Biochimica et Biophysica Acta 938 (1988) 167–180
Elsevier

BBA 73875

Membrane contents of distinct subpopulations of coated vesicles determined by scanning transmission electron microscopy

Clifford J. Steer [a], Margaret E. Bisher [b], Benes L. Trus [c], James F. Hainfeld [d], Joseph S. Wall [d] and Alasdair C. Steven [b]

[a] *Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases,* [b] *Laboratory of Physical Biology, National Institute of Arthritis, Musculoskeletal and Skin Diseases,* [c] *Computer Systems Laboratory, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD, and* [d] *Biology Department, Brookhaven National Laboratory, Upton, NY (U.S.A.)*

(Received 6 August 1987)
(Revised manuscript received 4 November 1987)

Key words: Clathrin; Coated vesicle; Scanning transmission electron microscopy; Receptor-mediated endocytosis; Molecular weight determination

In order to investigate the heterogeneity of clathrin-coated vesicles purified from rat liver, and to quantitate rigorously their membrane contents, we have analyzed scanning transmission electron micrographs of unstained coated vesicles before and after extraction with the non-ionic detergent Triton X-100, as well as of vesicles whose coats had been removed by dialysis against 10 mM or 100 mM Tris (pH 8.2). Their respective distributions of particle masses were thus determined and compared, in light of complementary biochemical quantitations of lipid and protein. Smaller coated particles, 25–45 MDa in mass and 60–80 nm in diameter, lose no mass when extracted with Triton, and disappear when their coats are dissociated. We conclude that they do not contain membrane vesicles, although they have dense, presumably proteinaceous, cores. They may represent particles generated during tissue homogenization or, possibly, a storage form of clathrin. The remaining 70% contain bona fide vesicles: these particles are 75–150 nm in diameter, and their average mass is about 80 MDa, of which 48 MDa is contributed by coat proteins, 10–12 MDa by phospholipid and cholesterol, and 20–22 MDa by vesicle-associated proteins. Their vesicles are of two types: smaller, denser, vesicles that contain substantial amounts of internalized material, and larger, less dense, vesicles that do not. The distinction between them may, in view of other findings, reflect a difference between coated vesicles derived respectively from the Golgi and the plasma membrane.

Introduction

Coated vesicles (CV) are transient organelles which mediate the transfer of material between membrane-bound compartments of the eukaryotic cell [1,2]. The principal constituent of their distinctive outer shell [3,4] is an M_r 180000 protein called clathrin [5]. The basic oligomer, or 'triskelion', is a clathrin trimer that also contains three 'light chains', $M_r \approx 30000$–36000 [6]. A coat con-

Abbreviations: CV, coated vesicles; LCV, liver coated vesicles; BCV, brain coated vesicles; T-LCV, Triton X-100-treated LCV; UCV, uncoated vesicles; Mes, 4-morpholineethanesulfonic acid; STEM, scanning transmission electron microscopy.

Correspondence: A.C. Steven, Bldg. 6, Rm. 114, National Institutes of Health, Bethesda, MD 20892, U.S.A.

sists of triskelions polymerized into a polyhedral surface lattice of hollow hexagons and pentagons [4,7]. Together with associated proteins [8,9], it encases a vesicle derived from the membrane of origin [10].

CV populations are highly heterogeneous both in situ and after isolation from various tissues. This variability reflects both polymorphism of the clathrin surface lattices whereby triskelions may polymerize into coats of different sizes, and variable amounts of internal contents within CV of the same size [11]. The possibility that CV of particular size classes may have distinct chemical or functional properties is an intriguing one. However, the mechanisms that determine the sizes of CV in vivo are poorly understood, and only recently has progress been made towards fractionating subclasses of CV and defining their molecular properties [12,13]. However, inferences concerning native CV based on in vitro studies are compromised by the likelihood that isolates also include artifactual clathrin particles.

