Instability of coated vesicles in concentrated sucrose solutions

(clathrin/preparation of coated vesicles/membrane fluorescent probe/reassociation of coated vesicles)

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ABSTRACT A method of preparing homogeneous coated vesicles that eliminates the high sucrose concentrations heretofore used is presented. It is shown that sucrose at high concentrations dissociates the coat from coated vesicles. This reaction can explain the presence of empty coats observed with preparations obtained with high concentrations of sucrose. The protein and membrane lipid components have been analyzed by the intrinsic tryptophan and extrinsic diphenylhexatriene fluorescence, respectively. Analysis of mixtures of coated vesicles and baskets resolved the contributions of the two species to the fluorescence curves.

The role of coated pit regions of plasma membranes in receptor-mediated endocytosis (of lipoproteins, glycoproteins, and peptide and nonpeptide hormones) has been well documented (1–9). These regions also serve as a means of recycling membrane after transmitter release at the neuromuscular junction (10). Coated pits and coated vesicles (CVs) have been shown to be involved in secretory processes (11, 12) and in intracellular protein transfer (13).

Cellular processes involving coated structures are readily observable by electron micrography because the coat possesses a characteristic polygonal structure. Pearse isolated the protein responsible for the membrane coat from coated vesicles and called it clathrin (14, 15). Three types of polygonal coat structures were identified by Pearse and colleagues (16). Kanaseki and Kadota (17) suggested and later Heuser (18) inferred from an electron micrographic study that modifications in the organization of clathrin in coated pits resulted in their transition to CVs.

CVs have been prepared by most investigators by following the method of Pearse (14). An important step in the purification was centrifugation in a continuous sucrose gradient that reached 60%. We now report that exposure of coated vesicles to high sucrose concentrations results in the dissociation of the coat protein from the membrane. The CVs that are recovered when the sucrose is removed may be vesicles that are formed from the mixture of membranes and clathrin present in concentrated sucrose solutions.

We have prepared CVs by modifying various steps in the Pearse procedure (14) and using a $^1H_2O/^2H_2O$-8% sucrose step gradient instead of a 20–60% continuous sucrose gradient. This step gradient separates the CVs from other less dense membranous structures but does not dissociate them. Higher yields of more homogeneous CVs are obtained in less time than previously found by the Pearse method. In contrast, CVs prepared by using 5–60% sucrose gradients contain a large percent of clathrin baskets, which are presumably formed from dissociated coat protein after removal of sucrose (16, 19).

MATERIALS AND METHODS

Materials. 2-(N-Morpholino)ethanesulfonic acid (Mes) and EGTA came from Sigma; analytical grade magnesium chloride, sodium azide, and tetrahydrofuran were from Fisher. Sucrose was from Bethesda Research Laboratories, and 1,6-diphenylhexatriene (DPH) and Gold Label ^2H_2O were from Aldrich.

Isolation of CVs. A 1,600-g sample of defatted bovine brain was homogenized with an equal volume of 0.10 M Mes buffer, pH 6.5, containing 1 mM EGTA, 0.50 mM $MgCl_2$, and 3 mM NaN_3 (henceforth referred to as homogenization buffer) in a Waring blender with three 10-sec bursts at maximum speed, according to the procedure of Pearse (14). The homogenate was centrifuged for 50 min at 20,000 × g and the supernatant (≈2,000 ml) was spun in 24 tubes at 100,000 × g (34,000 rpm in a Beckman 35 rotor) for 1 hr to obtain a crude vesicle pellet (≈1 ml). The pellet in each tube was suspended in the homogenization buffer (≈15 ml per pellet) by using either a Dounce or a Polytron homogenizer for 5 sec at low speeds. The suspension was centrifuged at 10,000 × g for 10 min to eliminate aggregated material and the supernatant was centrifuged at 140,000 × g (37,000 rpm in a Beckman 45 Ti rotor) for 1 hr. The pellet in each tube was suspended in the homogenization buffer (≈35 ml per pellet) and the low-speed and high-speed centrifugations were repeated. The pellet obtained after the high-speed centrifugation was resuspended in 90 ml of buffer and centrifuged at 10,000 × g for 10 min.

