

Rapid Separation of Endosomes from Lysosomes Using a Thermo Scientific TV-865 Rotor

Barbara M. Mullock and J. Paul Luzio, Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK

Key Words

- Lysosome/Endosome Separation
- Isopycnic Separation
- Nycodenz gradient

Introduction

Cell-free systems are one way to study the molecular cell biology of membrane traffic pathways in eucaryotic cells⁽¹⁾. Endocytic pathways allow the receptor-mediated entry of large molecules into cells and their passage through membrane-bound intracellular compartments known as endosomes. The lysosomes are the destination of many endocytosed macromolecules, but the mechanisms by which molecules are routed via the appropriate parts of the endosomal system to the lysosomes are poorly understood.

We have, therefore, been using cell-free systems prepared from rat liver to examine the interaction with lysosomes of endosomes loaded with ¹²⁵I-labelled ligand⁽²⁾. Endosomes are loaded *in vivo* and reacted with lysosomes *in vitro* under various conditions. We have developed a method which rapidly and completely separates lysosomes and endosomes so that we can measure the radio label associated with each class of organelle. We achieve isopycnic separation by loading the reaction mixtures over Nycodenz gradients and centrifuging them. The Thermo Scientific TV-865 rotor allows us to examine 8 such mixture volumes simultaneously using a one hour centrifugation. Small (0.5 mL) reaction volumes are economical in their use of expensive reagents and reduce the amount of gradient material required, making the use of an expensive medium such as Nycodenz feasible.

Materials and Methods

Preparation of loaded post-mitochondrial supernatant

Rats were injected intravenously with ¹²⁵I-ASF, as described by Branch et al., 10 minutes before killing⁽⁴⁾. The livers were perfused *in situ* with cold 0.25 M sucrose, 10 mM N-tris (hydroxymethyl) methyl-2-aminoethane-sul-

phonic acid (TES), pH 7.4, homogenized in 3 volumes 0.25 M sucrose, 10 mM MTES, 1 mM MgCl (pH 7.4), and centrifuged at 1,500 x g for 10 minutes at 4°C. 0.5 mL aliquots of the post-mitochondrial supernatant were incubated at 37°C with 1.3 mM vanadium-free ATP, 10 mM phosphoenolpyruvate, and 35 U of pyruvate kinase in order to facilitate endosome/lysosome interaction. After incubation, the mixtures were chilled. The samples were then ready for centrifugation.

Centrifugation Procedure

Linear Nycodenz gradients were prepared from equal volumes of a solution containing 45% (w/v) Nycodenz (Nycomed UK), 10 mM TES and 1 mM EDTA (pH 7.4) and a solution containing 0.25 M sucrose, 10 mM TES and 1 mM EDTA (pH 7.4). Total volumes in each Thermo Scientific Ultracrimp® tube (P/N 03945) should be between 4.5 and 5.0 mL. A simple gradient maker has two open chambers connected via a tap at the base and an exit from one chamber to which a peristaltic pump is attached. This chamber is stirred and becomes the mixing chamber. Initially, the denser Nycodenz solution is placed in the mixing chamber and the sucrose in the second chamber. The tap between the chambers is opened and simultaneously the pump is started. With a multi-channel pump, we routinely make 4 gradients at once, pumping at a rate of approximately 1 mL/min/channel.



The mixture enters the centrifuge tubes through bent, large bore hypodermic needles which rest in the openings of the tubes and drip freely. It is difficult to arrange for the gradient mixture to run down the walls of vertical rotor tubes with their narrow openings, and we find that gradients made using free drips are perfectly satisfactory. Gradients are made in a 4°C room and allowed to sit overnight to smooth by diffusion at this temperature.

Reaction mixtures for analysis were approximately 0.5 mL. After incubation, they were chilled and loaded over the gradients using the peristaltic pump arrangement used for gradient making. The microtubes in which incubations were carried out were in turn connected to a narrow bore pump line by fitting in a rubber bung carrying a fine stainless steel tube of appropriate length to reach the tip of the microtube and a second shorter tube to admit air. Each mixture took about 1 minute to load. A brief pulse of water was sucked through the system to wash it between each sample. Tubes were crimped with the Ultracrimp tool and centrifuged in a TV-865 rotor for an hour at 50,000 rpm at 4°C. A slow start option was used.

