Isolation of Clathrin-Coated Vesicles by Differential and Density Gradient Centrifugation

Endocytosis of nutrients, signaling receptors, and other cell surface regulatory proteins via clathrin-coated vesicles (CCVs) is essential for normal cellular function. CCVs are also involved in the transport of proteins, such as lysosomal hydrolases, from the *trans*-Golgi network to the endosomal/lysosomal system. CCVs are relatively uniform in size and, at 50 to 100 nm in diameter, they are among the smallest membranous organelles. Moreover, they are encased in a dense, proteinaceous coat, which contributes to the formation of the vesicular structure. Protocols for isolating CCVs take advantage of the small size and high density of these organelles by using differential centrifugation coupled with velocity and equilibrium gradients to separate CCVs from contaminating membranes.

The first reliable protocol for isolating CCVs was described by Pearse (1975), working with pig brain. CCVs were enriched in a microsomal fraction prepared by differential centrifugation. They were subsequently separated from larger and less dense membranes using velocity and equilibrium sedimentation, respectively, on linear sucrose gradients. Pearse (1982) later introduced a protocol (for purification of CCVs from human placenta) in which Ficoll and D_2O were substituted for sucrose. Most protocols currently in use are derived from these original protocols.

In Basic Protocol 1, the authors describe a procedure for isolating CCVs from adult rat brain. This procedure, which is based on the protocol of Maycox et al. (1992), uses differential centrifugation coupled with Ficoll and D_2O-sucrose density gradient centrifugation. The application of an additional step involving velocity sedimentation in linear sucrose gradients, as originally described by Wasiak et al. (2002), is also outlined. In Alternate Protocols 1 and 2, the authors describe how the same basic approach can be applied to the isolation of CCVs from developing rat brain and cell lines, respectively.

When applied to other tissues, such as rat liver, the steps outlined in Basic Protocol 1 yield CCV preparations that exhibit only modest enrichment and purity. In Basic Protocol 2, therefore, the authors describe a fractionation procedure that has been used to purify CCVs from rat liver. This protocol, based on that of Pilch et al. (1983), involves differential centrifugation coupled with velocity and equilibrium centrifugation using discontinuous sucrose gradients.

Following fractionation, it is necessary to characterize CCV enrichment and purity. One important method for doing so is the analysis of equal protein aliquots of the various subcellular fractions by SDS-PAGE (*UNIT 6.1*) using Coomassie blue staining and/or immunoblotting (*UNIT 6.2*) with antibodies against specific CCV proteins (e.g., clathrin). Thus, for each protocol, we indicate fractions from which aliquots should be retained for analysis. The purity of CCVs is best assessed by electron microscopy (EM). In the Support Protocol, we describe an EM procedure, based on that of Baudhuin et al. (1967), that involves filtration of the isolated vesicle fractions onto nitrocellulose filters. This procedure ensures a random sampling of membranes in the pellet fraction and is thus appropriate for quantitative evaluation of CCV purity.

NOTE: All protocols using live animals must first be approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for laboratory animal care and use.

Contributed by Martine Girard, Patrick D. Allaire, Francois Blondeau, and Peter S. McPherson
Current Protocols in Cell Biology (2005) 3.13.1-3.13.31

NOTE: All prepared solutions, glassware, centrifuge tubes, and equipment should be precooled, and samples should be kept on ice throughout unless otherwise noted.

NOTE: It is recommended that the purification of CCVs be monitored using clathrin as a marker. Each fraction that should be included in subsequent analyses is named, and the need to reserve aliquots of various fractions in screw-cap tubes is indicated throughout the protocols that follow. All reserved aliquots should be snap-frozen in liquid nitrogen and stored at −70°C.

BASIC PROTOCOL 1

ISOLATION OF CCVs FROM ADULT RAT BRAIN USING DIFFERENTIAL AND DENSITY GRADIENT CENTRIFUGATION

In this protocol, adult rat brains are homogenized in MES buffer at pH 6.5 (buffer A) using a glass-Teflon homogenizer. Figure 3.13.1 provides a flow chart summarizing the subsequent steps used to isolate CCVs. A microsomal fraction is first generated from the homogenate by performing two differential centrifugation steps. Next, the CCVs are separated from larger microsomal contaminants by velocity sedimentation through a Ficoll-sucrose solution. The CCVs are subsequently separated from less dense membranes by pelleting through a sucrose cushion prepared in D_2O. These steps represent the basic procedure as described by Maycox et al. (1992). The D_2O pellets are further fractionated on linear sucrose gradients (20% to 50%) as originally described by Wasiak et al. (2002).

Materials

10 Sprague-Dawley rats (150 to 200 g each)
1× buffer A (see recipe) containing protease inhibitors (see recipes)
Ficoll-sucrose solution (see recipe)
Deuterium oxide (D_2O; heavy water)–sucrose solution (see recipe)
20% and 50% sucrose solutions in 1× buffer A (see recipe)

Glass-Teflon homogenizers (assorted sizes; Wheaton) fitted to a power head
Sorvall high-speed centrifuge equipped with SS-34 fixed-angle rotor (or equivalent)
Battery-operated pipet filler
Ultracentrifuge with fixed-angle (Sorvall T-865, Beckman 45Ti, or equivalent) and swinging-bucket (Sorvall AH-629, Beckman SW-28, or equivalent) rotors
25-G, $\frac{5}{8}$-in. needle
13-ml thin-walled centrifuge tubes
2-mm-diameter glass capillary tubes
Two-chamber gradient maker
Peristaltic pump

Additional reagents and equipment for standard protein assays (*APPENDIX 3H*)

NOTE: Protease inhibitors are added (to a concentration of 1×) to 1× buffer A within 30 min of buffer use, except for PMSF, which should be added immediately after the buffer comes into contact with a protein sample.

Prepare a crude homogenate

1. Sacrifice 10 Sprague-Dawley rats (150 to 200 g), by decapitation or, alternatively, by CO_2 asphyxiation followed by decapitation.

   The sacrificing of rats must be supervised or carried out by an experienced animal technician according to specific animal care protocols at the investigator's institution.

2. Open the rats' skulls with scissors, and remove and weigh the brains. After weighing, transfer the brains to a beaker and add enough ice-cold 1× buffer A so that they are completely covered.

   Ten rat brains typically weigh 15 to 20 g.

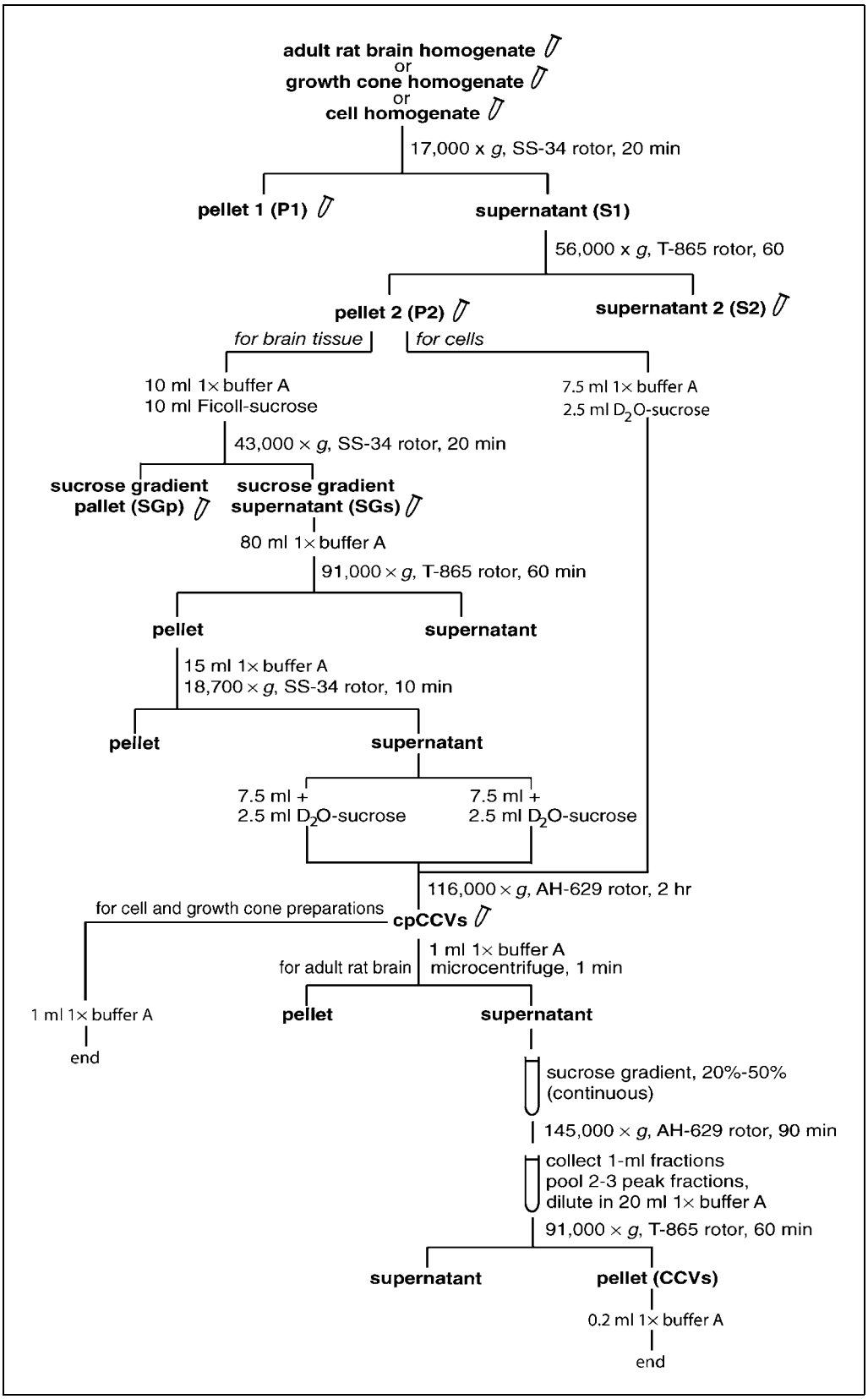

Figure 3.13.1 Flow chart summarizing Basic Protocol 1 and Alternate Protocol 2. The flow chart also covers Alternate Protocol 1 after the lysis of growth cones. The stylized microcentrifuge tube denotes fractions from which an aliquot should be retained for analysis by SDS-PAGE. CCVs, clathrin-coated vesicles; cpCCVs, cusion-pellet clathrin-coated vesicles.

3. Once all of the brains have been removed and weighed, decant and discard the 1× buffer A and transfer the brains to the mortar of a 55-ml glass-Teflon homogenizer.

   A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process all of the rat brains that have been collected.

4. Add 5 ml of 1× buffer A for every gram of rat brain in the homogenizer. Homogenize using 10 strokes of the Teflon pestle with the power head set at 1500 rpm.

5. Pool homogenates in a 250-ml graduated cylinder and add ice-cold 1× buffer A to a final volume of 10 ml for every gram of rat brain present (e.g., if 20 g of rat brain is present, add ice-cold 1× buffer A to a final volume of 200 ml). Reserve a 0.5-ml aliquot of the pooled homogenate, but do not discard the rest.

Prepare a microsomal (P2) fraction by differential centrifugation

6. Transfer pooled homogenate to six 40-ml centrifuge tubes and centrifuge 20 min at 17,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

7. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a 250-ml glass beaker on ice.

8. Resuspend one of the pellets (P1; step 6) in 10 ml of 1× buffer A by pipetting up and down using a pipet filler and pipet. Transfer the suspension to the mortar of a 35-ml glass-Teflon homogenizer. Prepare an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

9. Transfer S1 (step 7) to eight 25-ml centrifuge tubes and centrifuge 60 min at 56,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

10. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot of S2 from one of the tubes and discard the rest.

11. Combine all pellets (P2; step 9) and use a pipet filler and pipet to resuspend in a total of 10 ml of 1× buffer A.

    The head of a small Teflon homogenizer can be used to scrape the pellets out of their individual centrifuge tubes so that they can be combined.

12. Transfer the resuspended P2 pellets to the mortar of a 35-ml glass-Teflon homogenizer and prepare an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

13. Transfer the homogenized material to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the material once through the needle by slowly and evenly applying pressure to the plunger. Reserve a 0.25-ml aliquot of the P2 suspension, but do not discard the rest.

Centrifuge microsomal fraction on a Ficoll-sucrose density gradient

14. Add 10 ml ice-cold Ficoll-sucrose solution to the P2 suspension (∼10 ml) and gently mix by pipetting up and down. Transfer to a 40-ml centrifuge tubes.