The presence of such particles also affects biochemical determinations of the membrane content of CV. Despite the development of increasingly refined purification procedures, the values recently reported for their mass fraction of phospholipid and cholesterol vary widely, from 0.07 [14] to 0.40 [15]. In general, high values have been suspected of being due to contaminating (lipid-rich) membrane fragments, whereas low values may have been biased by the inclusion of vesicle-free clathrin 'cages'. To date, it has not been possible to resolve this tissue experimentally.

The recently developed technique of scanning transmission electron microscopy [16] affords an approach to both of the above problems through its capability of measuring particle masses on an individual basis rather than from bulk sampling of a purportedly homogeneous and monodisperse suspension. By compiling distributions of mass measurements for particular morphological classes of particles, distinct subpopulations may be defined and characterized. By measuring the mass depletion of CV-related particles that have been extracted with detergents, or stripped of their coats by manipulation of pH and ionic strength, average membrane contents may be determined for the various subpopulations. Because these measurements are confined to coated particles, this determination is not biased by contributions from contaminants. Furthermore, the incidence of membrane-free cages may be quantitated since these particles should form a subpopulation that loses little or no mass upon detergent extraction.

Experimental procedures

Materials. Sucrose (ultrapure) was purchased from Bethesda Research Laboratories, Rockville, MD; Mes from Calbiochem Behring Corp., La Jolla, CA; deuterium oxide (^2H_2O) from the Aldrich Chemical Co., Milwaukee, WI; Triton X-100 from Research Products International Corp., Elk Grove Village, IL, and Bio-Beads SM-2 from Bio-Rad, Richmond, CA. All other chemicals were reagent grade. Male Sprague-Dawley rats weighing 200–250 g were obtained from Taconic Farms, Germantown, NY.

Isolation of coated vesicles. LCV were isolated according to the procedure of Nandi et al. [17] as modified by Steer et al. [18]. In a typical preparation done at 4°C, 1 kg of livers from approx. 100 rats was homogenized in an equal volume of buffer A (0.1 M Mes/1 mM EGTA/0.5 mM $MgCl_2$/0.02% (w/v) sodium azide (pH 6.5)) in a Waring blender at maximum speed (four 10 s bursts). The homogenate was put through several high-speed (100 000 × *g*) and low-speed (12 000 × *g*) centrifugations to remove contaminating material. 6-ml aliquots of the CV-enriched suspension were layered on to 6 ml of a 17% sucrose/^2H_2O solution maintained at pH 6.5 with the same buffer salts as in buffer A. The step gradient was centrifuged at 125 000 × *g* (32 000 rpm, Beckman SW40) for 2 h at 16°C with the brake off. The CV-containing pellets were resuspended in buffer A and centrifuged at 12 000 × *g* for 10 min to remove aggregates. Final purification was achieved by Sephacryl S-1000 gel filtration column chromatography (Pharmacia, Piscataway, NJ). Approx. 8 ml of the CV suspension (4–5 mg protein/ml) were applied to a 2 × 85 cm column prewashed in buffer A, eluted in this buffer at a flow rate of 45 ml/h, and collected in 2-ml fractions. The CV eluted in a well-defined peak [18] of which appropriate fractions were pooled and pelleted by centrifugation at 100 000 × *g* for 60 min. The supernatant was

aspirated and the clear pellet of LCV was covered with buffer A and allowed to resuspend overnight, at a final protein concentration of 2–3 mg/ml.

Extraction of coated vesicles with Triton X-100. A 5 ml aliquot of purified CV was made 1% in Triton X-100 and incubated at 4°C for 30 min. The clarified suspension was then centrifuged through an 8% ^2H_2O/buffer A gradient containing the same concentration of Triton X-100 for 2 h, $100000 \times g$ at 8°C. The T-LCV were then washed twice by resuspending the pellet in buffer A to a total volume of 15 ml, incubating for approx. 15 min, then pelleting by centrifugation at $100000 \times g$ for 60 min. Finally, the particles were allowed to resuspend overnight to a concentration of about 1 mg/ml protein buffer A. In some experiments, the washed T-LCV were dialyzed overnight against Bio-Beads SM-2 to remove any residual detergent, but the resulting scanning transmission electron microscopy mass determinations were not significantly different from those of samples which were not so dialyzed.

