Velocity Sedimentation of Organelles at Low Centrifugal Force in an Isokinetic Gradient

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Mast-cell granules and polystyrene microspheres (0.600 and 1.011 μm in diameter) were sedimented in a previously described [Pretlow (1971) *Anal. Biochem.* **41**, 248–255] isokinetic gradient in a low-speed centrifuge. For the analytical velocity sedimentation of organelles, this gradient offers several advantages over gradients that are commonly used for the sedimentation of organelles: (a) the density gradient ($0.0008 \text{ g} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1}$) is small, and the effective densities of organelles will change relatively little during sedimentation; (b) the densities at all points in the gradient (1.017–1.027 g/ml) are less than those in gradients commonly used for the sedimentation of organelles, the effective densities of sedimenting organelles are consequently relatively large, and the effect of density as a determinant of velocity of sedimentation is less limiting than in conventional gradients; (c) the small slope of the gradient is associated with a relatively slow increase in the viscosity encountered by the sedimenting organelle; (d) the iso-osmotic gradient is not significantly affected by the gradient medium (Ficoll), and the osmolarity can be adjusted to the desired value by the selection of an appropriate salt solution as the solvent for the Ficoll; (e) the gradient will be isokinetic for particles of densities similar to most organelles. An ultracentrifuge is not required for work with this gradient.

In 1971 Pretlow described a linear isokinetic gradient, which varied from 2.7 to 5.5% Ficoll (polysucrose, average mol.wt. 400000; Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) over a distance of 13 cm; this gradient has been used for the purification of many different kinds of cells (Pretlow *et al.*, 1975, 1976). As discussed previously (Pretlow, 1971; Pretlow *et al.*, 1969), the two principal physical parameters that determine their rates of sedimentation are density and diameter.

This gradient is particularly suited for the characterization of particles by velocity sedimentation, because it has a very slight density gradient ($0.0008 \text{ g} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1}$). Because the viscosity of aqueous Ficoll increases exponentially with its concentration (Pretlow *et al.*, 1969), the slow increase in Ficoll concentration is associated with a slow increase in viscosity. Since rapidly sedimenting particles do not decelerate as rapidly as they do in steeper ($\text{g} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1}$) gradients, they are more completely separated from less-rapidly-sedimenting particles.

In selecting gradients for a particular purpose, one must be aware of the advantages and disadvantages that will be associated with specific gradients.

Although slight gradients such as that used in the present paper permit more complete separations than are obtained in steeper gradients, slight gradients are not as well suited for preparative purposes since slight gradients will support smaller total masses than steep gradients. The isokinetic gradient used in this report is a slight gradient. As discussed in detail previously (Pretlow, 1971), isokinetic gradients offer the advantage that the investigator can calculate the experimental conditions, i.e. centrifugal force and duration of centrifugation, which (Pretlow, 1971) ‘... gives the maximum purification of the desired ...’ isokinetically sedimenting particle without the need to determine the densities of the sedimenting particles or the need for a computer-generated solution of the equation of motion for sedimenting particles. Having determined the experimental conditions that permit maximum purification of a particular particle in the slight isokinetic gradient used here under conditions that permit isokinetic sedimentation, if one wishes to obtain a wider separation of two particles in the gradient by placing the gradient closer to the centre of the centrifuge (Pretlow & Boone, 1968), one can calculate the alteration of centrifugal force or duration of centrifugation necessary to compensate for the altered distance from the centre of the centrifuge, again without the need for a computer.

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With our published computer program (Pretlow *et al.*, 1975) for the integration of the sedimentation equation, we predicted that particles in the size range of organelles should sediment in this isokinetic gradient, because of its low viscosity and density, even at relatively low centrifugal forces in a low-speed centrifuge. We now report the separation of organelles in the previously described isokinetic gradient (Pretlow, 1971) in a low-speed centrifuge. The low-speed centrifuge offers the additional advantage over most ultracentrifuges that the gradient can be located at a greater distance from the centre of revolution. In our study of the effect of the distance between the density gradient and the centre of revolution (Pretlow & Boone, 1968; Pretlow *et al.*, 1975), we found the swirling, i.e. the changes described by others as 'rotational movements of the fluid' associated with changes in angular velocity (de Duve *et al.*, 1959), during deceleration of the centrifuge to be less severe as the gradient is removed a greater distance from the centre of revolution.

Materials and Methods

Particles for separation

Two sizes (0.600 and 1.011 μm) of polystyrene microspheres (Duke Standards Co., Palo Alto, CA, U.S.A.) were diluted with Joklik's modification of minimum essential medium (Grand Island Biological Co., Grand Island, NY, U.S.A.) and layered in sample volumes of 5.5 ml over the isokinetic gradient. Initially, the concentration of polystyrene microspheres was adjusted by counting them with the light-microscope in an erythrocyte counting chamber. Subsequently, we found that the concentration of microspheres of each of the two sizes as determined in a counting chamber was a linear function of A_{410} over the range of concentrations used in our experiments; and, thereafter, the concentration of microspheres was monitored at 410 nm with a Beckman DB-GT spectrophotometer (Beckman Instruments, Palo Alto, CA, U.S.A.). In preliminary experiments, we found that both sizes of latex particles sedimented isokinetically as would be anticipated from the density (1.060 g/ml) of polystyrene.