15. Centrifuge 20 min at 43,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

16. Using a pipet filler and pipet, remove the supernatant (sucrose gradient supernatant, or SGs) and transfer to a 100-ml graduated cylinder.

17. Resuspend the pellet (sucrose gradient pellet, or SGp) in 5 ml of 1× buffer A by pipetting up and down. Transfer the suspension to the mortar of a 10-ml glass-Teflon

homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

18. Dilute the SGs from step 16 (20 ml) by adding 80 ml ice-cold 1× buffer A. Transfer to six 25-ml centrifuge tubes.

19. Centrifuge diluted SGs 60 min at 91,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

20. Using a pipet filler and pipet, remove and discard all supernatants.

21. Combine all pellets and use a pipet filler and pipet to resuspend in a total of 15 ml of 1× buffer A.

    The head of a small Teflon homogenizer can be used to scrape the pellets out of their individual centrifuge tubes so that they can be combined.

22. Transfer the resuspended material to the mortar of a 30-ml glass-Teflon homogenizer and homogenize using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

23. Transfer the homogenized material to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the material once through the needle by slowly and evenly applying pressure to the plunger, and collect in a 40-ml centrifuge tube.

24. Centrifuge 10 min at 18,700 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

25. Using a pipet filler and pipet, remove the supernatant (still referred to as the SGs) and transfer to a 50-ml graduated cylinder. Reserve a 0.5-ml aliquot and discard pellet.

Pellet CCVs through sucrose cushion

26. Split SGs into 2 aliquots of 7.5 ml and transfer each aliquot to a 13-ml thin-walled centrifuge tube designed for a Sorvall AH-629 swinging-bucket rotor.

27. Underlay each SGs sample with 2.5 ml D_2O-sucrose solution in the following way.

    a. Use a piece of Tygon tubing to attach a 5-ml syringe barrel to a 2-mm-diameter glass capillary tube.

    b. Load the 5-ml syringe barrel with D_2O-sucrose solution.

    c. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the centrifuge tube containing the 7.5-ml sample.

    d. Slowly eject 2.5 ml D_2O-sucrose solution into the centrifuge tube from the syringe.

28. Centrifuge samples 2 hr at 116,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

29. Using a pipet filler and pipet, remove and discard the supernatants.

30. Combine pellets (cushion-pellet CCVs, or cpCCVs) and resuspend in a total of 1 ml of 1× buffer A.

    The head of a small Teflon homogenizer can be used to scrape the pellets out of their individual centrifuge tubes so that they can be combined.

31. Transfer suspension to the mortar of a 3-ml glass-Teflon homogenizer.

32. Generate even cpCCV suspensions using 3 strokes of a Teflon pestle (from the glass-Teflon homogenizer) with the power head set at 1500 rpm. After homogenization, transfer the suspended material to a single microcentrifuge tube. Reserve a 100-µl aliquot of the homogenized suspension, but do not discard the rest.

The protocol can be stopped at this point, with the cpCCVs having been collected; if so, the cpCCV suspension can be divided into aliquots (to eliminate the need for repeated freezing/thawing in the future), transferred to screw-cap tubes, snap-frozen in liquid nitrogen, and stored at −70°C for up to 1 year. Otherwise, proceed to linear sucrose gradient fractionation.

Perform linear sucrose gradient fractionation

33. Transfer the cpCCV suspension to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the suspension once through the needle by slowly and evenly applying pressure to the plunger, and collect suspension in a microcentrifuge tube.

34. Microcentrifuge the cpCCV suspension 1 min at maximum speed, 0° to 4°C.

35. In a 13-ml thin-walled centrifuge tube designed for a Sorvall AH-629 swinging-bucket rotor, prepare a 12-ml linear gradient from equal volumes of 20% and 50% sucrose in 1× buffer A using a two-chamber gradient maker. Use a peristaltic pump to ensure an even flow rate (from the gradient maker to the centrifuge tube) of ∼2 to 3 ml/min.

36. Using a Pasteur pipet, remove the supernatant and gently layer it onto the top of the linear gradient. Discard pellet.

37. Centrifuge 90 min at 145,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

38. Collect approximately twelve fractions of 1 ml from the centrifuged gradient in the following way.

    a. Use a piece of Tygon tubing to attach a 2-mm-diameter glass capillary tube to a peristaltic pump.
    b. Gently position the exposed end of the capillary tube so that it rests on the bottom of the tube containing the centrifuged sucrose gradient.
    c. Activate the peristaltic pump and collect each fraction at a flow rate of ∼2 to 3 ml/min.

39. Use a standard protein assay (*APPENDIX 3H*) to determine which of the isolated fractions yield the highest protein signals.

    The highest protein signals should be found near the middle of the gradient (around fractions 6 to 8). The peak protein signal is directly correlated with the peak concentration of CCVs.

40. Pool the 2 to 3 fractions that yield the highest protein signals and dilute to a total volume of 20 ml in 1× buffer A. Transfer to a 25-ml centrifuge tube.

41. Centrifuge the pooled and diluted sample 60 min at 91,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

42. Decant and discard supernatant. Resuspend pellet (which contains CCVs) in 0.2 ml of 1× buffer A.

43. Transfer the resuspended CCVs to the mortar of a 1-ml glass-Teflon homogenizer and generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

44. Transfer the suspension to a screw-cap tube, snap-freeze in liquid nitrogen, and store at −70°C for up to 1 year.

    This CCV suspension can be divided into aliquots before freezing to eliminate the need for repeated freezing/thawing in the future.

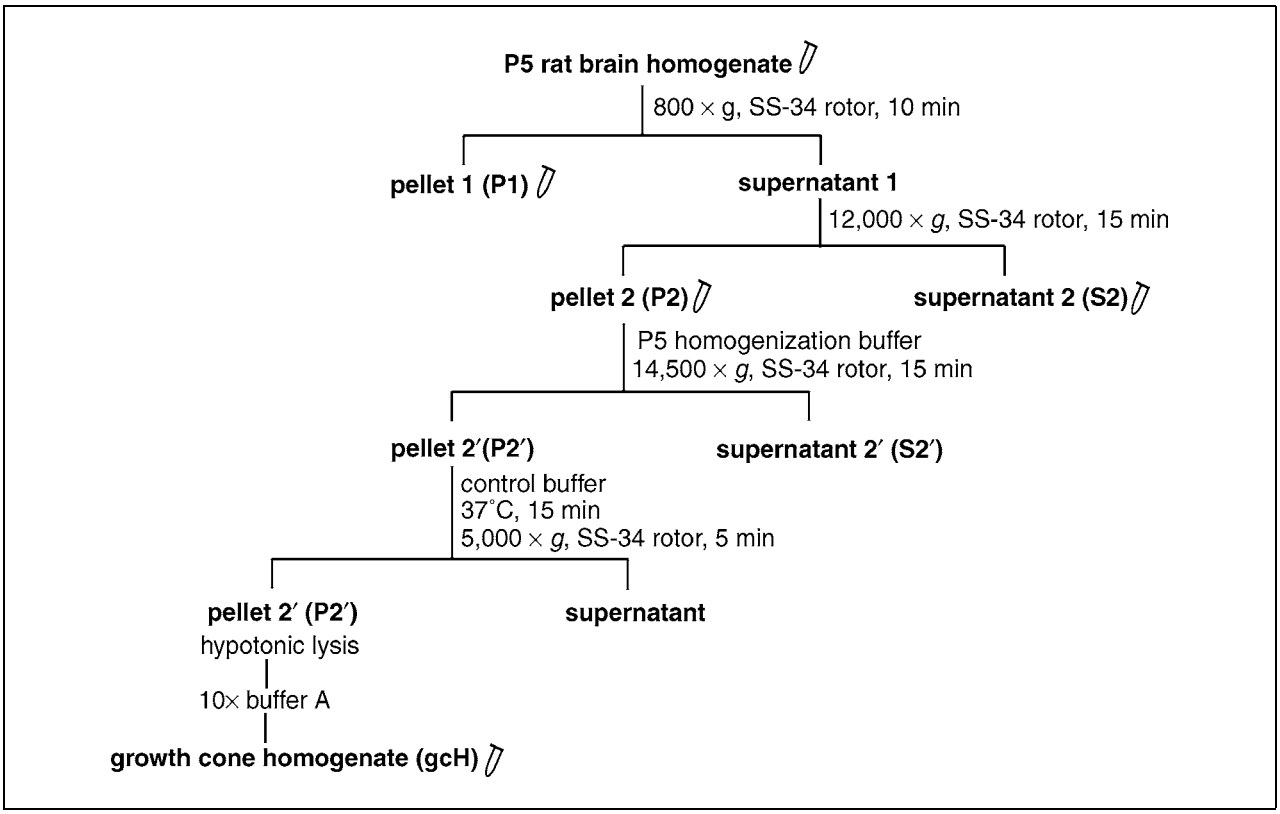

Figure 3.13.2 Flow chart summarizing the generation of crude growth cones in Alternate Protocol 1. The stylized microcentrifuge tube denotes fractions from which an aliquot should be retained for analysis by SDS-PAGE.

ISOLATION OF CCVs FROM DEVELOPING RAT BRAIN USING DIFFERENTIAL AND DENSITY GRADIENT CENTRIFUGATION

ALTERNATE PROTOCOL 1

This protocol is similar to Basic Protocol 1. It is derived from a procedure developed by Maycox et al. (1992) to isolate CCVs from synaptosomes, nerve terminals that are pinched off during homogenization. However, because Alternate Protocol 1 starts with P5 (postnatal day 5) rat brains, CCVs are being isolated primarily from growth cones, nerve terminal structures that guide growing neurites prior to synapse formation (Saito et al., 1992). Figure 3.13.2 presents a flow chart for the initial steps of the protocol. P5 rat brains are homogenized in HEPES buffer containing 0.3 M sucrose (P5 homogenization buffer). With gentle homogenization and isotonic conditions, the growth cones remain intact and are pelleted out at moderately low *g* forces. Next, the crude growth cones are lysed by hypotonic shock to release CCVs, and buffer A is added to stabilize the clathrin coats. The lysate is then subjected to a series of differential and density gradient centrifugation steps as described in Basic Protocol 1 and outlined in Figure 3.13.1.

Additional Materials *(also see Basic Protocol 1)*

15 litters of P5 rat pups
P5 homogenization buffer (see recipe) containing protease inhibitors (see recipes)
Control buffer (see recipe) containing protease inhibitors (see recipes)
10× buffer A (see recipe)

NOTE: Protease inhibitors are added (to a concentration of 1×) to P5 homogenization buffer, control buffer, and 1× buffer A within 30 min of buffer use, except for PMSF, which should be added to a given solution immediately after the solution comes into contact with protein samples.

Prepare crude growth cones

1. Sacrifice 15 litters of P5 rat pups by decapitation or, alternatively, by CO_2 asphyxiation followed by decapitation.

   The sacrificing of rats must be supervised or carried out by an experienced animal technician according to specific animal care protocols at the investigator's institution.

2. Open the rats' skulls with scissors, and remove and weigh the brains. After weighing, transfer the brains to a beaker and add enough ice-cold P5 homogenization buffer so that they are completely covered.

   In 15 litters, there will be ~200 pups total. The total wet weight of the brains collected will be ~110 g.

3. Once all brains have been removed and weighed, decant and discard the P5 homogenization buffer and transfer the brains to the mortar of a 55-ml glass-Teflon homogenizer.

   A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process all of the rat brains that have been collected.

4. Add 5 ml P5 homogenization buffer for every gram of rat brain in the homogenizer. Homogenize using 10 strokes of the Teflon pestle with the power head set at 900 rpm.

5. Pool homogenates in a 1000-ml graduated cylinder. Reserve a 0.5-ml aliquot of the pooled homogenate.

6. Transfer pooled homogenate to sixteen 40-ml centrifuge tubes and centrifuge 10 min at 800 × *g*, 0° to 4°C, in a Sorvall SS-34 (or equivalent) fixed-angle rotor.

   The SS-34 rotor fits eight tubes, so two rounds of centrifugation will be necessary.

7. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a glass beaker on ice.

   It is recommended that the supernatants be removed at the centrifuge, as transport of the tube can disturb the pellet, which is very soft.