Preparation of uncoated vesicles (UCV). A 5 ml aliquot of purified CV was dialyzed overnight against either 10 mM or 100 mM Tris-HCl (pH 8.2) and then centrifuged at $100000 \times g$ for 60 min in a Beckman 65 rotor. The supernatant containing dissociated protein was removed and the pellet of uncoated vesicles overlaid with 1 ml of 100 mM Tris (pH 8.2). Within several hours, the uncoated vesicles were resuspended with no evidence of aggregation. Repeat dialysis was ineffective in removing any further clathrin.

Protein and lipid analysis. Protein was determined according to the method of Lowry et al. [19] or that of Bradford [20], with bovine serum albumin as standard. Total cholesterol was determined using a colorimetric assay (Sigma Chemical Co., St. Louis, MO: kit no. 350). Sample was added directly to the assay mix and absorbance at 500 nm was recorded. Turbidity from the sample (less than 3% of the total absorbance) was subtracted after remeasuring the absorbance in the presence of ascorbic acid. Phospholipid was determined by the method of Fiske and SubbaRow [21] after extraction of the membrane lipid [22]. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [23] using a 9% gel system. The gels were stained with either Coomassie blue R-250 or silver [24]. The amount of protein in individual bands was determined by colorimetric measurement of the Coomassie blue stain eluted from the gel with 25% pyridine [25], applying to each band a background correction calculated from the amount of staining in an equal area of an unloaded gel track.

Digital quantitation of gel electrophoregrams. Photographic negatives (on 4×5 inch sheets of Kodak Type 4162 film) were digitized using a Perkin-Elmer 1010MG scanning microdensitometer [26] with a 100 μm square aperture and a 100 μm raster step. The resulting two-dimensional matrices of density values were then subjected to a nonlinear mapping to compensate for non-linearities between the photographically recorded optical density and the density of stain on the gel [11]. For each gel track, a linear background was calculated by averaging laterally over the densities of empty tracks on either side, and this baseline was subtracted from the matrix. These data were then integrated in the lateral dimension to obtain one-dimensional traces such as those shown in Fig. 5. The total densities associated with specific bands or zones of the gel were determined by appropriate numerical integration. These image processing operations were performed using the PIC program system [27], running on a VAX 11/780 computer.

Scanning transmission electron microscopy. This was performed with the STEM at Brookhaven National Laboratory [28], operating at 40 keV, with its liquid-N_2 cold-stage maintaining a specimen temperature of −150°C. At focus, the diameter of the electron probe is about 0.25 nm, and digital images of 512×512 pixels were recorded at sampling intervals of 1.0, 2.0 or 4.0 nm, using the signal from the large-angle dark-field detector, which collects about 40% of all elastically scattered electrons. Most mass measurements were made using micrographs recorded with the 4.0 nm sample step, corresponding to average electron doses of about 100 electrons/nm^2.

In specimen preparation, suspensions of particles at 0.1–0.4 mg/ml were adsorbed for 1 min to thin carbon film substrates, washed (four cycles of 1 min each) with 10 mM ammonium acetate (a volatile salt that sublimes in vacuo), pH 6.5 (LCV and T-LCV) or pH 8.0 (UCV). Excess fluid was

drawn off and the specimens frozen by immersion in liquid-N_2 slush. Freeze-drying at constant sublimation rate over 6–8 h was performed as described [29].