After centrifugation, the crimped top was cut from each tube with a scalpel. The tube was placed vertically in a plastic holder which allowed a 1 mm diameter stainless steel tube to be passed centrally and vertically from the top to the bottom of the tube and clamped in place. The gradient was

then sucked out, dense end first, using a peristaltic pump at a rate of approximately 1 mL/min. 5 drop (approximately 100 μ L) fractions were collected in lidless microtubes using a small fraction collector. Although this method of gradient collection is usually said to be poorer than upward displacement or tube piercing, we have found it gives reproducible results in addition to being quick and simple. Upward displacement devices are not usually designed to work with vertical rotor tubes which lack the flat tops of swing-out rotor tubes, while tube piercing methods can very easily lead to leakage.

Results

Radioactivity in the various fractions was determined in an NE-1600 gamma counter, and refractive indices of at least some fractions were measured to establish the exact density gradient in each tube. Enzyme assays can also be carried out if desired. Reproducibility of peaks with respect to density is excellent, although the precise fraction number at which a given density is found will vary slightly from tube to tube, particularly if four gradients were initially prepared from the same mixing vessel.

Figure 1 shows a typical separation of endosomal radiolabel from the lysosomal enzyme N-acetyl-B-glucosaminidase in unincubated rat liver post-mitochondrial supernatant. After incubation at 37°C with an ATP-regenerating system, much of the radioactivity has moved to the thelyosomal position.

Discussion

The Thermo Scientific TV-865 vertical ultracentrifuge rotor makes it possible to analyze the distribution of material between endosomal and lysosomal fractions in 8 different reaction mixtures in a total time of little more than 3 hours from the end of the reaction to having measurements of counts across each gradient. The rotor can be similarly used for rapid fractionations of small, precious samples using other gradient media such as sucrose, for example, the rate-zonal separation of antibody receptor complexes carried out by colleagues in this laboratory⁽³⁾. It is essential to put in a small cushion to prevent any solid material reaching the wall of the tube during centrifugation, if the denser of the two solutions used for the gradient is not sufficiently dense. 0.75 mL of 45% Nycodenz containing appropriate

buffer and ion was, therefore, placed in the tubes before making 10-30% sucrose gradients exactly as described above for Nycodenz gradients.

The only difficulty observed arose when very viscous media were used. The Nycodenz gradients described above give identical separations whether run in 5 mL or 40 mL vertical tubes. However, the 1-22% Ficoll gradients which we use to separate different types of endosomes from each other^(4,5) give anomalous results in the small tubes, presumably as a result of difficulties over reorientation in a narrow tube. With this single exception, we find that the TV-865 rotor gives us all the advantages of centrifugation in a vertical rotor, i.e., very rapid and efficient separation with minimal damage to subcellular particles, coupled with economy in the use of expensive density media, ability to fractionate small reaction volumes without undue dilution, and the extra speed resulting from the shorter times needed to pump off small gradients.

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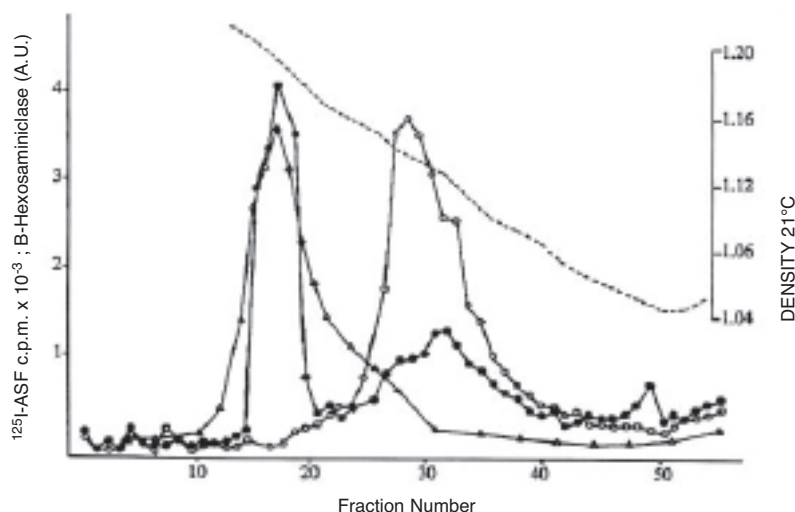


Figure 1:

Distribution of endosomal radiolabel and N-acetyl-B-glucosaminidase on a small Nycodenz gradient
 ○ - Unincubated rat liver post-mitochondrial supernatant containing labeled endosomes.
 ● - The same post-mitochondrial supernatant after incubation at 37°C with an ATP-regenerating system.
 △ - N-acetyl-B-glucosaminidase, a lysosomal enzyme.

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