8. Resuspend one of the pellets (P1; step 6) in 5 ml P5 homogenization buffer. Transfer the suspension to the mortar of a 10-ml glass-Teflon homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

9. Transfer S1 to sixteen 40-ml centrifuge tubes and centrifuge 15 min at 12,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

   The SS-34 rotor fits eight tubes, so two rounds of centrifugation will be necessary.

10. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot from one of the tubes and discard the rest.

11. Combine all pellets (P2; step 9) and use a pipet filler and pipet to resuspend in a total of 400 ml P5 homogenization buffer. Reserve a 0.5-ml aliquot of the P2 suspension.

12. Transfer the P2 suspension to sixteen 40-ml centrifuge tubes and centrifuge 15 min at 14,500 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

13. Using a pipet filler and pipet, remove and discard all supernatants.

14. Gently pipet 10 ml control buffer up and down over each pellet until only the soft, beige outer component of the pellet (P2′) is resuspended, leaving behind a dense, red core. Pool all of these suspensions in a 500-ml beaker and add control buffer up to a total volume of 200 ml.

The soft, beige outer component, which is weakly adherent to the walls of the centrifuge tube, contains the growth cones. The dense, red core that is left behind is mainly mitochondrial and is strongly adherent to the walls of the tube.

15. Incubate the pooled P2′ suspension 15 min in a 37°C water bath.

Lyse growth cones

16. Transfer the P2′ suspension to sixteen 40-ml centrifuge tubes and centrifuge 5 min at 5000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

17. Using a pipet filler and pipet, remove and discard all supernatants.

18. Combine all pellets and resuspend in a total of 20 ml of 1× buffer A.

19. Transfer suspension to a 500-ml graduated cylinder and add 160 ml ice-cold water. Immediately thereafter, homogenize the diluted suspension (in aliquots not exceeding the capacity of the homogenizer mortar) in a 55-ml glass-Teflon homogenizer using 10 strokes of the Teflon pestle with the power head set at 1500 rpm. Pool homogenized aliquots in a 500-ml graduated cylinder.

    All 160 ml of ice-cold water should be added at once in order to rapidly dilute the sample and lyse the growth cones.

    A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process the entire suspension.

20. Immediately after homogenization, add 20 ml of 10× buffer A to the suspension. Reserve a 0.5-ml aliquot of this suspension (referred to as the growth cone homogenate, gcH), but do not discard the rest.

    The addition of 10× buffer A stabilizes the clathrin coats that are present in the sample.

21. Proceed from Basic Protocol 1, step 6 through step 32, treating the gcH as if it were the homogenate suspension obtained in Basic Protocol 1, step 5. Reserve aliquots as directed throughout this portion of the Basic Protocol.

ISOLATION OF CCVs FROM CELL LINES

ALTERNATE PROTOCOL 2

In this protocol, as in Basic Protocol 1, cells are homogenized in 1× buffer A. As outlined in the flow chart in Figure 3.13.1, differential centrifugation is used to generate a microsomal fraction, and enrichment for CCVs is performed by centrifuging this microsomal fraction through a sucrose cushion prepared in D_2O.

Additional Materials *(also see Basic Protocol 1)*

HEK-293, COS-7, or HeLa cells
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Rubber stopper cut in half lengthwise (to yield straight edges along top and bottom)
Additional reagents and equipment for mammalian cell culture (*UNIT 1.1*)

NOTE: Protease inhibitors are added (to a concentration of 1×) to 1× buffer A within 30 min of buffer use, except for PMSF, which is added immediately after the buffer comes into contact with a protein sample.

Prepare a cell extract

1. Grow 5 dishes (diameter, 10 cm) of HEK-293, COS-7, or HeLa cells to confluence in a 37°C incubator (*UNIT 1.1*).

2. Remove confluent cells from incubator. Using a vacuum source, aspirate culture medium from cells.

3. Wash cells twice, each time by gently adding 10 ml room temperature PBS to each dish of cells and then using a vacuum source to aspirate the PBS.

4. Add 4 ml of 1× buffer A to each plate. Detach cells from plate surface by scraping the bottom of the plate with the straight edge of the cut rubber stopper (i.e., the edge where the flat face that results from cutting and the bottom face of the stopper meet).

5. Transfer cell suspensions to the mortar of a 55-ml glass-Teflon homogenizer and homogenize using 10 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the homogenate.

Prepare a microsomal (P2) fraction

6. Transfer the homogenate to one to two 40-ml centrifuge tubes and centrifuge 20 min at 17,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

7. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a glass beaker on ice.

8. Resuspend one of the pellets (P1; step 6) in 5 ml of 1× buffer A. Transfer the suspension to the glass mortar of a 10-ml glass-Teflon homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

9. Transfer S1 to two 25-ml centrifuge tubes and centrifuge 60 min at 56,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

10. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot from one of the tubes and discard the rest.

11. Combine all pellets (P2; step 9) and use a pipet filler and pipet to resuspend in a total of 7.5 ml of 1× buffer A.

12. Transfer the resuspended P2 pellets to the mortar of a 10-ml glass-Teflon homogenizer and generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

13. Transfer the homogenized material to a syringe barrel equipped with a 25-G, $\frac{5}{8}$-in. needle. Pass the suspension through the needle once by slowly and evenly applying pressure to the plunger, and collect in a 13-ml thin-walled centrifuge tube designed for a Sorvall AH-629 swinging-bucket rotor. Reserve a 0.25-ml aliquot.

Pellet CCVs through sucrose cushion

14. Underlay the sample with 2.5 ml D_2O-sucrose solution in the following way.

    a. Use a piece of rubber tubing to attach a 5-ml syringe barrel to a 2-mm-diameter glass capillary tube.

    b. Load the 5-ml syringe barrel with D_2O-sucrose solution.

    c. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the centrifuge tube containing the 7.5-ml sample.

    d. Slowly eject 2.5 ml D_2O-sucrose solution into the centrifuge tube from the syringe.

15. Centrifuge sample 2 hr at 116,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

16. Using a pipet filler and pipet, remove and discard the supernatant. Resuspend the pellet (CCVs) in 0.25 ml of 1× buffer A.

17. Transfer the resuspended CCV pellet to the mortar of a 1-ml glass-Teflon homogenizer and generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm.

18. Transfer the suspension to a screw-cap tube, snap-freeze in liquid nitrogen, and store at −70°C for up to 1 year.

    This CCV suspension can be divided into aliquots before freezing to eliminate the need for repeated freezing/thawing in the future.

ISOLATION OF CCVs FROM ADULT RAT LIVER USING DIFFERENTIAL AND DENSITY GRADIENT CENTRIFUGATION

BASIC PROTOCOL 2

In this protocol, adult rat livers are homogenized in HEPES buffer, pH 7.4, containing 0.25 M sucrose (liver homogenization buffer) using a glass-Teflon homogenizer. Figure 3.13.3 provides a flow chart summarizing the subsequent steps used to isolate CCVs. First, a microsomal fraction is obtained from the homogenate by two differential centrifugation steps. (MES buffer, pH 6.5, is added after the first centrifugation step in order to stabilize the clathrin coat.) Next, CCVs are separated from larger microsomal contaminants by velocity sedimentation through discontinuous sucrose gradients. The CCVs are subsequently separated from less dense membranes by equilibrium sedimentation on discontinuous sucrose gradients, and they can be further purified by performing an additional velocity sedimentation step.

Basic Protocol 2 is based on the procedure described by Pilch et al. (1983), as modified from Blitz et al. (1977).

Materials

Ten Sprague-Dawley rats (150 to 200 g)
Liver homogenization buffer (see recipe) containing protease inhibitors (see recipes)
10× and 1× MES buffers (see recipes) containing protease inhibitors (see recipes)
5%, 10%, 20%, 30%, 40%, 45%, 50%, and 55%, and 60% (w/v) sucrose solutions in 1× MES buffer (see recipe)
Glass-Teflon homogenizers (assorted sizes; Wheaton) fitted with power heads
Sorvall high-speed centrifuge equipped with SS-34 fixed-angle rotor (or equivalent)
Battery-operated pipet filler
Ultracentrifuge with fixed-angle (Sorvall T-865, Beckman 45Ti, or equivalent) and swinging-bucket (Sorvall AH-629, Beckman SW-28, or equivalent) rotors
16- and 36-ml polypropylene tubes for Sorvall AH-629 (or equivalent) swinging-bucket rotor
2-mm-diameter glass capillary tubes

NOTE: Protease inhibitors are added to liver homogenization buffer and 1× MES buffer within 30 min of buffer use, except for PMSF, which is added to a given solution immediately after the solution comes into contact with protein samples.

Prepare crude homogenate

1. Starve 10 Sprague-Dawley rats overnight. After overnight starvation, sacrifice rats by decapitation or, alternatively, by CO_2 asphyxiation followed by decapitation.

   The starvation and sacrificing of rats must be supervised or carried out by an experienced animal technician in accordance with specific animal care protocols at the investigator's institution.

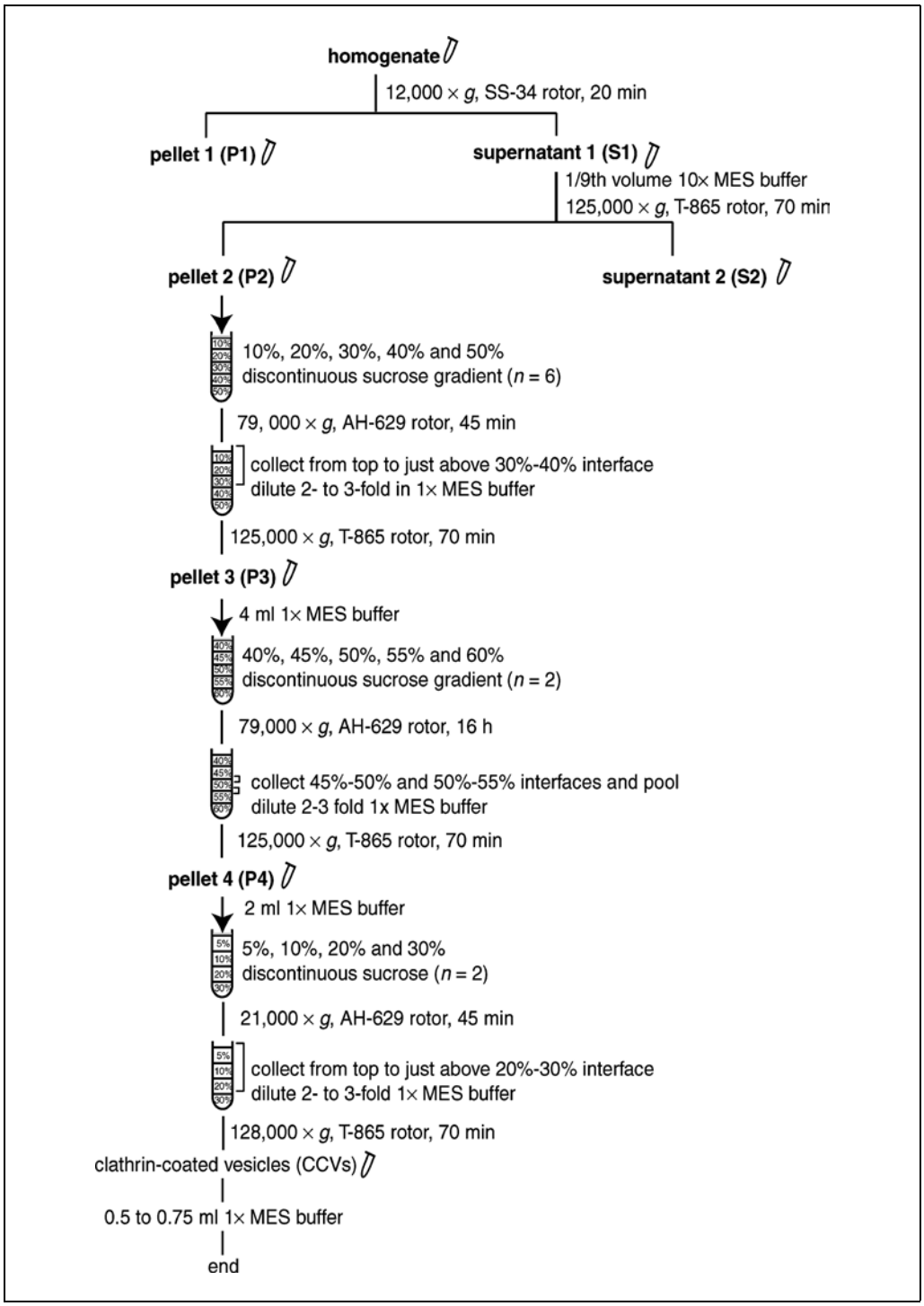

Figure 3.13.3 Flow chart summarizing Basic Protocol 2. The stylized microcentrifuge tube denotes fractions from which an aliquot should be retained for analysis by SDS-PAGE.