Centrifugation

The gradients were centrifuged in an MSE LR-6 centrifuge. The gradients were contained in 100 ml polycarbonate centrifuge tubes (tube no. 2806; International Equipment Co., Needham Heights, MA, U.S.A.), which were centrifuged in 1 litre swinging buckets with adapters. After centrifugation of particles (both polystyrene microspheres and mast-cell granules), the gradients were collected with the

previously described (Pretlow & Boone, 1969) gradient-tapping cap. The first fractions from the gradients were equal to and represented the volumes of the unseparated sample suspensions layered over the gradients; all subsequent fractions contained 4 ml.

Mast-cell granules

For convenience and because of their constant physical properties, we used polystyrene microspheres for the experiments in which we tested the feasibility of separating subcellular-sized particles in the isokinetic gradient in a low-speed centrifuge. We have performed preliminary experiments with mast-cell granules. Mature mast cells (stages III and IV as defined previously) were purified by velocity sedimentation in linear gradients of Ficoll in tissue-culture medium by a slight modification of the method that we reported previously (Pretlow & Cassady, 1970). The purified mast cells were pooled, pelleted, resuspended in 2 ml of Joklik's medium, and disrupted by sonication in a 10 ml centrifuge tube in an ice/water bath with a Bronwill Biosonic IV Ultrasonic Probe (VWR Scientific, San Francisco, CA, U.S.A.) at an energy setting of 30% of 'low' for 30 s (Lagunoff *et al.*, 1964; Uvnas, 1974). The suspended mast-cell granules were sedimented for 10 min in the isokinetic gradient at 4.0°C in the MSE LR-6 centrifuge with a centrifugal force of 390g measured at the sample/gradient interface. Slides for examination with the light-microscope were prepared with the Cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, U.S.A.) and stained with Wright stain. For electron microscopy, granules were sedimented to form a pellet for 15 min with a centrifugal force of 4400g, fixed in 4.0% glutaraldehyde buffered in 0.1 M-sodium phosphate, pH 7.4, for 1 h, washed in 0.1 M-sodium phosphate buffer, pH 7.4, post-fixed in 1.0% osmium tetroxide in 0.1 M-sodium phosphate buffer, pH 7.4, dehydrated in graded alcohols, and embedded in Spurr epoxy resin (Polysciences, Warrington, PA, U.S.A.). Sections for light-microscopy were cut at 1 μm and stained with Toluidine Blue (Trump *et al.*, 1961). Sections for electron microscopy were cut with a diamond knife (E. I. Du Pont De Nemours and Co., Wilmington, DE, U.S.A.), doubly stained with lead citrate and uranyl magnesium acetate, and examined in a Philips EM-200 electron microscope.

Results and Discussion

To determine experimentally whether or not isokinetic sedimentation would occur as would be anticipated from the density of the polystyrene particles (1.060 g/ml), polystyrene particles of each

Fig. 1. Separation of 0.600 and 1.011 μm microspheres by velocity sedimentation

Microspheres were separated in the isokinetic gradient in the MSE LR-6 centrifuge (a) by centrifugation for 134 min with a centrifugal force of 780g measured at the sample/gradient interface 13.7 cm from the centre of revolution. When the experiment was altered only by centrifuging in the Sorvall RC-3 centrifuge (b), moving the sample/gradient interface to a location 8.0 cm from the centre of revolution, and centrifuging for 75 min with a centrifugal force of 1400g (the same $\omega^2 tr$ value), the microspheres were again separated. When the sample/gradient interface is located 8.0 cm from the centre of revolution, this gradient is no longer isokinetic, and the observed separation is consistent with our earlier observation (Pretlow & Boone, 1968) that particles are more widely separated as gradients are moved closer to the centre of revolution. The modal location of the smaller particle continues to be in fraction 4; however, the larger particle has sedimented farther than when the sample/gradient interface was 13.7 cm from the centre of revolution.

size (0.600 and 1.011 μm) were centrifuged separately in isokinetic gradients with a centrifugal force of 780g measured at the sample/gradient interface for several different periods of time. The sedimentation of the particles in the gradients varied with the duration of centrifugation, and the locations of the particles in the gradients proved that isokinetic sedimentation had occurred. In the illustrative experiment that we report here, centrifugation was carried out in the MSE LR-6 centrifuge for 134 min with a centrifugal force of 780g measured at the sample/gradient interface. Under these conditions, the two populations of microspheres were separated (Fig. 1); examination of the separated particles with high magnification by light-microscopy showed that the rapidly sedimenting population (fractions 7–10) contained only the 1.11 μm microspheres and that the less-rapidly-sedimenting population (fractions 3–5) contained only the 0.600 μm microspheres.