High-sucrose preparation. About 10 ml of the above supernatant was placed on a discontinuous sucrose gradient of 32%, 40%, 48%, 53%, and 60% sucrose (28 ml). After cooling to 5°C solutions were centrifuged for 18–20 hr at an average of 75,000 × g (27,000 rpm in a Beckman SW 27 rotor). A white band of CVs appearing at 48–50% sucrose was collected, diluted with 3 vol of homogenization buffer, and then centrifuged at 120,000 × g (Beckman 65 rotor) for 1 hr. The pellet of CVs was suspended in homogenization buffer and centrifuged at 120,000 × g for 1 hr. The pellet was stored in homogenization buffer at 5°C and resuspended when needed.

Low-sucrose preparation. Five to six milliliters of supernatant was layered over 6–7 ml of an 8% sucrose ^2H_2O solution (having the same buffer salts as in homogenization buffer) and centrifuged at about 80,000 × g (28,000 rpm in a Beckman SW 40 or SW 41 rotor) for 2 hr at 20°C. The pellet containing CVs was collected, washed, and resuspended in the homogenization buffer. The suspension was spun at 20,000 × g for 10 min to

eliminate heavy particulate material and the supernatant was used for further experiments. The pellet could also be stored at 5°C in this condition for several weeks before resuspension and clarification.

It should be noted that there is a considerable saving in time and effort in the low-sucrose preparation, principally due to the difference in the number and duration of sucrose gradient centrifugations. Pearse sediments the crude CV pellets on three separate sucrose gradients for 2 hr, 16 hr, and 1 hr (14). These three steps have been condensed into one sucrose gradient step of 8% sucrose in ^2H_2O, which lasts only 2 hr. Pearse mentions that the processing of 100 g of tissue took about 28 hr (14). Our time would be closer to 6–7 hr for a similar quantity of tissue.

From 1,600 g of brain processed at a time, we have obtained 5.9 mg of protein [analysis by the Lowry method (20) with serum albumin as a standard] in CVs prepared from 100 g of tissue. Pearse has reported a value of 3.1 mg of protein in CVs from 100 g of pig tissue (14). In the Pearse preparations, the vesicles were not pure because they contained a significant percentage of baskets (16, 19).

Sucrose Density Gradient Analysis. Continuous sucrose density gradients were prepared from either 10% and 30% or 10% and 60% sucrose solutions in homogenization buffer. Small volumes of 1 mM DPH in tetrahydrofuran was added to the sucrose solutions to give a final DPH concentration of 1 μM. Sucrose solutions containing DPH were stirred vigorously for 30–60 min before the gradients were made. CVs were made in 1 μM DPH and then incubated for more than an hour before they were layered on the sucrose gradient.

Membrane Lipid and Protein Analysis. The presence of protein and membrane lipid in the sucrose gradient fractions was monitored by measuring the fluorescence of tryptophan (excitation, 280 nm; emission, 340 nm) and DPH (excitation 366 nm; emission, 430 nm), respectively. DPH has been used as a fluorescent label to study the viscosity of membranes of different cell types. We have shown that the fluorescence of DPH increases in the presence of CVs or uncoated vesicles but does not change in the presence of protomer clathrin (8S) or clathrin baskets. We are therefore able to distinguish between clathrin baskets, which are devoid of membrane, and CVs. The emission at 340 nm monitors all the proteins containing tryptophan. Although the quantum yields of proteins vary, the emission intensity of a mixture of proteins can serve as an indicator of protein concentration.

Sucrose Density Gradients of Mixtures of CVs and Baskets. We have prepared solutions containing a constant concentration of CVs and a variable concentration of baskets—i.e., 0%, 10%, 20%, and 30%. These concentrations are based on the protein absorption of both types of macromolecules. The four solutions were sedimented at the same time in the same rotor. The fluorescence was measured on all fractions without changing the settings on the fluorometer for tryptophan or DPH. Consequently, the tryptophan and DPH curves can be compared directly with each other. The tryptophan intensity of the sample of CVs without added baskets was set to the same value as the DPH intensity at their peaks. Both sets of points then fell on the same curve in all the fractions except the very low and very high, in which intensities were too small to give much precision. The intensities of tryptophan fluorescence in the three mixtures were then adjusted by the identical factor as in the control solution. In this way any difference in the tryptophan and DPH curves would represent the contribution of the added baskets to the tryptophan fluorescence curve.