Conventional transmission electron microscopy. Quality control of specimens prior to STEM experiments was performed by conventional electron microscopy of preparations negatively stained with

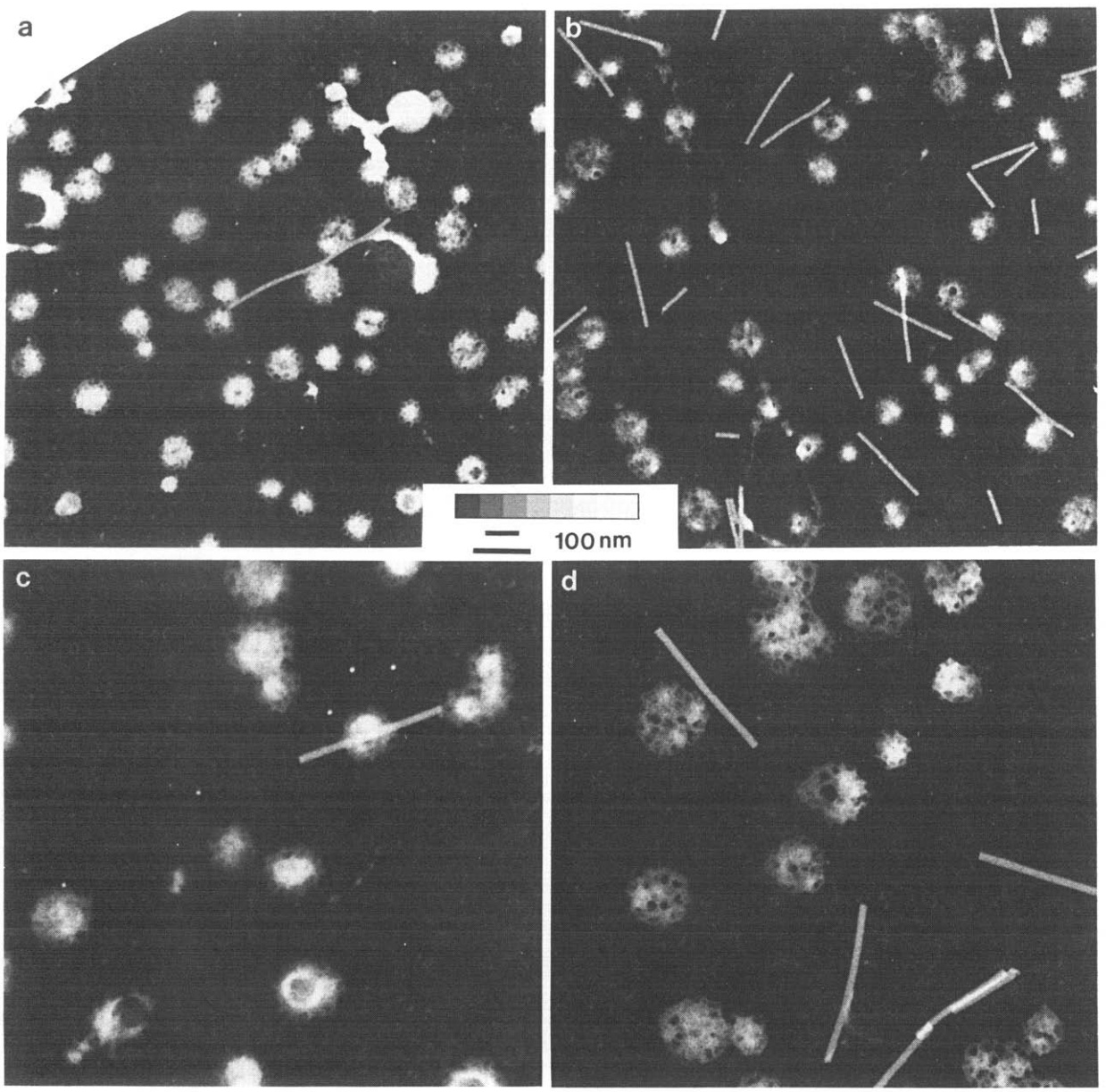

Fig. 1. Effect of extraction with the detergent Triton X-100 on the structures of coated vesicles purified from rat liver (LCV). The particles are visualized without staining by dark-field scanning transmission electron microscopy after specimen preparation by freeze-drying. Triton-extracted particles (b and d) are compared with controls (a and c) in digital images that have been precisely calibrated to convey the same scale of density increments. The rod-shaped particles are tobacco mosaic virions included in these experiments as internal mass standards.

uranyl acetate, using a Philips EM400T electron microscope.