2. Open the rats' abdominal cavities and remove their livers. Transfer the livers to a chilled beaker containing ~100 ml liver homogenization buffer.
3. Once all brains have been removed and weighed, decant and discard the liver homogenization buffer. Cut the livers into small pieces with scissors and weigh.

   Ten rat livers typically weigh 60 to 100 g.

4. Transfer the liver tissue to the mortar of a 55-ml glass-Teflon homogenizer.

   A large glass-Teflon homogenizer generally has a capacity of 50 ml, so it is likely that multiple homogenizations will be required to process all of the rat liver tissue that has been collected.

5. Add 2 ml liver homogenization buffer for every gram of rat liver in the homogenizer. Homogenize using 10 to 15 strokes of the Teflon pestle with the power head set at 1500 rpm.

6. Pool homogenates in a 500-ml graduated cylinder and add ice-cold liver homogenization buffer to a final volume of 4 ml for every gram of rat liver present (e.g., if 100 g of rat liver is present, add ice-cold liver homogenization buffer to a final volume of 400 ml). Reserve a 0.5-ml aliquot of the pooled homogenate, but do not discard the rest.

Prepare a microsomal (P2) fraction by differential centrifugation

7. Transfer the pooled homogenates to eight to ten 25-ml centrifuge tubes and centrifuge 20 min at 12,000 × *g*, 0° to 4°C, in a Sorvall SS-34 fixed-angle rotor.

8. Using a pipet filler and pipet, remove the supernatant (S1) from each tube, being careful not to disturb the pellet, and combine all supernatants in a 500-ml graduated cylinder on ice.

9. Resuspend one of the pellets (P1; step 7) in 25 ml of 1× MES buffer. Transfer the suspension to the mortar of a glasss-Teflon homogenizer. Generate an even suspension using 5 to 10 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.5-ml aliquot of the suspension and discard the rest.

10. Add 1/9th volume of 10× MES buffer to the pooled S1 from step 8. Reserve a 0.5-ml aliquot.

11. Transfer the pooled S1 (with MES buffer added) to eight to ten 25-ml centrifuge tubes and centrifuge 70 min at 125,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

    The SS-34 rotor fits eight tubes, so two rounds of centrifugation may be necessary.

12. Using a pipet filler and pipet, remove the supernatant (S2) from each tube. Reserve a 0.5-ml aliquot from one of the tubes and discard the rest.

13. In the following way, combine the pellets (P2; step 11) and resuspend them in a maximum of 30 ml of 1× MES buffer.

    a. Scrape the pellets out of their centrifuge tubes using the head of a small Teflon homogenizer (adding a combined total of 15 to 20 ml of 1× MES buffer to the tubes to facilitate pellet removal), and transfer the pellets (along with the added 1× MES buffer) to a chilled beaker. Resuspend the transferred pellets using a 1-ml pipet tip.

    b. Add a combined total of 10 to 15 ml of 1× MES buffer to the tubes from which the P2 pellets were removed, use the head of a small Teflon homogenizer to scrape the remnants of the pellets from the walls of these tubes, and then transfer the contents of the tubes to the chilled beaker from substep a.

       This procedure ensures maximum recovery of the P2 pellet, which is very thick.

14. Transfer the P2 suspension to the mortar of a 30-ml glass-Teflon homogenizer. Generate an even suspension using 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.25-ml aliquot of the homogenized P2 suspension.

Isolate CCVs from P2 microsomes

15. Prepare six discontinuous sucrose gradients (sucrose concentrations: 10%, 20%, 30%, 40%, and 50%; 5 ml per layer), with each gradient being constructed in the following way.

    a. To a 36-ml polypropylene tube designed for a Sorvall AH-629 swinging-bucket rotor, add 5 ml of a 10% sucrose solution.

    b. Use a piece of Tygon tubing to attach a 10-ml syringe barrel to a 2-mm-diameter glass capillary tube.

       Alternatively, a syringe equipped with a blunt-end pipetting needle can be used to dispense the gradient solutions.

    c. Load the syringe barrel with the 20% sucrose solution.

    d. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the 36-ml polypropylene tube in which the sucrose gradient is being constructed.

    e. Slowly eject 5 ml of the 20% sucrose solution. Discard the sucrose solution remaining in the syringe barrel.

    f. Repeat substeps c to e three times, first using a 30% sucrose solution, then using a 40% sucrose solution, and finally using a 50% sucrose solution.

    g. Mark all interfaces with an indelible marker.

       It is important to mark all interfaces with an indelible marker, as this will facilitate sample recovery.

16. Using a Pasteur pipet, gently layer 5 ml of the P2 suspension on top of each of the six discontinuous sucrose gradients prepared in step 15.

17. Centrifuge gradients 45 min at 79,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

18. For each gradient, using a Pasteur pipet and starting from the top of the tube, collect all of the liquid down to (but not including) the interface between the 30% and 40% sucrose solutions. Transfer collected liquid to a 250-ml graduated cylinder.

19. Add sufficient 1× MES buffer to dilute the collected sample 2- to 3-fold.

20. Transfer diluted sample to eight to twelve 25-ml centrifuge tubes and centrifuge 70 min at 125,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

21. Decant and discard all supernatants. Resuspend pellets (P3) in a total of 4 ml of 1× MES buffer and combine.

    The head of a small Teflon homogenizer can be used to scrape the pellets from their individual centrifuge tubes so that they can be combined.

22. Transfer P3 suspension to the mortar of a 10-ml glass-Teflon homogenizer and generate an even suspension using 3 to 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.25-ml aliquot of the P3 suspension.

23. Prepare two discontinuous sucrose gradients (sucrose concentrations: 40%, 45%, 50%, 55%, and 60%; 5.5 ml per layer) using the procedure outlined in step 15.

    Note that the gradients constructed in this step differ from those constructed in step 15 only in terms of their layer volumes and sucrose concentrations.

24. Using a Pasteur pipet, layer 2 ml of the P3 suspension on top of each of the two discontinuous sucrose gradients.

25. Centrifuge gradients 16 hr at 79,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

26. From each gradient, collect the interface between the 45% and 50% sucrose solutions and the interface between the 50% and 55% sucrose solutions in the following way.

    a. Using a Pasteur pipet and starting from the top of the tube, remove and discard all of the contents of the tube down to a point just above the interface between the 45% and 50% sucrose solutions.

    b. Gently remove the material at the interface between the 45% and 50% sucrose solutions and transfer it to a glass beaker on ice.

    c. Continue by removing and discarding the contents of the tube down to a point just above the interface between the 50% and 55% sucrose solutions.

    d. Gently remove the material at the interface between the 50% and 55% sucrose solutions and add it to the material from the interface between the 45% and 50% sucrose solutions.

    e. Determine the volume of the collected material using a pipet filler and pipet, and then return the material to the glass beaker.

27. Dilute the sample 2- to 3-fold with 1× MES buffer.

28. Transfer diluted sample to four to eight 25-ml centrifuge tubes and centrifuge 70 min at 125,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

29. Decant and discard supernatants. Resuspend pellets (P4) in a total of 2 ml of 1× MES buffer and combine.

    The head of a small Teflon homogenizer can be used to scrape the pellets from their individual centrifuge tubes so that they can be combined.

30. Transfer P4 suspension to the mortar of a 10-ml glass-Teflon homogenizer and generate an even suspension using 3 to 5 strokes of the Teflon pestle with the power head set at 1500 rpm. Reserve a 0.25-ml aliquot of the P4 suspension.

31. Prepare two discontinuous sucrose gradients (sucrose concentrations: 5%, 10%, 20%, and 30%; 3.0 ml per layer), with each gradient being constructed in the following way.

    a. To a 16-ml polypropylene tube designed for a Sorvall AH-629 swinging-bucket rotor, add 3.0 ml of a 5% sucrose solution.

    b. Use a piece of Tygon tubing to attach a 10-ml syringe barrel to a 2-mm-diameter glass capillary tube.

        Alternatively, a syringe equipped with a blunt-end pipetting needle can be used to dispense the gradient solutions.

    c. Load the syringe barrel with the 10% sucrose solution.

    d. Gently position the exposed end of the attached capillary tube so that it rests at the bottom of the 16-ml polypropylene tube in which the sucrose gradient is being constructed.

    e. Slowly eject 3.0 ml of the 10% sucrose solution. Discard the remaining sucrose solution in the syringe barrel.

f. Repeat substeps c to e twice, first using a 20% sucrose solution and then using a 30% sucrose solution.

g. Mark all interfaces with an indelible marker.

It is important to mark all interfaces with an indelible marker, as this will facilitate sample recovery.

32. Using a Pasteur pipet, layer 1 ml of the P4 suspension on top of each of the two discontinuous sucrose gradients.

33. Centrifuge gradients 45 min at 21,000 × *g*, 0° to 4°C, in a Sorvall AH-629 swinging-bucket rotor.

34. For each gradient, using a Pasteur pipet and starting from the top of the tube, collect the contents of the tube down to a point just above the interface between the 20% and 30% sucrose solutions. Transfer collected material to a 100-ml graduated cylinder.

35. Dilute the sample 2- to 3-fold with 1× MES buffer.

36. Transfer diluted sample to two to four 25-ml centrifuge tubes and centrifuge 70 min at 128,000 × *g*, 0° to 4°C, in a Sorvall T-865 fixed-angle rotor.

37. Decant and discard supernatants. Using a pipettor, resuspend pellets (CCVs) in a total of 0.5 to 0.75 ml of 1× MES buffer and combine.

38. Transfer the suspension to a screw-cap tube, snap-freeze in liquid nitrogen, and store at −70°C for up to 1 year.

This CCV suspension can be divided into aliquots before freezing to eliminate the need for repeated freezing/thawing in the future.

SUPPORT PROTOCOL

ANALYSIS OF CCV PURITY BY ELECTRON MICROSCOPY

This protocol is derived from the method of Baudhuin et al. (1967). It is specifically designed to analyze subcellular particles generated by centrifugation and to ensure random sampling throughout a membrane pellet. Isolated membrane fractions that have been enriched for CCVs are fixed using glutaraldehyde and then applied to Millipore filters, generating very thin pellicles of packed particles. The CCVs and contaminating membranes in these samples are further fixed with osmium tetroxide and stained with tannic acid and uranyl acetate (Simionescu and Simionescu, 1976), after which the filters are embedded in Epon for EM analysis.

Materials

Fresh CCVs in 1× buffer A or 1× MES buffer (see Basic Protocol 1, Basic Protocol 2, Alternate Protocol 1, or Alternate Protocol 2)
1× buffer A (see recipe), filtered
1× MES buffer (see recipe), filtered (use only if CCVs were prepared using Basic Protocol 2)
25% (w/v) glutaraldehyde solution
Nitrogen gas
Sodium cacodylate buffer (see recipe), 4°C
Osmium tetroxide solution (see recipe), 4°C
Tannic acid solution (see recipe), 4°C
Sodium sulfate solution (see recipe), 4°C
Uranyl acetate solution (see recipe), 4°C, filtered
Maleate buffer (see recipe), 4°C

50%, 70%, 90%, 95%, and 100% (all v/v) ethanol
Propylene oxide
Epon solution (see recipe)
3:1, 1:1, and 1:3 (all w/w) Epon solution (see recipe)/propylene oxide
Platform rocker
Filter unit (Baudhuin et al., 1967; also see annotation to step 3), including 13-mm-diameter Millipore filter discs (pore size, 0.22 μm)
Glass vial
Small paraffin-coated polypropylene containers (Peel-A-Way disposable embedding molds; Polysciences) with (see recipe) and without a coating of hardened Epon on the inside bottom surface

NOTE: Osmium tetroxide, glutaraldehyde, and propylene oxide must be handled and disposed of in accordance with protocols approved by Institutional Chemical Safety and Chemical Waste Committees. Uranyl acetate is radioactive and therefore must be handled and disposed of in accordance with protocols approved by an Institutional Radiation Safety Committee, as well as in accordance with protocols approved by Institutional Chemical Safety and Chemical Waste Committees.

NOTE: All steps involving propylene oxide should be performed in a fume hood, as this compound is highly volatile.