Protein Concentration. Clathrin concentration was measured by its absorbance at 280 nm, $A_{280}^{1\%} = 10.9$. The concentration of protein in CVs was determined by diluting in con-
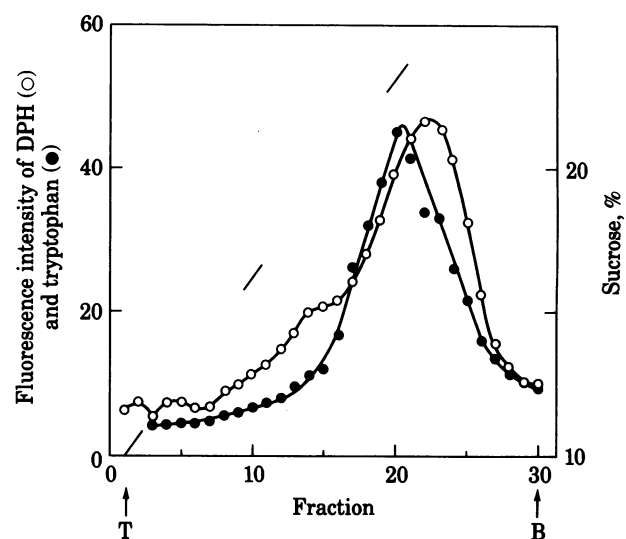

FIG. 1. Sucrose gradient centrifugation (10–30%) of CVs prepared at high sucrose concentrations. Centrifugation (SW 41 rotor) was at 27,000 rpm for 110 min at 20°C. ○, DPH fluorescence; ●, tryptophan fluorescence; T, top of the gradient; B, bottom.

centrated guanidinium chloride solutions to eliminate their turbidity and measuring at 280 nm. The same extinction coefficient was used as for clathrin.

RESULTS

We first present an analysis of the homogeneity of CV preparations obtained by centrifugation in high- and low-sucrose gradients. The protein and membrane lipid composition of CVs in a high (10–60%) sucrose gradient is later shown for comparison.

Analysis in 10–30% Gradients. *CVs prepared in a high-sucrose step gradient (10–60% sucrose).* Analysis of the sedimentation pattern of the membrane lipid and protein components of CV preparations by the specific fluorescent labels DPH and
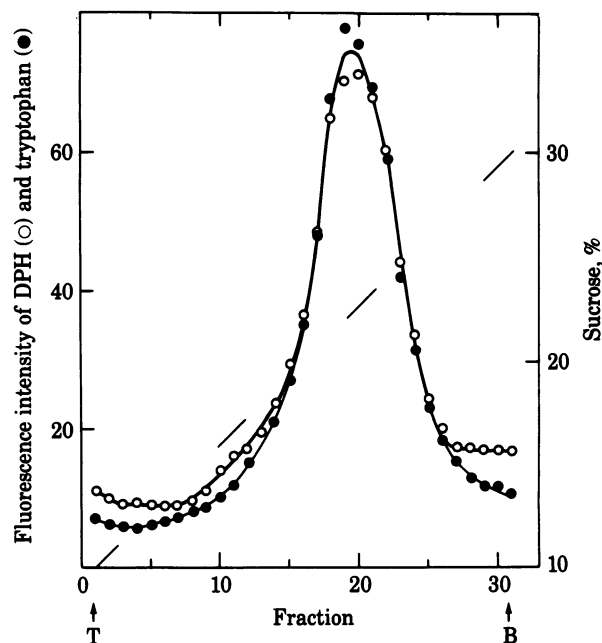

FIG. 2. Sucrose gradient centrifugation (10–30%) of CVs prepared in a low-sucrose step gradient. Centrifugation (SW 41 rotor) was at 27,000 rpm for 110 min at 20°C. ○, DPH fluorescence; ●, tryptophan fluorescence.

tryptophan, respectively, indicated that the distribution of the two labels did not represent the behavior of a single sedimenting species, because the peak of the tryptophan fluorescence was centered about two fractions behind the peak of the DPH fluorescence (Fig. 1). The greater asymmetry of the tryptophan band and its overlap with the faster-sedimenting DPH band suggest that the tryptophan band is a composite of two protein bands: a faster-sedimenting one that is associated with the DPH band and a slower-sedimenting one that represents protein free of membrane. The faster-sedimenting band therefore reflects the sedimentation behavior of CVs, whereas the slower-sedimenting band is due to clathrin baskets. We have reported the formation of two sizes of baskets—i.e., 150S and 300S—by polymerizing clathrin protomer. The baskets in this CV preparation should be the 150S size because we have found that CVs have an average sedimentation coefficient of 200 S (unpublished data).

