STABILITY REGION OF ALBUMIN ZONES IN SUCROSE DENSITY GRADIENTS AT 1 G

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SUMMARY

A method is described to study the stability of zones in liquid density gradients. The definition of a stable zone is formulated. Application of this method to the particular case of inverted-layered albumin zones in constant sucrose gradients at 1g revealed that zone capacity is not predicted by available theory. Instability sets in at gradient loads too low to explain the phenomenon by density inversion. In contrast to shelf-layered zones, inverted gradient-layered zones show complete and lasting stability below some critical load. This stability limit was evaluated quantitatively as a fraction of theoretical prediction and shown to depend on strength of the supporting gradient. For a wide range of gradient strengths a stability region could thus be delineated.

Application of sample material as an inverted gradient and selection of working conditions compatible with the stability region is therefore advised for practical density gradient work.

Application of a homogeneous sample zone on or into a liquid column stabilized by a density gradient can be accompanied by a phenomenon of hydrodynamic disturbances termed droplet-sedimentation (1) or streaming (2). If a rigorous quantitation of transport processes in density gradients is aimed at, this phenomenon has to be understood. It is also expected that separation efficiency in density gradient methodology will be increased considerably if this disturbance is eliminated. Related to this is the problem of zone capacity (3), i.e. the amount of material that can be stabilized in a zone of given dimensions in a given density gradient.

Quantitative models describing zone stability and capacity have been developed by Svensson et al. (4). These equations are based on the principle that a density increase in the direction of the gravity vector has to be maintained everywhere and at

every moment in the liquid column. Experimental tests of these models have been conducted since and, although the experimental approaches were rather different, have led to the general conclusion that, at least in the 1 g situation, an overall increase in density does not suffice to guarantee complete stability of the zone (5-8). For shelf-layered zones a theory has recently been proposed to account for this discrepancy (9). An experimental investigation by the present authors (7) on the stability of albumin zones in constant sucrose density gradients at one gravity disclosed the possibility to obtain fully stable zones when incorporated as inverted gradients. As for shelf-layered zones, instability developed at a zone load far below the value predicted by the available model.

A method is described which permits the quantitative study of the stability of sample zones in liquid density gradients. Application to the particular case of inverted gradient-layered albumin zones in constant sucrose gradients revealed that full stability can be obtained below some critical concentration of zone material. Limiting values could be determined fairly accurately and were shown to depend on the gradient strength of the supporting medium.

MATERIALS AND METHODS

Sucrose (Analar p.a.) was dried in vacuo at 70°C for 4 h. Solutions in water (twice distilled and deionized) were prepared on a weight basis. Crystallized bovine plasma albumin (Armour) was used as such. Albumin solutions in water were prepared on a weight basis and the apparent specific volume determined by pycnometry. An average value of 0.74_9 was obtained, independent of concentration. Concentration values were then corrected assuming a partial specific volume of 0.735 (25°C) (10).

Constant sucrose density gradients were formed in cellulose centrifuge tubes (inner diameter: 1") with the aid of a density gradient forming device of own design, allowing an automatic introduction of the sample zone as an inverted gradient (12). Following incorporation of the albumin zone, a water overlay was applied on top of the gradient column.

Analysis of the initial concentration distribution of the protein was performed by continuous extinction measurement at 280 nm during formation of the gradient and incorporation of the

zone. After standing for 45 min the tube content was lifted and analysed in the same way. Records of initial and final concentration distributions were thus obtained (Fig. 1).

All experiments were performed at 25°C (±0.05°C).

At zero time, total zone width was 0.6 cm in all experiments (Fig. 1).

CRITERION OF STABILITY AND EVALUATION OF RESULTS

A solute zone in a liquid column is considered as being stable only when it is clear that zone broadening is due to diffusion alone i.e. no indications for convective transport should be present during or after formation of the solute zone.

The ratio of final and initial zone widths taken at one quarter of the peak maxima was selected as the parameter describing zone spreading and will be referred to as the zone width ratio.

Sucrose density gradient values were expressed as density increments per unit lenght ($g.ml^{-1}.cm^{-1}$). Densities were derived from tables relating concentration and density of sucrose solutions (11) with the use of Everett's interpolation formula. The sucrose concentration at the upper zone boundary was fixed at 5% (w/w) in all experiments irrespective of gradient strength.

The maximum concentration of albumin that theoretically can be held stable as an inverted gradient in a given zone width by a particular constant density gradient was calculated from the following expression, adapted from the Svensson model for a migrating zone (4):

$$C_m = \frac{h.\frac{d\rho}{dx}}{1 - \bar{v}.\rho_t}$$

where C_m : upper theoretical limit of albumin concentration in $g.ml^{-1}$ at the upper zone boundary compatible with the absence of density inversion in the zone.

h : zone width in cm (0.6 cm in all experiments).

$\frac{d\rho}{dx}$: density gradient value of supporting medium in $g.ml^{-1}.cm^{-1}$.

$\bar{v}$: partial specific volume of albumin in $ml.g^{-1}$ ($0.735\ ml.g^{-1}$).

ρ_t : density of the upper zone boundary in the absence of albumin in $g.ml^{-1}$ ($1.017_8\ g.ml^{-1}$ in all experiments).

For a fixed sucrose concentration at the upper boundary of the zone and a fixed zone width, C_m is a direct measure of the maximum theoretical load of a particular density gradient of constant strength. Relative gradient load is defined as the ratio C/C_m where C stands for the albumin concentration actually realized. No volume change on mixing was assumed.