Mass measurements: digital image processing. Digital STEM micrographs were analyzed for the purpose of particle mass determinations using the PIC system [27]. Micrographs were displayed on a video monitor, and the centers of particles were designated interactively by the operator using a Summagraphics graphpen/tablet system. The cumulative densities were then integrated within circumscribing circles of sufficiently large radii to ensure inclusion of the entire particles. Background subtractions were effected using a procedure similar to that described by Hainfeld et al. [30]. Particle masses were then calibrated relative to those of tobacco mosaic virus particles (131 kDa/nm) which were included in these preparations as internal standards. To achieve unbiased samplings of the particle mass distributions, all particles that were entirely contained within the field and were not clumped or visibly disrupted, were measured. Histograms and other statistical procedures were performed using the MLAB program [31], running on a DEC-10 computer.

Results

Effect of extraction with Triton X-100 on the morphology and masses of LCV

Treatment of LCV with this non-ionic detergent results in a profound change in their appearance as visualized unstained by dark-field STEM (Fig. 1). Whereas all untreated LCV appear as roundish, dense particles of varying diameters (50–150 nm), whose clathrin surface lattices are visible mainly around their peripheries, T-LCV preparations contain two markedly different classes of particles. The first are relatively large (100–150 nm), and of generally low density, and their polyhedral clathrin lattices are usually visible over most of their surface areas. Usually they contain eccentrically positioned local concentrations of density: we refer to them as 'large, non-dense particles'. Particles of the second class are typically smaller and more compact (hence, 'small dense particles'). They are indistinguishable from the smaller LCV present in control samples (cf. Figs. 1a, b). The relative proportions of these two classes, about 70% and about 30% respec-

TABLE I

STATISTICS FOR DISTRIBUTIONS OF MASSES FOR VARIOUS TYPES OF PARTICLE ANALYZED BY QUANTITATIVE SCANNING TRANSMISSION ELECTRON MICROSCOPY

Particle	Number of measurements	Number of experiments	Average mass (MDa)	Mass (MDa)					
				min.	20%	40%	60%	80%	max.
Liver coated vesicles (LCV)	750	5	68.0	21.5	38.1	54.8	72.7	91.9	263.5
Triton-X-100-extracted LCV (T-LCV) [a]	1159	5	54.0	23.9	38.8	48.8	57.8	67.4	123.7
(i) 'Large non-dense particles' [a]	806 (70%)	5	61.5	25.6	49.5	56.7	63.5	70.2	123.7
(ii) 'Small dense particles' [a]	353 (30%)	5	37.8	23.9	30.8	34.7	38.7	44.9	76.0
Uncoated vesicles (10 mM Tris (pH 8.2))	711	3	39.8	12.9	26.7	33.5	40.8	49.1	113.0
(i) 'Floppy disks'	434 (61%)	3	38.5	12.9	26.7	32.7	40.0	48.3	106.3
(ii) 'Nuggets'	267 (39%)	3	41.5	16.1	29.6	34.8	42.4	50.4	113.0
Uncoated vesicles (100 mM Tris (pH 8.2))	447	2	43.1	14.5	28.8	34.5	41.6	52.5	174.9
(i) 'Floppy disks'	223 (50%)	2	37.1	14.5	26.8	31.6	37.5	45.5	102.8
(ii) 'Nuggets'	224 (50%)	2	43.9	19.6	31.2	36.6	45.0	57.0	84.5

[a] Data corrected for residual detergent binding.

tively, are highly reproducible between different experiments with the same preparation, and among independent preparations. We have measured the masses of several hundred particles of both types (Table I and Fig. 2). The masses of 'large non-dense particles' range from about 38 to about 105 MDa, averaging 68 MDa, whereas those of small dense particles vary from 27 to 65 MDa, and average 42 MDa. The mass distributions obtained with different T-LCV preparations were somewhat less reproducible than those of particles which had not been exposed to detergent (LCV, UCV). We attribute this property to the retention of varying amounts of Triton (see below), despite the rigorous washing procedures employed.