The fluorescent profiles of CVs prepared in high sucrose gradients usually reveal a minor shoulder on the less dense side of the peak with a larger DPH to tryptophan fluorescence ratio than that of the peak value (Fig. 1). This shoulder may be due to contamination by CVs that have lost their coat or by other types of membranous material.

CVs prepared in a low-sucrose step gradient (²H₂O-8% sucrose). The relationship of the fluorescent profiles of the two labels was very different with CVs prepared by this low-sucrose

modification. In this case the fluorescence intensities of both labels fit on a single, symmetrical curve (Fig. 2) except for minor deviations on the low-density side of the gradient. It is clear from the close agreement (i.e., constant ratio) of the DPH and tryptophan fluorescent bands that few baskets and very little uncoated vesicle membranes are present. Moreover, the ratio of membrane lipid to protein should be constant in all sedimenting species of CVs except for those represented by the small DPH shoulder on the low-density side of the gradient. In accord with the symmetrical distribution of fluorescent labels we have also found a symmetrical distribution of sedimentation coefficients from velocity experiments (unpublished data). The electron micrograph of this preparation of CVs is shown in Fig. 3.

Analysis in 10–60% Gradients. In the preparation of CVs most investigators have followed the original procedure of Pearse and recovered the CV fraction at 50–55% sucrose concentration. We have now analyzed for membrane and protein distribution directly in fractions of the 10–60% gradient of CV preparations obtained from both a continuous 10–60% and a step ¹H₂O/²H₂O-8% sucrose gradient. We have not noticed any significant difference in the sedimentation profiles in 10–60% gradients with CVs prepared in either type of gradient. We have observed that the distribution of the two fluorescent labels is completely different in the fractions of the 10–60% gradient when compared with that found in the 10–30% gradient with
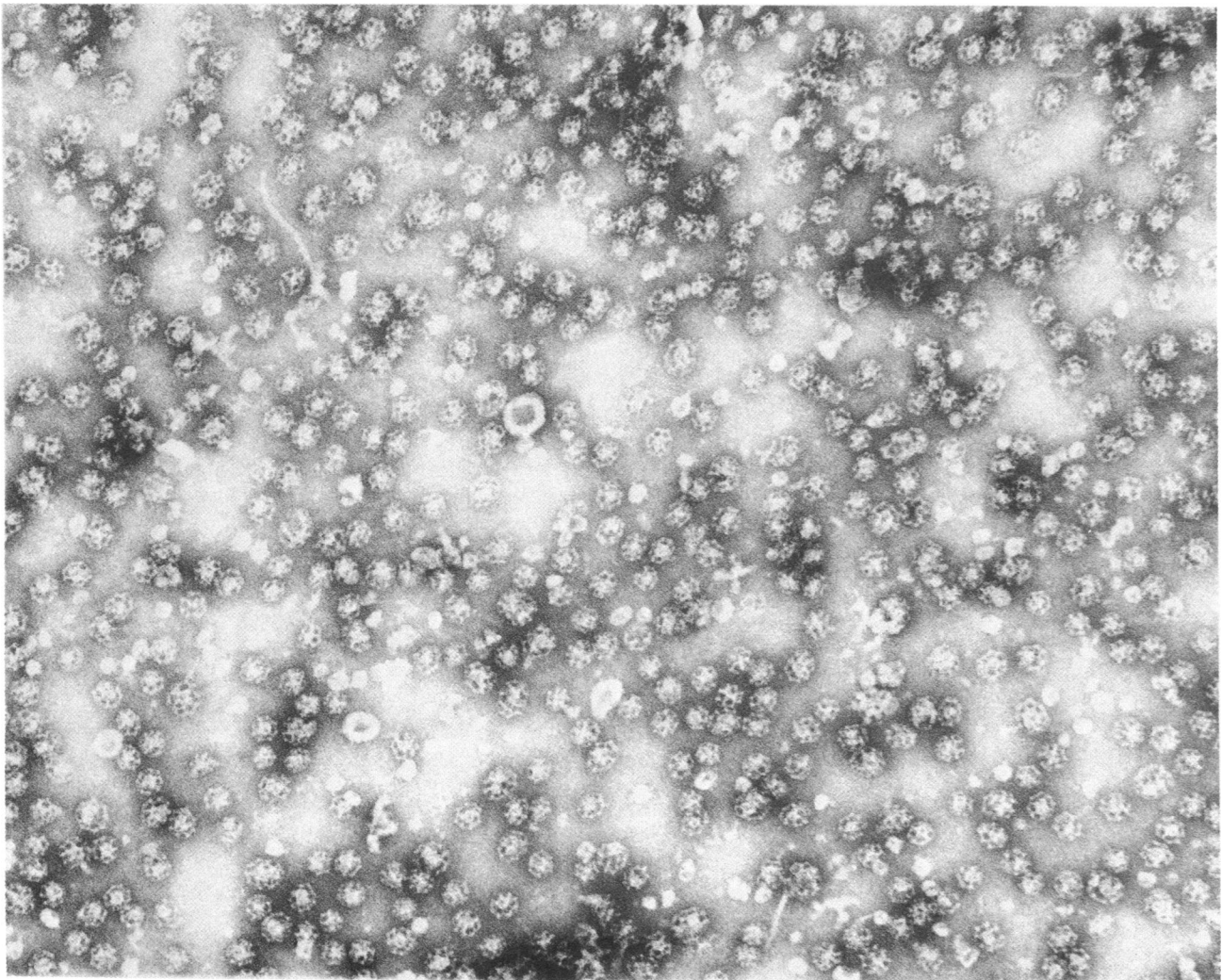