RESULTS AND DISCUSSION

Application of a zone as an inverted gradient in a density gradient column has been suggested by Svensson (13) and also advocated by Britten and Roberts (12). From a theoretical point of view, application of zones in this way offers definite advantages. The large gradients in density and viscosity occurring at the lower boundary of shelf-layered zones are avoided. Furthermore, according to Fick's law, differences in diffusion coefficients between both solutes cannot give rise to density inversion in constant concentration gradients.

Analysis of experimental inverted layered zones showed that full stability can be realized in practice, but only below some critical load of the gradient (Fig. 1).

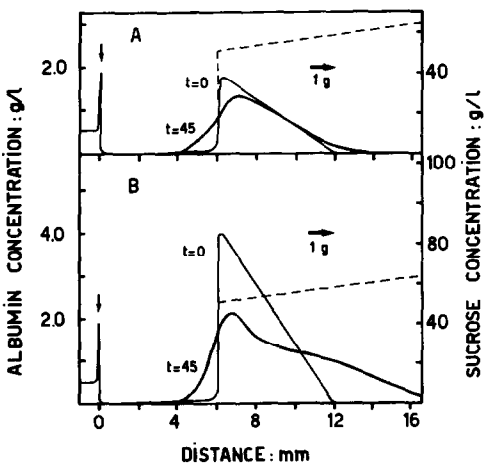

Fig. 1. Experimental records of inverted-layered albumin zones on constant sucrose gradients.
The field of gravitation is directed to the right. Between meniscus, indicated by a vertical arrow, and upper zone boundary a water overlay is applied to avoid complications due to reflected diffusion. Experimental curves of initial and final albumin concentration distributions were matched using the meniscus deflection as the reference. Full lines: albumin concentration distributions (experimental curves). Broken line: initial sucrose con-

centration distribution (calculated curve).
A. Stable zone: deduced from comparison of initial (t = 0 min) and final (t = 45 min) concentration profiles. Initial albumin density gradient in the lower boundary: -1.0×10^{-3} g.ml^{-1}.cm^{-1}. Supporting sucrose density gradient: 5.0×10^{-3} g.ml^{-1}.cm^{-1}. Zone load: 20% of theoretical maximum.
B. Unstable zone: experiment identical to A except for a higher zone load: 40% of theoretical maximum. Albumin density gradient in the lower boundary: -2.0×10^{-3} g.ml^{-1}.cm^{-1}.

In order to establish more accurately the transition conditions, repetitive experiments with different sample concentrations in a density gradient of the same strength were performed. The zone width ratio computed from the experimental records was plotted as a function of relative gradient load (Fig. 2). As is apparent from the biphasic nature of the curve, there clearly exists a limiting value above which instability develops. A fairly accurate estimate of this limit could be obtained by extrapolating the steep section of the curve to the abcissa.

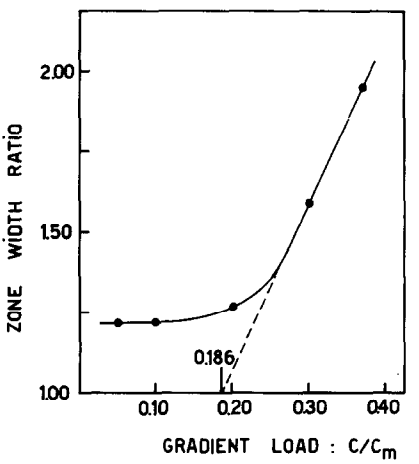

Fig. 2. Dependence of zone width ratio on gradient load. Inverted layered albumin zones supported by a constant sucrose gradient.
Significance of co-ordinate parameters are explained in the text. The upper limit of stability was determined by graphical extrapolation as indicated by the broken line. Supporting sucrose density gradient: 25.0×10^{-3} g.ml^{-1}.cm^{-1}.

Repitition of this set of experiments at different strengths of the supporting gradient revealed that the limiting value, as defined above, is gradient strength dependent (Fig. 3). The highest relative stable load was obtained at low values of gradient strength. Nevertheless the limiting value always remained far below the theoretical maximum. The curve delineates a stability region for inverted gradient-layered zones.

Although quantitative measurements have been carried out only on albumin zones in sucrose density gradients, occasional observations suggested the same general behaviour for other systems. Qualitative experimental evidence furthermore suggested

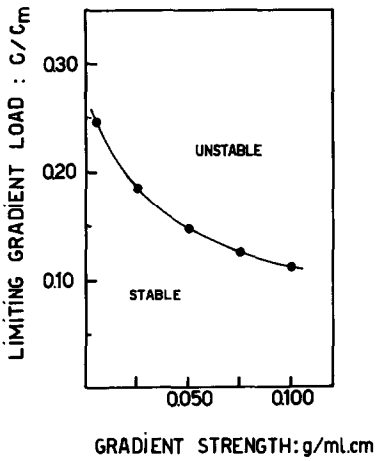

Fig. 3. Dependence of upper limit of stability on strength of supporting sucrose gradient.
For constant density gradients of different values, the upper limit of stability was determined as outlined in Fig. 2 and plotted as a function of gradient strength. The graph thus obtained delineates a stability region which may be useful for practical application.

that the stability region will be larger as the molecular weight ratio of sample constituent and gradient forming solute approaches unity.

It seems that application of the present findings in actual work would enable the elimination of the most important constraint on quantitative treatment of zone migration in density gradient methodology. It is also expected that resolution will be substantially improved by respecting working conditions compatible with the stability region. The experimental results in-

dicate that, in addition to the principle of an overall increase in density, another factor must be taken into consideration in any attempt to develop an adequate theoretical model describing stability of inverted-layered zones. It seems very unlikely that the observed instabilities in the unstable zones were due to local density inversion as the primary cause.

Difficulties in the prediction of stability of inverted-layered sample zones for a particular experimental system due to the present lack of an adequate theoretical model can be overcome by the empirical delineation of a stability region, as described.

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