Self-generated gradients (macromolecules/macromolecular complexes)

1. Background

Iodixanol, like solutions of heavy metal salts (e.g. CsCl), can form a gradient from a solution of uniform density under the influence of the centrifugal field. Once the solute begins to sediment through the solvent a concentration gradient is formed which is opposed by back-diffusion of the solute. With a sufficiently high RCF, at equilibrium, the sedimentation of the solute is exactly balanced by the diffusion and the gradient is stable. It is possible to calculate the time for a self-generating gradient to reach equilibrium and it is described by the following equation:

$$t = k\,(r_b - r_t)^2$$

t is the time in hours; r_b and r_t the distance from the centre of rotation to the bottom and top of the gradient respectively and k is a constant, which depends on the diffusion coefficient and viscosity of the solute and on temperature [1]. The slope of the gradient is given by the equation:

$$\rho_r - \rho_i = \frac{1.1x10^{-2}\,xQ^2}{2\beta^o}(r_c^2 - r^2)$$

where ρ_r is the density at a point r cm from the axis of rotation, ρ_i is the starting density of the homogeneous solution, r_c is the distance in cm from the axis of rotation where the density of the gradient = ρ_i , Q is the rotor speed in rpm and β^o is a constant depending on the solute [1].

The shape of the gradient that is formed for a particular solute thus depends on the following factors:

- sedimentation path length of the rotor
- time of centrifugation
- speed of centrifugation
- temperature

The big advantages of the use of any self-generated gradient are the ease of sample handling (the sample is simply adjusted to the required starting concentration of iodixanol) and the great reproducibility of the gradient density profile under a particular set of centrifugation parameters.

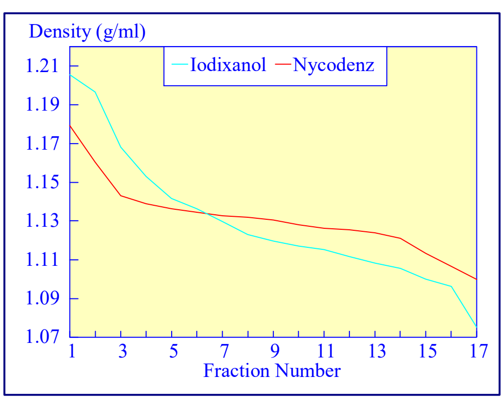


Figure 1 Comparison of 20% (w/v) iodixanol and Nycodenz® in 0.25 M sucrose: 20° fixed-angle rotor (11.5 ml) at 270,000g for 3 h at 4° C

2. Self-generated gradient formation

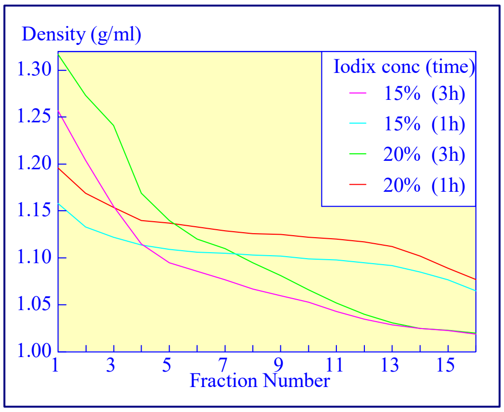
Iodixanol is able to form useful self-generating gradients in 1-4 h depending on the centrifugation speed and the rotor [2]. Figure 1 compares the gradient density profile generated from 20% (w/v) iodixanol and 20% (w/v) Nycodenz® in 0.25 M sucrose in a 20° fixed-angle rotor at 270,000g_{av} for 3 h at 4°C. Clearly a steeper gradient is formed from the iodixanol and this is a function of the higher molecular mass of iodixanol (approx. twice that of Nycodenz®): it therefore sediments rather more rapidly and diffuses more slowly.

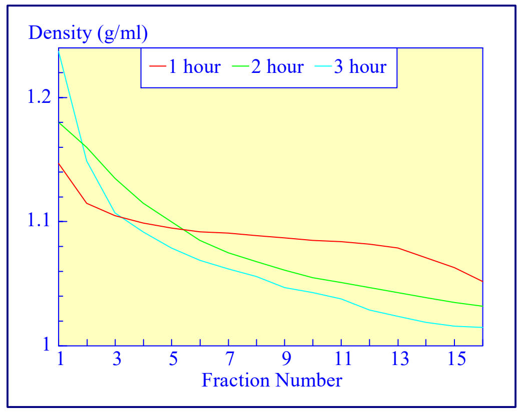
2a. Types of rotor

Swinging-bucket rotors, which have rather long sedimentation path lengths, are little used for the formation of self-generating gradients. The shorter sedimentation path length rotors are much better suited to this task. Vertical and near-vertical rotors are particularly useful, although some fixed-angle rotors (preferably those with shallow angles of 20-24°) may be used.