Fix sample in glutaraldehyde

1. Add 0.2 mg of CCV fractions (as measured using a standard protein assay; *APPENDIX 3H*) to a microcentrifuge tube. Dilute to a final volume of 0.9 ml in ice-cold, filtered 1× buffer A or ice-cold, filtered 1× MES buffer, depending on which buffer the CCV fractions are suspended in.

2. Add 100 μl of ice-cold 25% glutaraldehyde solution. Incubate sample overnight at 4°C with gentle rocking.

   Once the glutaraldehyde solution has been added, the sample can be kept at 4°C for up to 72 hr.

   The authors have only processed fresh CCV fractions using this protocol; thus, they perform steps 1 and 2 immediately following the preparation of CCVs.

Filter sample

3. Wash the filter unit with double-distilled water.

   The filter unit is a custom-built apparatus modeled on the one described by Baudhuin et al. (1967). In brief, a Millipore filter is placed between two Teflon O-rings and then seated on top of a support screen at mid-height in a stainless steel centrifuge tube, and a Plexiglas chamber with a cylindrical opening of matching diameter is fitted to the top of this tube. After the sample of interest has been applied to the filter, the Plexiglas chamber can be closed with a plug (held securely to the Plexiglas chamber by a screw) that allows the filter unit to be connected to a tank of nitrogen gas.

4. Install a 13-mm-diameter Millipore filter (pore size, 0.22 μm) in the filter unit.

5. Apply 1× buffer A to the filter, and adjust the nitrogen flow rate such that 2 ml of the buffer passes through the filter in 30 sec.

6. Apply the fixed sample from step 2 to the filter.

7. Using a Pasteur pipet, gently layer 4 ml of 1× buffer A on top of the fixed sample.

   The 1× buffer A must be layered on top of the fixed sample very gently. The difference in density between the 1× buffer A and the fixed sample prevents mixing of the two layers.

8. Close the filter unit and apply constant nitrogen pressure (using the flow rate determined in step 5). When 2 ml of liquid has passed through the filter (as estimated by eye), cut off the nitrogen flow and open the filter unit.

   Allowing 2 ml of liquid to pass through the filter will ensure that the fixed membranes are collected on the filter while also ensuring that the filter does not dry out. As membranes collect on the filter, the rate at which liquid passes through will decrease to as low as 2 ml every 20 to 30 min.

9. Add 2 to 4 ml ice-cold sodium cacodylate buffer to a glass vial kept at 4°C. Using forceps, transfer the filter to this glass vial such that the sample side faces up.

   In this step and all subsequent steps, take care to ensure that the filter does not dry out.

10. Wash the filter three times, each time by adding 2 to 4 ml ice-cold cacodylate buffer, incubating 15 min at 4°C, and then removing the buffer.

    To avoid damaging the filter, add and remove the cacodylate buffer gently.

    All wash and incubation times presented here are intermediate recommended times and can be changed by ~30% without affecting sample preparation.

Fix sample in osmium tetroxide

11. After removing the last cacodylate buffer wash, add 2 ml ice-cold osmium tetroxide solution. Incubate 1 hr at 4°C, and then remove the solution.

12. Wash the filter three times, each time by adding 2 ml ice-cold cacodylate buffer, incubating 10 min at 4°C, and then removing the buffer.

Treat with tannic acid

13. After removing the last cacodylate buffer wash, add 2 ml ice-cold tannic acid solution. Incubate 1 hr at 4°C, replacing the old 2-ml aliquot of tannic acid solution with a new 2-ml aliquot every 20 min. At the end of the incubation period, remove all of the tannic acid solution.

14. Wash the filter twice, each time by adding 2 ml ice-cold sodium sulfate solution, incubating 10 min at 4°C, and then removing the solution.

Treat sample with uranyl acetate

15. After removing the last sodium sulfate wash, add 2 ml ice-cold, filtered uranyl acetate solution. Incubate in the dark for 2 hr at 4°C, and then remove the solution.

    Precipitate particles will appear as dark spots when the sample is viewed under EM. Thus, it is critical that the uranyl acetate solution be passed through a 0.22-μm filter prior to use. It is also critical that the sample be kept in the dark throughout the course of the incubation, as uranyl acetate will precipitate if it is exposed to light.

16. Wash the filter twice, each time by adding 2 to 4 ml ice-cold maleate buffer, incubating 10 min at 4°C, and then removing the buffer.

    During these two washes, handle the sample under the assumption that it is contaminated with uranyl acetate.

17. Add 2 to 4 ml ice-cold maleate buffer. Incubate overnight at 4°C, and then remove the buffer.

Dehydrate sample

18. Add 2 to 4 ml of ice-cold 50% ethanol to the vial containing the filter. Incubate 10 min at 4°C.
19. Replace the 50% ethanol with 2 to 4 ml of ice-cold 70% ethanol. Incubate 10 min at 4°C.
20. Replace the 70% ethanol with 2 to 4 ml of ice-cold 90% ethanol. Incubate 10 min at 4°C.
21. Replace the 90% ethanol with 2 to 4 ml of ice-cold 95% ethanol. Incubate 10 min at 4°C, and then remove the ethanol solution.
22. Wash the filter twice more, each time by adding 2 to 4 ml of ice-cold 95% ethanol to the vial, incubating 10 min at 4°C, and then removing the ethanol solution.
23. Add 2 to 4 ml of ice-cold 100% ethanol to the vial. Incubate 10 min at 4°C, and then remove the ethanol.
24. Wash the filter once by adding 2 to 4 ml of ice-cold 100% ethanol to the vial, incubating 10 min at 4°C, and then removing the ethanol.

Treat sample with propylene oxide

25. Wash the filter once more by adding 2 to 4 ml of ice-cold 100% ethanol to the vial and incubating 10 min at 4°C. At the end of the 10-min incubation period, fill a small, paraffin-coated polypropylene (Peel-A-Way) disposable embedding mold with propylene oxide and transfer the filter to this mold.

    Do not transfer the filter to an empty mold and then add propylene oxide, as this will cause the filter to fragment.

26. Incubate the filter in propylene oxide for 1 to 2 hr at room temperature.

    Propylene oxide is highly volatile. Therefore, the propylene oxide in the mold will need to be replenished periodically to replace any liquid that has evaporated over the course of the incubation.

    Incubation in propylene oxide makes the filter extremely fragile, and in many cases, the filter will break into pieces as a result. In the remaining steps, the filter should be treated very gently, so as to minimize fragmentation.

Embed samples

27. Gently pour the filter and propylene oxide into a Peel-A-Way embedding mold whose inside bottom surface has been coated with a layer of hardened Epon.
28. Replace the propylene oxide solution in the Epon-coated mold with 2 to 4 ml of 1:3 (w/w) Epon solution/propylene oxide. Incubate 1 hr at room temperature.
29. Replace the 1:3 Epon solution/propylene oxide with 2 to 4 ml of 1:1 (w/w) Epon solution/propylene oxide. Incubate 1 hr at room temperature.
30. Replace the 1:1 Epon solution/propylene oxide with 2 to 4 ml of 3:1 (w/w) Epon solution/propylene oxide. Incubate 1 hr at room temperature.
31. Replace the 3:1 Epon solution/propylene oxide with 2 to 4 ml Epon solution. Incubate 76 hr at 60°C to generate a hard block surrounding the sample.
32. Trim the block, and then section and analyze using standard EM protocols (Baudhuin et al., 1967).

REAGENTS AND SOLUTIONS

Use deionized distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Buffer A

For 10× stock

To 80 ml H_2O, add:
19.52 g 2-(*N*-morpholino)ethanesulfonic acid (MES; 1 M final)
5 ml of 200 mM ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA; 10 mM final)
0.25 ml of 2 M $MgCl_2$ (5 mM final)
Adjust to pH 6.5 with 10 N NaOH
Add H_2O to 100 ml
Store up to 4 weeks at 4°C

For 1× working solution

To 400 ml H_2O, add 50 ml of 10× buffer A
Adjust to pH 6.5 with 1 N NaOH if necessary
Add H_2O to 500 ml
Store up to 2 days at 4°C

To prevent the appearance of dust or other particles in the sample to be analyzed, pass 1× buffer A through a 0.22-μm filter before use.

Control buffer

To 400 ml H_2O, add:

10 ml of 1 M HEPES, pH 7.4 (20 mM final)
16 ml of 4 M NaCl (128 mM final)
0.6 ml of 2.5 M KCl (3 mM final)
0.3 ml of 2 M $MgCl_2$ (1.2 mM final)
0.05 ml of 1 M $CaCl_2$ (0.1 mM final)
18.8 g glucose (11 mM final)
Add H_2O to 500 ml
Store up to 24 hr at 4°C

D_2O-sucrose solution

To 5 ml deuterium oxide (D_2O; heavy water), add:

0.8 g sucrose (8% w/v final)
1 ml of 10× buffer A (see recipe)
Add D_2O to 10 ml
Prepare fresh and use immediately

Epon solution

12.6 g Epon 812 resin (specific density, 1.256 g/cm^3; MECA Lab)
6.7 g dodecenylsuccinic anhydride (specific density, 1.024g/cm^3; MECA Lab)
6.6 g nadic methyl anhydride (specific density, 1.237 g/cm^3; MECA Lab)
0.3 g 2,4,6-tris[(dimethylamino)methyl]phenol (DMP30; specific density, 0.977 g/cm^3; MECA Lab)
Make solution fresh and use immediately

With the exception of DMP30, the components used to make Epon are very viscous and thus hard to measure by volume. Therefore, in the preparation of Epon, measurement of these components by weight is recommended.

The recipe provided here yields 26.2 g Epon, which is sufficient for the preparation of four samples in the Support Protocol.

Ficoll-sucrose solution

To 20 ml of 1× buffer A (see recipe), add:
3.75 g sucrose (12.5% w/v final)
3.75 g Ficoll 400 (12.5% w/v final)
Add 1× buffer A to 30 ml
Stir or gently rock until sucrose and Ficoll 400 are completely dissolved
Prepare fresh and use immediately

Liver homogenization buffer

To 800 ml H_2O, add:
10 ml of 1 M HEPES, pH 7.4 (10 mM final)
85.6 g sucrose (0.25 M final)
Adjust to pH 7.4 if necessary
Add H_2O to 1000 ml
Store up to 24 hr at 4°C

Maleate buffer

To 400 ml H_2O, add:
4 g NaOH
11.6 g maleic acid (50 mM final)
Adjust pH to 6 with 1 N HCl
Add H_2O to 500 ml
Store up to 3 months at 4°C

MES buffer

For 10× stock
To 150 ml H_2O, add:
39.04 g MES (1 M final)
20 ml of 200 mM EGTA (20 mM final)
1.5 ml of 2 M $MgCl_2$ (15 mM final)
Adjust to pH 6.5 with 10 N NaOH
Add H_2O to 200 ml
Store up to 4 weeks at 4°C

For 1× working solution
To 400 ml H_2O, add 50 ml of 10× MES buffer
Adjust to pH 6.5 with 1 N NaOH
Add H_2O to 500 ml
Store up to 2 days at 4°C

Osmium tetroxide solution

To 1 ml of 4% (w/v) osmium tetroxide, add (in the order presented):
2 ml of 3% (w/v) potassium ferrocyanide
1 ml of 0.2 M sodium cacodylate buffer (see recipe)
Prepare fresh and use immediately

P5 homogenization buffer

To 800 ml H_2O, add:
10 ml of 1 M HEPES, pH 7.4 (10 mM final)
102.9 g sucrose (0.3 M final)
Add H_2O to 1000 ml
Store up to 24 hr at 4°C

Peel-A-Way disposable embedding mold with Epon-coated inside bottom surface

Add just enough Epon solution (see recipe) to cover the bottom of a Peel-A-Way disposable embedding mold (Polysciences). Incubate 76 hr at 60°C.

Protease inhibitors

Aprotinin, 10,000× stock:
To 1 ml H_2O, add 5 mg aprotinin powder (5 mg/ml final)
Store up to 6 months at −20°C
Benzamidine, 100× stock:
To 100 ml H_2O, add 1 g benzamidine powder (64 mM final)
Pass solution through a 0.22-μm filter
Store up to 6 months at 4°C
Leupeptin, 10,000× stock:
To 1 ml H_2O, add 5 mg leupeptin powder (5 mg/ml final)
Store up to 6 months at −20°C
Phenylmethylsulfonyl fluoride (PMSF), 500× stock:
To 100 ml of 100% ethanol, add 1 g PMSF powder (10 mg/ml final)
Store up to 3 months at room temperature

Sodium cacodylate buffer

To 400 ml H_2O, add:
21.4 g sodium cacodylate (0.2 M)
Adjust pH to 7.4 with 1 N HCl
Add H_2O to 500 ml
Store up to 3 months at 4°C
Dilute 1:1 with H_2O immediately prior to use in the Support Protocol (final sodium cacodylate concentration, 0.1 M)

Sodium sulfate solution

To 80 ml of 0.1 M sodium cacodylate buffer (see recipe), add 1 g sodium sulfate (1% w/v final). Add 0.1 M sodium cacodylate buffer to 100 ml. Prepare fresh and use immediately.