Having demonstrated experimentally that particles with constant stable physical properties and with

sizes similar to organelles could be separated in a low-speed centrifuge, we then observed the separation characteristics of mast-cell granules. After sedimentation in the isokinetic gradient for 10 min with a centrifugal force of 390g measured at the sample/gradient interface (13.7 cm from the centre of revolution), mast-cell granules were found in fractions 2–7. The granules in our preparation, like those described by Uvnas (1974) prepared under similar conditions, contained 'a mixture of membrane-bound and membrane-free particles ...' When fractions contained insufficient material for embedding for electron microscopy, adjacent fractions were combined and embedded together. The membrane-bound granules stained more intensely than membrane-free granules when 1 μm sections were stained with Toluidine Blue (Figs. 2 and 3). The membrane-bound granules were concentrated in fractions that contained more-rapidly-sedimenting granules (fractions 3 and 4, Fig. 3), whereas membrane-free particles were concentrated in fractions with less-rapidly-sedimenting

Fig. 2. *Mast-cell granules from fraction 2 after separation in the isokinetic gradient*

The intensely stained granules are membrane-bound when examined with the electron microscope, and the less intensely stained granules are membrane-free (stained with Toluidine Blue; magnification $\times 1000$).

Fig. 3. *Mast-cell granules from fractions 3 and 4 from the same gradient used for Fig. 2*

It appears that the granules in fractions 3 and 4 are slightly larger than those in fraction 2. If we assume that the granules are of equal density, this would be expected. In all experiments, it appeared that membrane-bound granules were concentrated in the fractions that contained more-rapidly-sedimenting granules. Granules in fractions 3 and 4 were pooled to obtain sufficient material to be processed for electron microscopy (stained with Toluidine Blue; magnification $\times 1000$).

particles (fraction 2, Fig. 2). Granules, which had not been obtained as monomers, intact cells and fragments of cells sedimented more rapidly than those in the fractions that contained concentrated membrane-bound or membrane-free granules. Ultrastructurally, both the membrane-bound and membrane-free granules (Fig. 4) were similar to those in the electron micrographs published by Uvnas (1974). Since our objective in these experiments was limited to determining the sedimentation characteristics of organelles in a low-speed centrifuge in gradients with the properties described above, we have not characterized the purified granules in detail.

Because the MSE LR-6 centrifuge is not commonly available in the United States, we repeated our experiment in the Sorvall RC-3 machine, with the HG-4L rotor (E. I. Du Pont De Nemours and Co.). Despite the fact that this rotor has the largest diameter of the available rotors for the RC-3 machine, it is not as large as the rotor that we use for the MSE LR-6 machine; and it is not possible to position the gradient with the sample gradient interface 13.7 cm from the centre of revolution as required for isokinetic centrifugation in this gradient (Pretlow, 1971). Consequently, we placed the

gradient as far (8.0 cm) from the centre of revolution as possible and adjusted the speed and duration of centrifugation so that the $\omega^2 tr$ (Pretlow *et al.*, 1975; Pretlow & Boone, 1968) products would be identical at the sample gradient interfaces in the two rotors (see the legend to Fig. 1). Consistent with our previous report (Pretlow & Boone, 1968), the two populations of microspheres were more widely separated and the shapes of the peaks were slightly different when the gradients were moved closer to the centre of revolution.

We have demonstrated that particles in the range of sizes commonly encountered in organelles can be sedimented in a low-speed centrifuge at relatively low centrifugal forces in a previously described (Pretlow, 1971) isokinetic gradient. The purification of cell organelles in density gradients has generally been accomplished in the past in much steeper ($\text{g} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1}$) gradients than that used for the

Fig. 4. Ultrastructural appearance of granules in pooled fractions 3 and 4
Magnification $\times 19000$.

separations reported here. The low concentration of Ficoll and the slight slope of this isokinetic gradient make separations possible at lower centrifugal forces than would be required for gradients with higher concentrations of Ficoll and steeper slopes. The relatively low range of densities in this gradient makes velocity of sedimentation relatively more dependent upon the diameters and less dependent upon the densities of sedimented particles. Although there is slight variability among different batches of Ficoll, as we have noted previously (Pretlow *et al.*,

1975), the highest concentration of Ficoll (5.5%, w/v) in this isokinetic gradient should add no more than 2.5 mosm to the osmotic pressure of the solvent; thus, for the work reported here, the osmolarity of the solvent (300 mosm in the case of the culture medium we used) determines the osmotic pressure in this iso-osmotic density gradient. The constant osmotic pressure in this gradient is an additional advantage as compared with many gradients that are currently used for the purification of organelles.

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