FIG. 3. Electron micrograph of coated vesicles (no. 92) prepared in 8% sucrose/²H₂O step gradient. See Fig. 2 and text for details. (×70,000.)

either type of CV preparation. Whereas in the 10–30% gradients, the DPH and tryptophan bands overlapped or were superimposable (depending on the method of preparation), in the 10–60% gradients the major peaks of the two labels were far apart and the distribution of each label was at least bimodal.

The distribution of tryptophan and DPH labels of CVs (two different 10–60% sucrose preparations) sedimented for 90 hr and for 18 hr in 10–60% gradients are shown in Figs. 4 and 5, respectively. The major tryptophan fluorescent bands occur at 47% sucrose after 18 hr (Fig. 5) and at 55% after 90 hr (Fig. 4). The tryptophan peak at 55% sucrose (Fig. 4), which is largely free of DPH emission, represents emission from clathrin baskets. This was shown by sedimenting a solution of clathrin baskets for 90 hr in the same gradient because the peak was found at 55% sucrose.

A major DPH band (usually the major band) occurs at ≈29% sucrose concentration after 18 hr and at ≈33% after 90 hr. In the region of these DPH bands the emission originates largely from uncoated vesicles because there is very little tryptophan emission. [There is frequently a shoulder or minor peak on the low-density side (i.e., 24% sucrose) of the major DPH peak.] There is also a second DPH band (45% sucrose at 18 hr, 46–48% at 90 hr) that overlaps the major tryptophan band. This band presumably represents the CVs that are not dissociated at the high sucrose concentrations used in the gradient. Though the area under this DPH peak varies considerably, it is usually much smaller than the area remaining in the DPH profile. It is evident that only a small percentage of the DPH fluorescence is associated with CVs in 10–60% sucrose gradients. These CVs may represent a more stable fraction of these organelles.

One other feature of these fluorescence patterns needs comment. This is a tryptophan peak that occurs at ≈34% sucrose after 18 hr (Fig. 5) and at ≈35% sucrose after 90 hr and that corresponds to the position of 8S clathrin. The height of this peak has varied considerably from one CV preparation to another, but it is always much smaller than the major tryptophan band. One can see a small shoulder in fraction 14 (Fig. 4) that may represent 8S clathrin. When native clathrin (8S) and clathrin baskets were sedimented in 10–60% gradients for 90 hr, tryptophan peaks were observed at 39% and 53%, respectively.
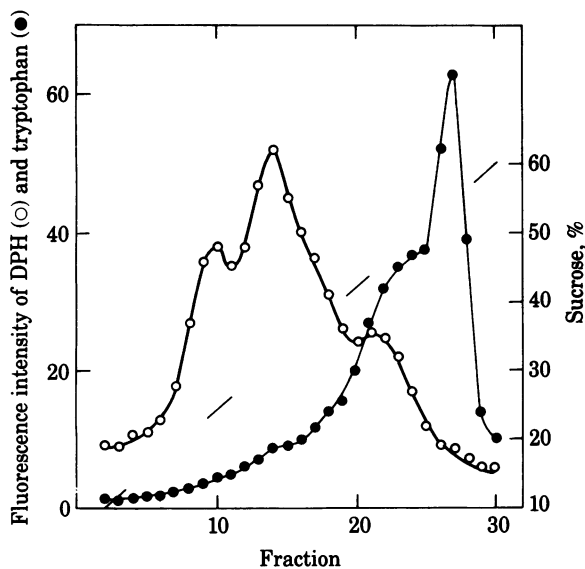