Gradients generated in the Beckman TLN100 near-vertical rotor (for the TLX120 table-top ultracentrifuge) which accommodates tubes of 3.5-4.0 ml, the Beckman VTi65.1 vertical rotor (for an appropriate floor-standing ultracentrifuge) which accommodates tubes of approx 11.0 ml (but which can be adapted down to smaller volumes) and the Beckman NVT65 (a near vertical rotor of similar tube capacity to that of the VTi65.1) are particularly useful for iodixanol self-generated gradients. The TLN100 and VTi65.1 rotors have approximately the same sedimentation path length (about 17 mm), that of the NVT65 is marginally longer (approx 25 mm); consequently under the same centrifugation conditions, they generate rather similar gradient profiles.

Figures 2 (left) and 3 (right) Figure 2: Effect of time and iodixanol (in 0.8% NaCl) concentration; Beckman TLN100 rotor (3.9 ml) at 353,000g at 15°C. Figure 3: Effect of time with 12.5% iodixanol (in 0.8% NaCl); Beckman TLN100 rotor (3.9 ml) at 353,000g at 15°C.





2b. Time of centrifugation

After 1 h at 15-18°C, centrifugation at approx 350,000g_{av}, gradients generated in the TLN100 are S-shaped (i.e. they contain a relatively shallow region in the middle) and span a relatively narrow density range, while after 3 h, gradients are considerably steeper and cover a much wider density range. Figure 2 compares two starting concentrations of iodixanol at these two times, while Figure 3 compares three times (1, 2 and 3 h) using a 12.5% (w/v) iodixanol starting concentration with the same rotor. The exponential nature of the gradient becomes more apparent with time but times greater than 3 h result in little further change in the shape of the gradient at 350,000g, indicating that an equilibrium point has been reached.

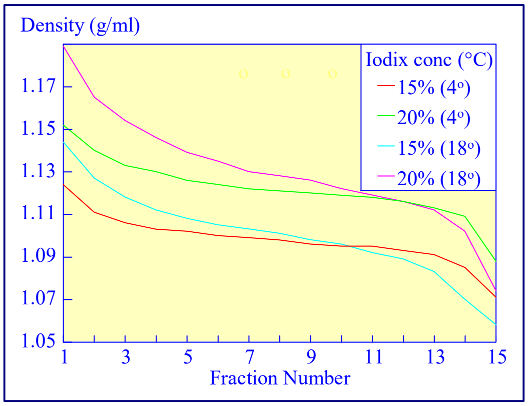
2c. Temperature

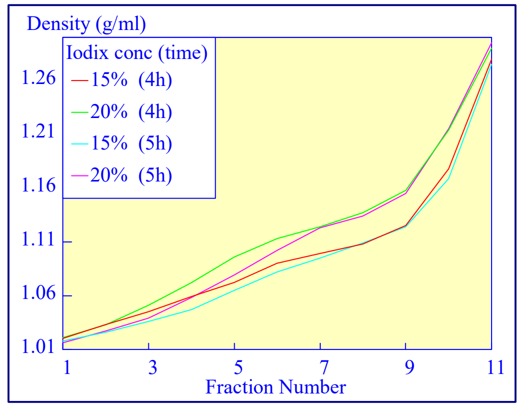
Higher temperatures tend to promote the formation of steeper gradients, although this effect is more apparent at shorter times of 1 h than at longer times of centrifugation. Figure 4 compares the formation of gradients at 4°C and 18°C in the NVT65 rotor at two iodixanol concentrations after centrifugation at approx 340,000g_{av} for 1 h. At 4°C, using 0.25 M sucrose as osmotic balancer, the gradients approach equilibrium more slowly: the excellent gradient profiles produced in the VTi65.1 with 15% or 20% (w/v) iodixanol at 4 h are very similar to those at 5 h (Figure 5), compare with Figure 2 (using NaCl as osmotic balancer at 15°C).

2d. Iodixanol concentration

Other than changing the density range covered by the gradient (Figures 2 and 4-8) the starting concentration of iodixanol has rather little effect on the rate of gradient formation or shape of gradient profile. The shape of the gradient can be made more linear at lower RCFs by using two layers of iodixanol (e.g. 10% and 30%, w/v) rather than a single uniform concentration (20%, w/v).

Figures 4 (left) and 5 (right) Figure 4: Effect of temperature and iodixanol (in 0.8% NaCl) concentration; Beckman NVT65 rotor (11.2 ml) at 340,000g for 1 h. Figure 5: Effect of time and iodixanol (in 0.25 M sucrose) concentration; Beckman VTi65.1 rotor (11.2 ml) at 353,000g at 4°C.