Sucrose solution, 20% or 50% (w/v), in 1× buffer A

To a 50-ml conical tube containing 25 ml of 1× buffer A (see recipe), add 10 g (20% final) or 25 g (50% final) sucrose. Add 1× buffer A to 50 ml. Stir or gently rock until sucrose is completely dissolved. Prepare fresh and use immediately.

Sucrose solutions in 1× MES buffer

Add the following amounts of sucrose to individual 50-ml conical tubes, each containing 25 ml of 1× MES buffer (see recipe).
2.5 g (5% final)
5 g (10% final)
10 g (20% final)
15 g (30% final)
20 g (40% final)
22.5 g (45% final)
25 g (50% final)
27.5 g (55% final)
Add 1× MES buffer to 50 ml
Prepare fresh and use immediately

Tannic acid solution

To 80 ml of 0.1 M sodium cacodylate buffer (see recipe), add:
1 g tannic acid (1% w/v final)
0.1 M sodium cacodylate buffer to 100 ml
Prepare fresh and use immediately

Uranyl acetate solution

To 100 ml H_2O, add:
25 ml maleate buffer (see recipe)
2 g uranyl acetate (2% w/v final)
Store up to 3 months at 4°C in the dark

To prevent the appearance of dust or other particles in the sample to be analyzed, pass uranyl acetate solution through a 0.22-μm filter prior to use.

COMMENTARY

Background Information

Homogenization and differential centrifugation

CCVs are generally purified in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffers at mildly acidic pH, as originally described by Pearse (1975). These buffers usually contain $MgCl_2$ and EDTA, although the inclusion of these chemicals appears to be primarily a matter of convention (Daiss and Roth, 1983). MES buffers are used at mildly acidic pH, as the stability of the clathrin coat is sensitive to pH. Clathrin coats are also sensitive to protonated amines, and Tris-containing buffers are therefore avoided (Keen et al., 1979). In fact, Tris-containing buffers are routinely used to strip clathrin coats following the isolation of CCVs; this technique is useful in determining whether a protein of interest is associated with the coat or with the vesicle fraction. Moreover, Tris-mediated stripping of clathrin from isolated CCVs is often used in the purification of clathrin. Keen et al. (1979) describe protocols for stripping clathrin coats from CCVs using Tris and other buffers.

Due to their small size, CCVs are resistant to shear forces, and high–shear force methods of homogenization are therefore acceptable (Daiss and Roth, 1983). Protocols originally described by Pearse (1975) used a Waring blender for the homogenization of pig brain tissue. Subsequent protocols for isolating CCVs from placental and lymphoma cells (Pearse, 1976; Pearse, 1982), calf brain (Blitz et al., 1977), rat liver (Pilch et al., 1983), and bovine adrenal cortex (Mello et al., 1980) used similar homogenization techniques. The protocols presented in this unit call for a Wheaton glass-Teflon homogenizer, as previously described by Maycox et al. (1992).

For adult brain and cultured cells, homogenization is performed directly in 100 mM MES, pH 6.5, containing 1 mM $MgCl_2$ and 0.5 mM EDTA (buffer A). The homogenizer power head is set at 1500 rpm, and the pestle is pushed through the sample rapidly and forcefully. These conditions are sufficient to lyse cultured cells and, in the case of brain tissue, to lyse nerve terminals as well as cell somata. When P5 rat brain samples are used, CCVs are isolated from crude growth cones using a protocol derived from the one described by Maycox et al. (1992) for purifying CCVs from synaptosomes. Thus, the homogenization step in that protocol is designed to lyse the somata of neurons but to leave growth cones (or synaptosomes) intact. To this end, homogenization is performed in isotonic sucrose using a glass-Teflon homogenizer. The power head is set at 900 rpm, and the pestle is passed through the sample slowly, using little force. Thus, populations of CCVs and other membrane components, as well as soluble proteins, are trapped inside the growth cones or synaptosomes. Spins of moderate force (12,000 to 14,500 × *g* in sucrose, 5000 × *g* in isotonic saline) are sufficient to pellet these membranes, whereas CCVs and other microsomal components from cell bodies are not pelleted at such *g* forces. After separation from membranes, the growth cones/synaptosomes are washed of peripherally associated vesicles and lysed using hypotonic shock. In Basic Protocol 2, homogenization is performed as in Basic Protocol 1, except that a neutral-pH homogenization buffer containing 0.25 M sucrose is initially employed, with MES buffer (100 mM

MES, pH 6.5; 2 mM $MgCl_2$; 1.5 mM EGTA) being added to the supernatant from the first centrifugation step.

In each protocol, the first step of purification involves differential centrifugation to generate crude microsomes, which are enriched for CCVs. Thus, the homogenates (or lysed growth cones, when P5 brain samples are used) are centrifuged at moderate *g* forces (12,000 to 17,000 × *g*), and the supernatants are collected and centrifuged at 56,000 to 125,000 × *g* (Figs. 3.13.1 to 3.13.3). These steps reduce contamination by nuclei, mitochondria, and other large organelles, such that the majority of CCV contamination comes from vesiculated endoplasmic reticulum, Golgi bodies, membranes of the endosomal/lysosomal system, and other transport vesicles, including synaptic vesicles when brain tissue samples are used.

Density gradients

The various forms of density gradient centrifugation employed in these protocols all take advantage of the small size and high density of CCVs to separate these vesicles from contaminating membranes present in the crude microsomal fractions. Pearse (1975) developed the first protocols of this type. In those protocols, microsomal membranes were applied to 5%-to-60% linear sucrose gradients and centrifuged at 50,000 × *g* for 2 hr (Pearse, 1975). Under velocity sedimentation conditions such as these, organelles will separate based on their size, and CCVs, because of their small size, have a low sedimentation rate. Pearse (1975) found that CCVs banded near the middle of the gradient, with larger contaminating membranes appearing at the bottom of the gradient. Once the CCVs were recovered by pelleting, they were centrifuged for 16 hr at 50,000 × *g* through a linear 20%-to-60% sucrose gradient (Pearse, 1975). Under equilibrium sedimentation conditions such as these, organelles have time to reach their isopycnic points and are thus separated based primarily on their densities. Because of their protein coat, CCVs have a high density (1.20 to 1.25 mg/ml; Daiss and Roth, 1983). Thus, as observed by Pearse (1975), they equilibrate at ~50% to 55% sucrose. Many of the small microsomal vesicles not removed in the velocity gradient step will equilibrate at lower sucrose concentrations and remain above the CCVs in the equilibrium gradient. Basic Protocol 2 takes advantage of these principles using an equilibrium sedimentation step sandwiched between two velocity sedimentation steps on discontinuous sucrose gradients.

One potential problem with sucrose density gradients is that osmotic stress encountered by an organelle at high sucrose concentrations can cause partial disruption of membranes. In fact, it was reported that the sucrose density gradient procedures originally described by Pearse (1975) caused partial disruption of CCVs, with some loss of contents (Pearse, 1982). Pearse (1982) thus introduced techniques for purifying CCVs using Ficoll and D_2O gradients. Maycox et al. (1992) adapted these procedures for the purification of CCVs from rat brain. As described in Basic Protocol 1 and Alternate Protocol 1, microsomal membranes are first fractionated by centrifugation in a Ficoll-sucrose solution in which the sucrose concentration is 12.5%. Centrifugation at 43,000 × *g* for 20 min leads to pelleting of larger microsomal membranes, while CCVs remain in the supernatant. The CCVs are then collected by centrifugation and pelleted through a cushion of 8% sucrose in D_2O. Only dense CCVs are pelleted under these conditions. This step is particularly important when working with brain tissue, since synaptic vesicles, which are abundant in brain, are major contaminating organelles in microsomal fractions. Maycox et al. (1992) were able to determine that these Ficoll-sucrose and D_2O-sucrose centrifugation steps were effective in separating CCVs from synaptic vesicles.

EM analysis of CCVs

Negative-stain electron microscopy (EM) is used routinely to examine CCV purity following subcellular fractionation (Pearse, 1975). Typically, a drop of a resuspended pellet is placed on a carbon-coated copper grid and negatively stained with 1% uranyl acetate, as originally described by Huxley (1963). However, the purity of CCVs may be overestimated using this procedure, as protein-coated vesicles are selectively retained on the grid (Daiss and Roth, 1983). Specimens prepared by fixing pellets prior to embedding and sectioning give a more faithful representation of the purity of CCVs (Daiss and Roth, 1983). However, as noted by Baudhuin et al. (1967), when particles in a given preparation have differing sedimentation coefficients or densities, the distribution of those particles within a pellet will be far from homogeneous. Thus, in the Support Protocol, we describe a procedure for analyzing CCV purity that is based on protocols developed by Baudhuin et al. (1967) and Simionescu and Simionescu (1976). Resuspended CCV fractions are fixed in glutaraldehyde and then passed through Millipore

nitrocellulose filters using nitrogen pressure (Baudhuin et al., 1967). This generates very thin pellicles of packed particles, with fraction heterogeneity solely in the direction perpendicular to the filter surface. The vesicles on the filters are then processed using osmium tetroxide and tannic acid treatments prior to staining with uranyl acetate (Simionescu and Simionescu, 1976). This procedure better preserves membranes as the samples go through the dehydration and embedding steps and results in increased contrast, especially for membranes, on EM (Simionescu and Simionescu, 1976). Taking pictures of large numbers of random EM fields and determining the percentages of CCVs in these fields should yield an unbiased estimate of CCV purity (Blondeau et al., 2004). However, it is possible that the Support Protocol leads to underestimation of CCV purity, since CCVs are small, and larger membranes may be preferentially retained on the filters.

Critical Parameters and Troubleshooting

Tissue source

The species of animal used for CCV preparations is an important consideration. The authors have isolated CCVs exclusively from rat. Rat is a convenient laboratory source of tissue, and the animals are easy to manipulate experimentally; for example, CCVs can be prepared from tissues following the injection of drugs or receptor ligands of interest. Moreover, rat proteins are more likely to be reactive with a broad range of commercial or investigator-generated antibodies. The authors recently performed a proteomic analysis of CCVs isolated from rat brain, with that study being aided by the relative completeness of rat databases (Blondeau et al., 2004). However, CCVs have been successfully isolated from a variety of species. For example, CCVs have also been isolated from the brains of rabbits, cows, and pigs (Pearse, 1975; Blitz et al., 1977; Woods et al., 1978); one advantage of using these species is that large amounts of tissue can be obtained. The use of human tissues is limited, given the relatively large amount of starting material required, although CCVs have been prepared from human placenta (Pearse, 1982).

Another advantage to using rat is that an extensive array of frozen tissues are available. The protocols described here use fresh tissue. However, the authors have also used commercially available frozen tissue from adult rat brain and liver. Based on Coomassie blue staining and immunoblot analysis of subcellular fractions, no obvious differences between fresh and frozen tissue in terms of the protein profiles of the CCVs isolated have been observed. However, Daiss and Roth (1983) have noted that fresh tissue yields a better product for studies of the assembly and disassembly of clathrin coats.

Vesicle isolation

Overall, the protocols presented here are reliable and reproducible. However, there are several points to note. Given the importance of mildly acidic pH to the stability of clathrin coats, the pH of each buffer should be checked immediately prior to starting the preparations. Tissues other than brain should be minced before homogenizing with a glass-Teflon homogenizer. During homogenization, the glass mortar should be held in such a way that the investigator does not warm the tissue with his or her hands. Also, when starting with adult brain synaptosomes (Maycox et al., 1992) or P5 growth cones (Alternate Protocol 1), the pestle should be moved through the sample with limited force in order to minimize shear forces. For the lysis of these structures, water should be added quickly to ensure hypotonic shock, and the pestle should then be passed through the samples forcefully.