FIG. 4. Sucrose gradient centrifugation (10–60%) of CVs prepared at high sucrose concentrations. Centrifugation (SW 41 rotor) was at 27,000 rpm for 90 hr at 5°C. ○, DPH fluorescence; ●, tryptophan fluorescence.
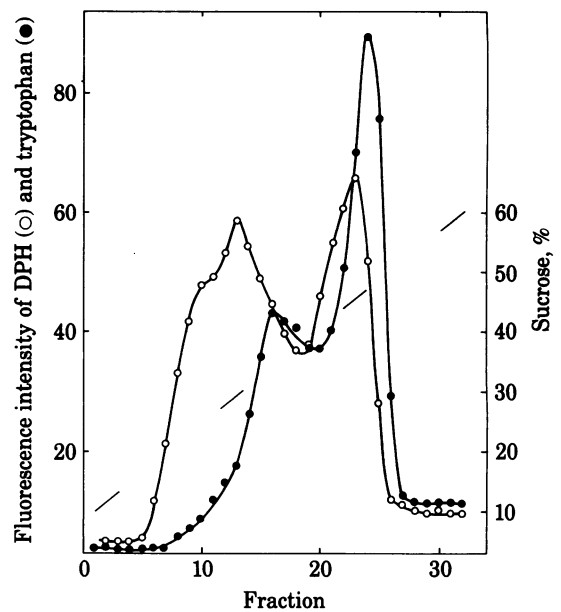

FIG. 5. Sucrose gradient centrifugation (10–60%) of CVs prepared in a low-sucrose step gradient. Centrifugation (SW 41 rotor) was at 27,000 rpm for 18 hr at 5°C. ○, DPH fluorescence; ●, tryptophan fluorescence.

It is clear that equilibrium conditions do not prevail for all components even after 90 hr of sedimentation.

Concentrated Sucrose Solutions of Coated Vesicles. At high rotor speeds, high pressures are generated in centrifugal cells. It is known that high pressures can dissociate proteins into their subunits and unfold globular proteins (21, 22). In order to distinguish between the effects of sucrose and those of pressure (in sucrose solutions) on the dissociation of CVs we have brought aqueous solutions of CVs prepared in $^1H_2O/^2H_2O$-8% step gradient to 15%, 40%, and 50% sucrose. After 2 hr, the 40% and 50% sucrose solutions were diluted to 15% sucrose and all three were sedimented on 15–30% gradients at 27,000 rpm for 110 min at 20°C. A single superimposable curve similar to that found in Fig. 2 was found for DPH and tryptophan emission for CVs exposed to 15% sucrose. For the CVs dissolved in 40% (or 50%) sucrose solutions, the two labels were displaced from each other and resembled the patterns found when CVs were prepared from 10–60% gradients (see Fig. 1). Longer periods of exposure to 40% or 50% sucrose solutions produced greater separation between the peaks of the DPH and tryptophan emission in 15–30% sucrose gradients. It is clear that the high sucrose concentration and not the pressure developed in the centrifuge is responsible for the dissociation of CVs into protein and membrane.