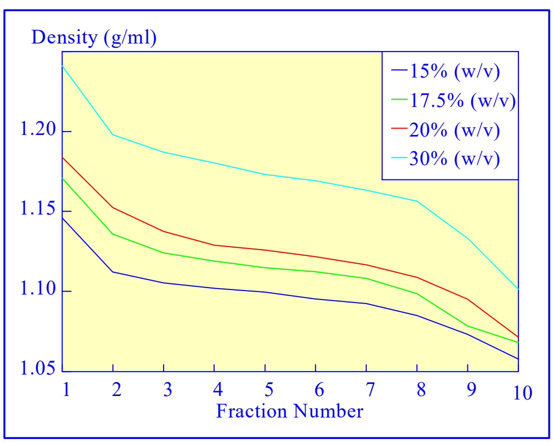


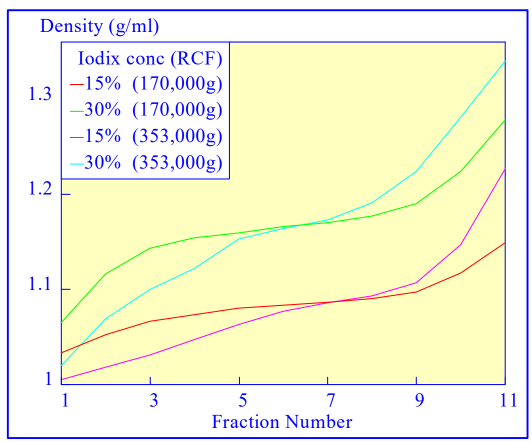


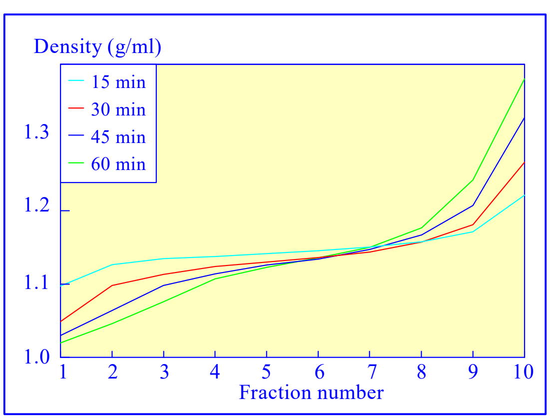
2e. RCF

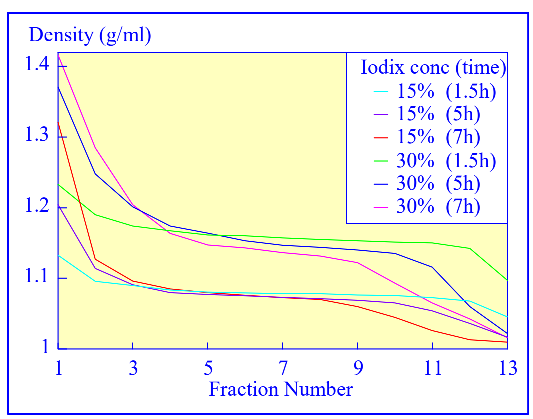
As the RCF decreases, the gradient becomes more shallow in the middle of the tube; the minimum RCF that produces a useful gradient will vary with the time and the rotor type. In the VTi65.1 vertical rotor, even at 170,000g_{av}, a useful shallow gradient is produced within 3 h (Figure 7). In very high performance rotors that can run at up to 150,000 rpm (and also have very short sedimentation path lengths – see next section), self-generated gradients can form in as little as 15 min (Figure 8)

Figures 6 (top left) and 7 (top right) Figure 6: Effect of iodixanol (in 0.25 M sucrose) concentration; Beckman VTi65.1 rotor (11.2 ml) at 353,000g for 1 h at 4°C. Figure 7: Effect of RCF and iodixanol (in 0.8% NaCl) concentration; Beckman VTi65.1 rotor (11.2 ml) at 353,000g for 3 h at 15°C. **Figures 8 (bottom left) and 9 (bottom right)** Figure 8: Effect of time with 25% (w/v) iodixanol (in 0.85% NaCl); Sorvall S150-AT (1.0 ml) at 710,000g at 16°C. Figure 9: Effect of time and iodixanol (in 0.8% NaCl) concentration; Beckman 80Ti rotor (13.5 ml) at 340,000g at 15°C.









2f. Sedimentation path length

The longer the sedimentation path length of the rotor, the greater the tendency to form S-shaped gradients. Figure 9 compares the gradient formed from 15% or 30% (w/v) iodixanol using the 80Ti fixed-angle rotor with a sedimentation path length of 43 mm (13.5 ml tube volume) at a series of times. At 70,000 rpm, (equivalent to 345,000g_{av}) approx 5 h is required to produce a useful gradient (compare with Figures 2-7).

3. References

1. Dobrota, M. and Hinton, R. (1992) *Conditions for density gradient separations* In: Preparative centrifugation - a practical approach (ed D. Rickwood) IRL Press at Oxford University Press, Oxford, UK, pp 77-142.
2. Ford, T., Graham, J. and Rickwood, D. (1994) *The preparation of subcellular organelles from mouse liver in self-generated gradients of iodixanol* Anal. Biochem., **220**, 360-366.

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