Ensure that pellets are completely scraped from the walls of centrifuge tubes. The head of a Teflon pestle is useful in this regard, and the authors generally rinse the walls of the tube with the resuspension buffer as well. Certain pellets, as noted in the protocols, are very soft, and it is important to remove the supernatant from these pellets at the centrifuge in order to avoid disturbing the pellet. Step gradients are easy to prepare, and with some practice, linear gradients can also be generated reproducibly using a conventional two-chamber gradient maker. When using high concentrations of sucrose, ensure that the solution in the front chamber of the gradient maker (i.e., the chamber with the exit port) is adequately stirred. An automatic gradient maker (for example, the BioComp system) that generates linear gradients following the layering of the densest and lightest solutions in a centrifuge tube can also produce reliable gradients.

EM analysis

When following the Support Protocol for the analysis of CCVs by EM, it is important to remember that several of the reagents are somewhat dangerous and must be handled with care. In addition, as noted in the annotation

to step 26, the filter becomes translucent and very fragile once treated with propylene oxide. Thus, it must be handled carefully to avoid losing sample. Even if the filter does fragment, keep all of the pieces that show a spot of brown color (the sample). Finally, do not exceed the recommended nitrogen flow rate, as doing so can cause the filter to collapse.

Anticipated Results

Basic Protocol 1

Using 10 rat brains with a combined wet weight of 15 to 20 g, Basic Protocol 1 yields approximately 0.5 mg of CCVs. The protocol can be stopped following the D_2O-sucrose cushion step (step 32), yielding 1 mg of cushion-pellet CCVs (cpCCVs). Figure 3.13.4A demonstrates the enrichment of clathrin heavy chain (CHC; as seen on Coomassie blue staining and immunoblotting) going from the crude homogenate to the cpCCV fraction. Other components of the clathrin coat, including the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor complexes, as well as the clathrin light chain (CLC), are readily detectable in the cpCCV fractions. Cosedimentation of these components is seen when the cpCCVs are fractionated by velocity sedimentation on linear 20%-to-50% sucrose gradients (Fig. 3.13.4B). These patterns are highly reproducible and can be used to easily and rapidly assess the quality of the preparation.

Figure 3.13.4C shows an EM image (Support Protocol) of CCVs following linear sucrose gradient centrifugation. When randomly chosen EM images from multiple preparations were counted, cpCCVs and CCVs were found to represent 64.9% ± 1.2% and 72.7% ± 1.7% (mean ± SD; $n = 3$ CCV preparations), respectively, of all membrane profiles (Blondeau et al., 2004). On sensitive tandem mass spectrometric analysis, CCVs prepared using this protocol were found to be essentially free of contamination by endoplasmic reticula, nuclei, mitochondria, Golgi bodies, endosomes, peroxisomes, and lysosomes (Blondeau et al., 2004).

Alternate Protocols 1 and 2

The Coomassie blue staining pattern of retained fractions from P5 rat growth cone CCV (gcCCV) preparations (Alternate Protocol 1) is shown in Figure 3.13.5A. As with adult CCV preparations, clathrin coat components, including α-, β-, γ-, and μ-adaptin and CLCs, are readily detectable in the gcCCVs. Immunoblotting for CHC in the same fractions shows clathrin enrichment (Fig. 3.13.5A). An EM image of gcCCVs is presented in Figure 3.13.5B. Counting of randomly sampled fields revealed that these vesicle fractions were 79% ± 2.3% (mean ± SD; $n = 4$ CCV preparations) pure. The exact same protocol applied to adult rat brain synaptosomes yields similar clathrin enrichment (data not shown). The authors routinely process the brains of P5 pups from ~15 litters (combined weight of brain tissue, ~110 g); doing so yields ~0.3 mg of gcCCVs.

Rothman and Fine (1980) have developed a protocol for the purification of CCVs from cultured CHO cells using the procedure of Pearse (1975). This procedure requires approximately 40 g of packed cells, corresponding to 24 liters of CHO cells in culture (Rothman and Fine, 1980). The procedure for the isolation of CCVs described in Alternate Protocol 2 is valuable, because it can be performed using as few as five plates of cells. The protocol can thus easily be applied to transfected cells to obtain valuable information regarding the mechanisms by which a given protein associates with CCVs (see Metzler et al., 2001, for example). This protocol, which leads to membranes that are significantly enriched for clathrin (Metzler

Figure 3.13.4 *(at right)* Summary of results from Basic Protocol 1. (**A**) Analysis of adult rat brain fractions on a Coomassie-stained gel (top) and on immunoblot with a clathrin heavy chain (CHC) antibody (bottom). The migratory positions of CHC, clathrin light chains (CLCs), and the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor protein complexes are indicated. P1, P2, and S2 are fractions obtained before isolation of the final clathrin-coated vesicle (CCV) fraction; see Basic Protocol 1 for additional details. (**B**) Cushion-pellet CCVs (cpCCVs) were prepared and fractionated on linear 20%-to-50% sucrose gradients, and 1-ml fractions were collected from the bottom of each gradient as described in Basic Protocol 1. Proteins from aliquots of fractions 1 through 11 were separated by SDS-PAGE and analyzed by Coomassie blue staining. The migratory positions of CHC, CLCs, and the α-, β-, γ-, and μ-adaptin subunits of AP-1 and AP-2 are indicated. (**C**) Fractions that yielded peak protein signals (i.e., CCV fractions; see Basic Protocol 1) were analyzed by EM as described in the Support Protocol. A representative image is shown. H, crude homogenate; SGp, suspension of pellet from Ficoll-sucrose density gradient centrifugation; SGs, supernatant from Ficoll-sucrose density gradient centrifugation.

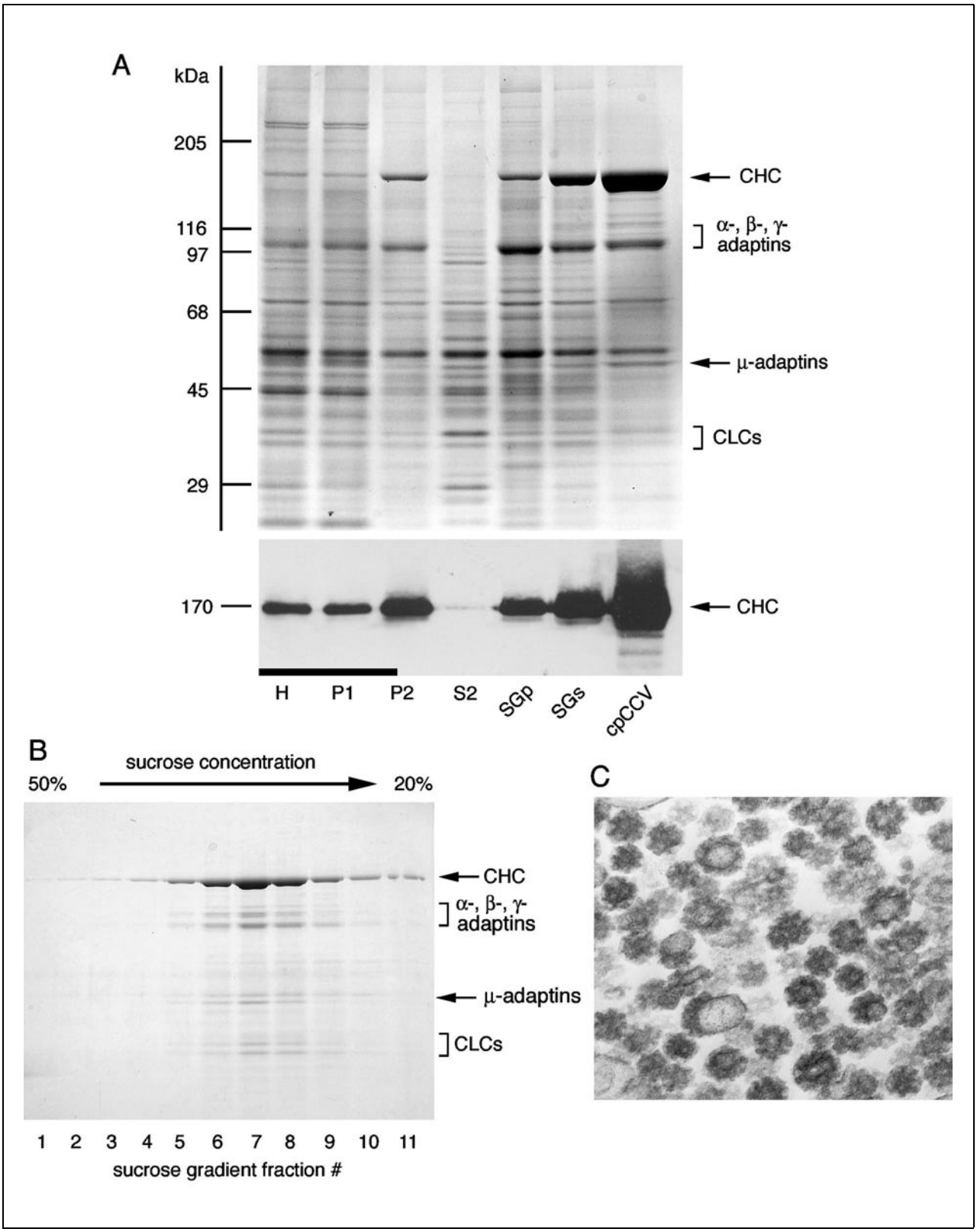

Figure 3.13.4 Legend at left.

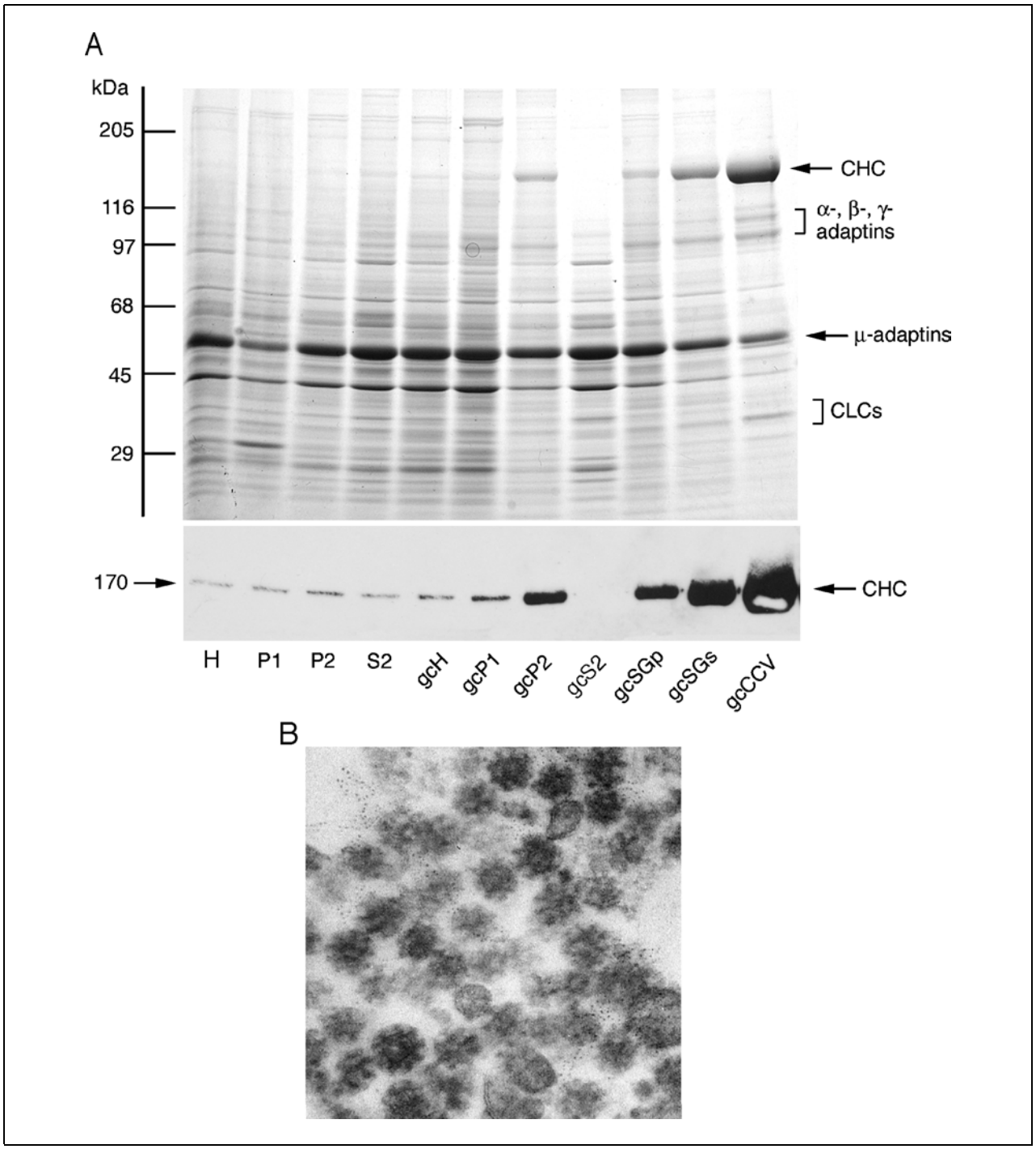

Figure 3.13.5 Summary of results from Alternate Protocol 1. (**A**) Analysis of P5 rat brain fractions on a Coomassie-stained gel (top) and on immunoblot with a clathrin heavy chain (CHC) antibody (bottom). The migratory positions of CHC, clathrin light chains (CLCs), and the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor protein complexes are indicated. P1, P2, S2, gcP1, gcP2, and gcS2 are fractions obtained before isolation of the final clathrin-coated vesicle (CCV) fraction; see Alternate Protocol 1 for additional details. (**B**) Growth cone clathrin-coated vesicles (gcCCVs) were analyzed by EM as described in the Support Protocol. A representative image is shown. H, crude homogenate; gcH, growth cone homogenate; gcSGp, suspension of pellet from Ficoll-sucrose density gradient centrifugation; gcSGs, supernatant from Ficoll-sucrose density gradient centrifugation.

