10^{-3}\,\text{g} \cdot \text{cm}^{-4} \\ \eta &= 1.6 \times 10^{-2}\,\text{g} \cdot \text{cm}^{-1} \cdot \text{s}^{-1} \\ r_p &= 1.95 \times 10^{-4}\,\text{cm} \\ \rho_{part} &= 1.09\,\text{g} \cdot \text{cm}^{-3} \end{aligned}$$

The theory yields the following results

$$(1/\tau)_{max} = 8 \times 10^{-12}[dn(z)/dz]\,\text{s}^{-1}.$$

Thus if the cell density in the band rises to 10^7/ml in 1 mm the instability will e fold every 20 min (1,250 s).

$$k\left(\frac{1}{\tau_{max}}\right) = 0.7 \times 10^2\,\text{cm}^{-1},$$

$$V_{lim} = 8 \times 10^{-12} n(z)_{max}\,\text{cm} \cdot \text{s}^{-1},$$

and with $n(z)_{max} = 10^7$/ml

$$V_{lim} = 8 \times 10^{-5}\,\text{cm} \cdot \text{s}^{-1} = 2.9\,\text{mm/h}.$$

Finally $V_{lim}/V_{sed} = 1$ when $n(z)_{max} = 5.5 \times 10^6$/ml.

Although there are insufficient data to make close comparisons with these theoretical predictions a number of conclusions may be drawn. The theory predicts that the instability will be a fine scale one. A value of $k[(1/\tau)_{max}]$ of 0.7×10^2 cm^{-1} implies streamers of approximately 0.4 mm diameter. The growth rate indicates that the instability should be macroscopically visible within minutes of establishing the cell band as is in fact observed. In addition, the estimate of cell number density at which the instability becomes important, i.e. $5.5 \times$

10^6/ml in the example considered, may be compared with the experimentally observed density at which streamers appear (1) of 4–15 × 10^6/ml.

In the above example diffusion of the cells may justifiably be ignored. For the sedimentation of much smaller particles diffusion may ensure complete stability. Applying the stability criterion

$$D_p \cdot (d\rho_s/dz) + D_s \cdot (d\rho_p/dz) > 0 \text{ stable}$$

to the sedimentation of hemoglobin in an ultracentrifuge and taking

$$D_p = 7 \times 10^{-7}\ \text{cm}^2 \cdot \text{s}^{-1}$$

$$D_s = 2.9 \times 10^{-6}\ \text{cm}^2 \cdot \text{s}^{-1}\ \text{(sucrose)}$$

$$d\rho_s/dz = 2 \times 10^{-2}\ \text{g} \cdot \text{cm}^{-4}$$

yields for the marginally stable situation

$$-d\rho_p/dz \leq 4.8 \times 10^{-3}\ \text{g} \cdot \text{cm}^{-4} \text{ stable.}$$

This result may be compared with that of Brakke (5) who observed that for a band thickness of 1.3 cm in a centrifuge tube of 16 mm diameter with a sucrose density gradient of 0.02 g · cm^{-4} stable separation occurred up to a critical hemoglobin load of 24.6 mg hemoglobin. Taking the partial specific volume of hemoglobin to be 0.75 these data yield

$$-d\rho_p/dz \leq 6.3 \times 10^{-3}\ \text{g} \cdot \text{cm}^{-4} \text{ stable}$$

in reasonable agreement with the theoretical estimate.

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