Sucrose Gradient Analysis of Mixtures of CVs and Baskets. From the agreement between the tryptophan and DPH fluorescence curves shown in Fig. 2, we have suggested that this preparation has few baskets. In order to verify the ability of this type of analysis to discriminate between CVs and baskets, we have sedimented on sucrose gradients (10–30%) mixtures of the two types of structures. In Fig. 6 is seen the tryptophan and DPH fluorescence curves of the solution containing 20% baskets. The displacement of the protein curve to lower fractions is clearly evident and resembles results obtained with the Pearse method of preparation of CVs—e.g., see Fig. 1. The smallest curve shown in Fig. 6 represents the difference between the tryptophan and DPH curves and reveals the contribution to tryptophan fluorescence of the added baskets. In Fig. 7, difference curves are presented for CV solutions containing
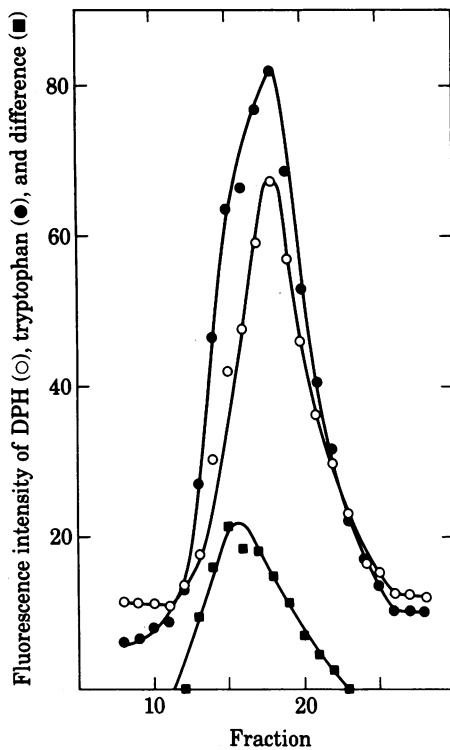
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FIG. 6. Sucrose gradient centrifugation (10–30%) of a mixture of CVs and baskets (20%). Centrifugation was at 27,000 rpm for 110 min at 20°C. o, DPH fluorescence; ●, tryptophan fluorescence; ■, difference in fluorescence intensities between the tryptophan and DPH fluorescence curves.

10%, 20%, and 30% baskets. The proportionality between maximal intensity and percent added baskets is evident. It appears that this method of analysis should be capable of detecting several percent contamination of CVs by baskets.

DISCUSSION

Considerable effort has been expended by numerous investigators in purifying and characterizing clathrin since its isolation by Pearse. Although clathrin is usually prepared from CVs, relatively few attempts have been made to purify further the CV preparation of Pearse. Electron micrographic examination of
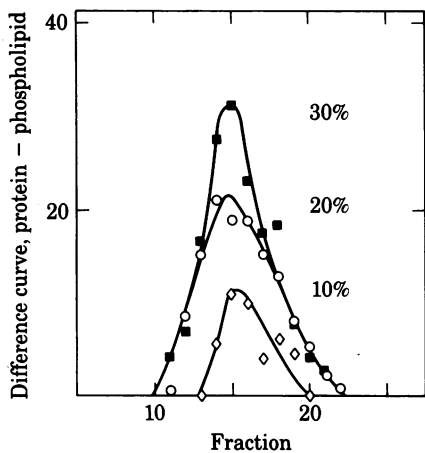

FIG. 7. Difference curves for mixtures of CVs and 10% (◇), 20% (○), and 30% (■) baskets. See Fig. 6 for further details.

the CVs prepared in different laboratories soon revealed that they contained large amounts of clathrin baskets (16, 19). Woodward and Roth (23) observed a broad, unsymmetrical size distribution of CVs sedimented in a 10–30% sucrose gradient when they measured the protein content by absorbance at 280 nm. Recently, Rubenstein et al. (24) resolved a CV preparation into coated and smooth vesicles and showed that the ATPase activity of the preparation was associated with the smooth vesicles. We have characterized CV preparations by evaluating both the protein and membrane lipid composition. If protein is present without membrane, as in clathrin baskets, it should be immediately evident (Figs. 6 and 7). If membrane fragments are present with little or no protein, it will be clear from the fluorescence ratio of the two probes.

We have been able to demonstrate by using this method that sedimentation in 10–60% sucrose gradients for 18 hr results in extensive dissociation of CVs into their lipid and protein components. Moreover, these components associate to reform CVs when sucrose is removed because sedimentation on nondissociating 10–30% sucrose gradients reveals only two species—i.e., CVs and 150S baskets. It appears therefore that CVs obtained from 10–60% gradients are largely composed of reconstituted vesicles. It remains to be shown that these preparations of CVs have the same properties as native CVs.

We have fractionated CVs in a single step gradient—i.e., $^{1}H_{2}O$ vs. $^{2}H_{2}O$-8% sucrose, which avoids the dissociating conditions of high sucrose concentrations. This step should prevent vesicles of higher lipid content than CVs from entering the $^{2}H_{2}O$-8% sucrose layer. It should be noted that the lipid content of CVs is very low for a membrane vesicle because about half of the total protein present in CVs is clathrin coat. Pearse has recently mentioned the deleterious effect of high sucrose concentration and changed to a $^{2}H_{2}O$/Ficoll gradient in preparing coated vesicles (8).

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