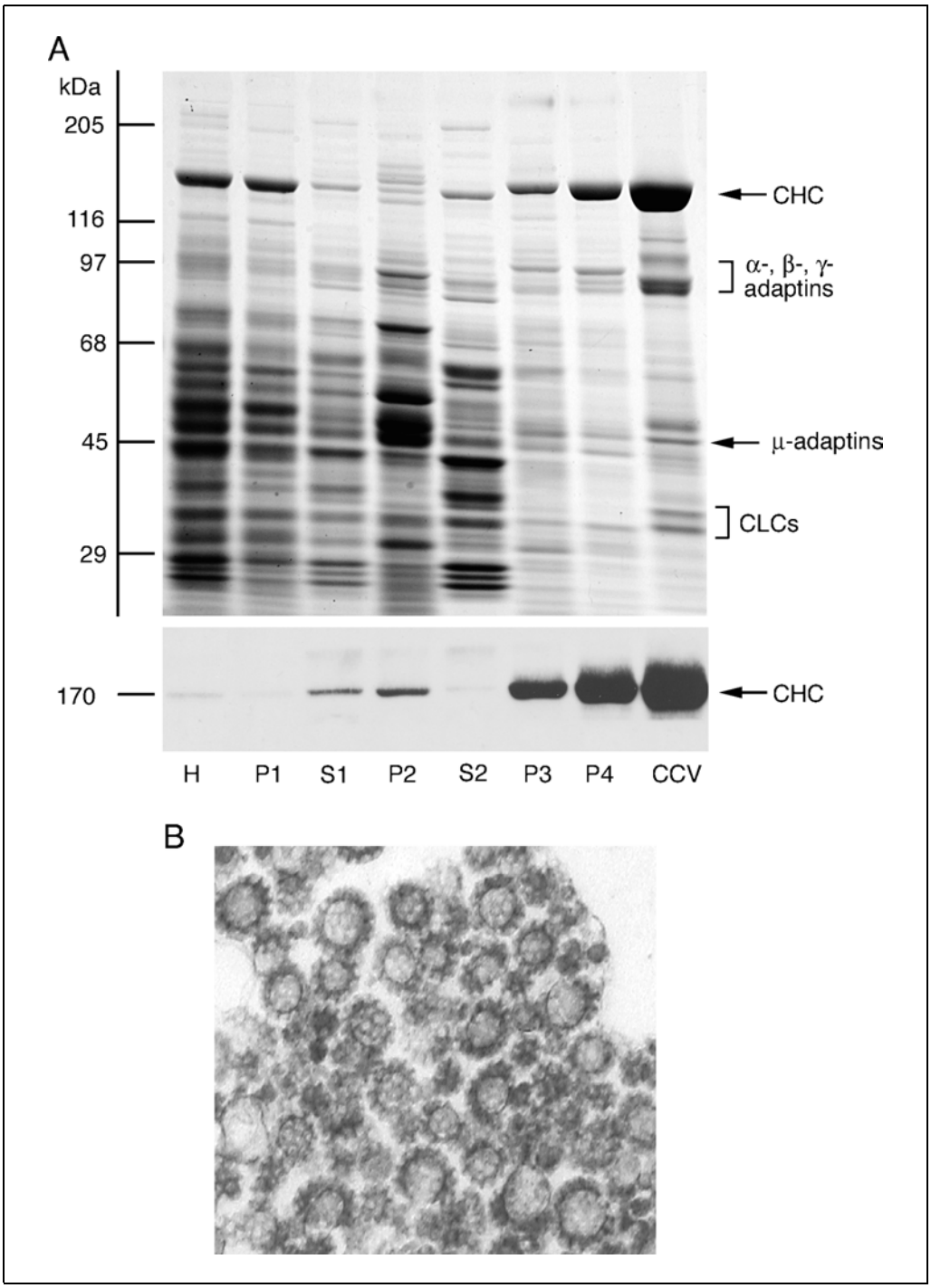

Figure 3.13.6 Summary of results from Basic Protocol 2. (**A**) Analysis of adult rat liver fractions on a Coomassie-stained gel (top) and on immunoblot with a clathrin heavy chain (CHC) antibody (bottom). The migratory positions of CHC, clathrin light chains (CLCs), and the α-, β-, γ-, and μ-adaptin subunits of the AP-1 and AP-2 clathrin adaptor protein complexes are indicated. P1, S1, P2, S2, P3, and P4 are fractions obtained before isolation of the final clathrin-coated vesicle (CCV) fraction; see Basic Protocol 2 for additional details. (**B**) Liver CCVs were analyzed by EM as described in the Support Protocol. A representative image is shown. H, crude homogenate.

et al., 2001), has been applied to COS-7 and HeLa cells, as well as HEK-293 cells. Five plates of confluent cells should yield ~0.5 mg of relatively crude CCVs.

Basic Protocol 2

Basic Protocol 2 starts with 10 rat livers (combined wet weight, 60 to 100 g) and yields 2 to 3 mg of CCVs. Figure 3.13.6A shows a Coomassie blue–stained gel of the fractions from a typical preparation. Major components of CCV coats, including CHCs, CLCs, and the various subunits of the AP-1 and AP-2 protein complexes, are easily detected in the CCV fraction. Enrichment for CHC throughout the preparation is monitored by Coomassie staining and immunoblotting (Fig. 3.13.6A). EM analysis of the CCV fraction revealed a nearly homogeneous CCV population (Fig. 3.13.6B), and random sampling of EM images showed that the fraction had a CCV purity of 89.2% ± 3.2% (mean ± SD; $n = 6$ CCV preparations).

Basic Protocol 2, which closely resembles the protocol originally described by Pearse (1975), should be applicable to the isolation of CCVs from a wide variety of tissues. The authors have used it to purify CCVs from rat kidney and brain, and they have observed similar enrichment for CHC in both cases. Similar or identical protocols have been used successfully to isolate CCVs from rabbit and calf brain (Blitz et al., 1977), human placenta (Pearse, 1982), lymphoma and CHO cells (Pearse, 1976; Rothman and Fine, 1980), and adrenal medulla and cortex (Pearse, 1976; Mello et al., 1980).

Time Considerations

There is no point at which any of the CCV purification protocols can be stopped and then resumed. Basic Protocol 1 will take ~14 hr, although this is shortened to ~10 hr if the last linear sucrose gradient step is omitted. Basic Protocol 2 takes ~35 hr. However, this includes a 16-hr (overnight) equilibrium sucrose gradient centrifugation. Basic Protocol 2 is thus very manageable over a 2-day period. Alternate Protocol 2 is the most straightforward and can be completed in ~6 hr, including the 2-hr centrifugation step. The most problematic protocol in terms of time management is Alternate Protocol 1. This protocol takes ~16 hr but contains no extended centrifugation steps. An important consideration with regard to this protocol is the time required to sacrifice and dissect brains from nearly 200 P5 rat pups.

The Support Protocol requires 6 days, not including the time needed for sectioning and EM observation. However, this time estimate includes 2 overnight incubations and 1 incubation of 76 hr. Days 2 and 3 both require hands-on time, with day 2 requiring ~8 hr for filtration and multiple wash steps.

Literature Cited

Baudhuin, P., Evrard, P., and Berthet, J. 1967. Electron microscopic examination of subcellular fractions: I: The preparation of representative samples from suspensions of particles. *J. Cell Biol.* 32:181-191.

Blitz, A.L., Fine, R.E., and Toselli, P.A. 1977. Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. *J. Cell Biol.* 75:135-147.

Blondeau, F., Ritter, B., Allaire, P.D., Wasiak, S., Girard, M., Hussain, N.K., Angers, A., Legendre-Guillemin, V., Roy, L., Boismenu, D., Kearney, R.E., Bell, A.W., Bergeron, J.J., and McPherson, P.S. 2004. Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. *Proc. Natl. Acad. Sci. U.S.A.* 101:3833-3838.

Daiss, J.L. and Roth, T.F. 1983. Isolation of coated vesicles: Comparative studies. *Methods Enzymol.* 98:337-349.

Huxley, H.E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. *J. Mol. Biol.* 77:281-308.

Keen, J.H., Willingham, M.C., and Pastan, I.H. 1979. Clathrin-coated vesicles: Isolation, dissociation and factor-dependent reassociation of clathrin baskets. *Cell* 16:303-312.

Maycox, P.R., Link, E., Reetz, A., Morris, S.A., and Jahn, R. 1992. Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. *J. Cell Biol.* 118:1379-1388.

Mello, R.J., Brown, M.S., Goldstein, J.L., and Anderson, R.G. 1980. LDL receptors in coated vesicles isolated from bovine adrenal cortex: Binding sites unmasked by detergent treatment. *Cell* 20:829-837.

Metzler, M., Legendre-Guillemin, V., Gan, L., Chopra, V., Kwok, A., McPherson, P.S., and Hayden, M.R. 2001. HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. *J. Biol. Chem.* 276:39271-39276.

Pearse, B.M. 1975. Coated vesicles from pig brain: Purification and biochemical characterization. *J. Mol. Biol.* 97:93-98.

Pearse, B.M. 1976. Clathrin: A unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 73:1255-1259.

Pearse, B.M. 1982. Coated vesicles from human placenta carry ferritin, transferrin, and immunoglobulin G. *Proc. Natl. Acad. Sci. U.S.A.* 79:451-455.

Pilch, P.F., Shia, M.A., Benson, R.J., and Fine, R.E. 1983. Coated vesicles participate in the receptor-mediated endocytosis of insulin. *J. Cell Biol.* 96:133-138.

Rothman, J.E. and Fine, R.E. 1980. Coated vesicles transport newly synthesized membrane glycoproteins from endoplasmic reticulum to plasma membrane in two successive stages. *Proc. Natl. Acad. Sci. U.S.A.* 77:780-784.

Saito, S., Fujita, T., Komiya, Y., and Igarashi, M. 1992. Biochemical characterization of nerve growth cones isolated from both fetal and neonatal rat forebrains: The growth cone particle fraction mainly consists of axonal growth cones in both stages. *Brain Res. Dev. Brain Res.* 65:179-184.

Simionescu, N. and Simionescu, M. 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy: I: Procedure, and evidence for mordanting effect. *J. Cell Biol.* 70:608-621.

Wasiak, S., Legendre-Guillemin, V., Puertollano, R., Blondeau, F., Girard, M., de Heuvel, E., Boismenu, D., Bell, A.W., Bonifacino, J.S., and McPherson, P.S. 2002. Enthoprotin: A novel clathrin-associated protein identified through subcellular proteomics. *J. Cell Biol.* 158:855-862.

Woods, J.W., Woodward, M.P., and Roth, T.F. 1978. Common features of coated vesicles from dissimilar tissues: Composition and structure. *J. Cell Sci.* 30:87-97.

Contributed by Martine Girard,
Patrick D. Allaire, Francois Blondeau,
and Peter S. McPherson
Montreal Neurological Institute
McGill University
Montreal, Quebec, Canada