The combined T-LCV mass measurements are compared with the LCV particle mass distribution in Figs. 3a, b and Table I. It seems plausible that 'small dense particles' are derived from the smaller LCV, and 'large non-dense particles' from the larger and more massive LCV. However, we cannot compare their mass distributions directly with those of the LCV subpopulations from which they are derived because the latter do not fall into two

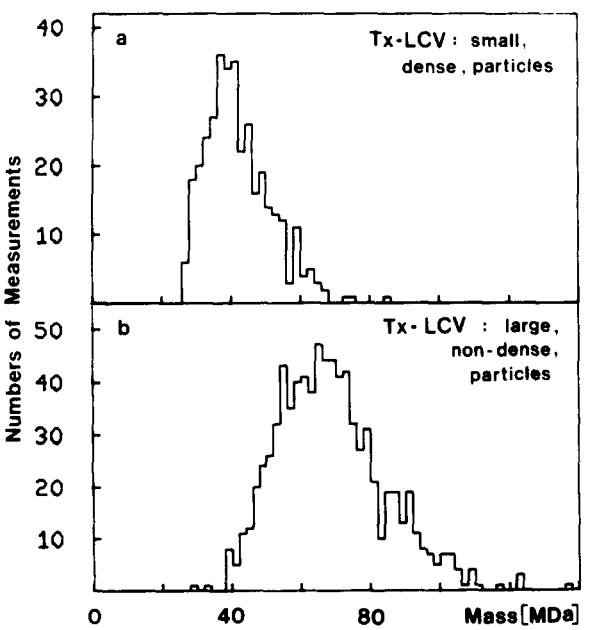

Fig. 2. Histograms representing the statistical distributions of masses determined for the two morphological types of particle observed in dark-field STEM micrographs of Triton-extracted LCV.

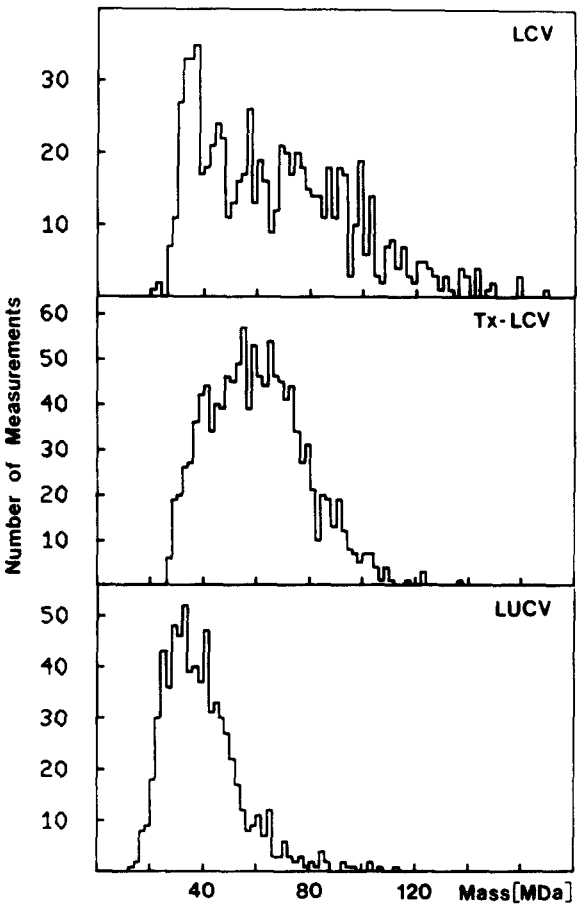

Fig. 3. Histograms representing unbiased samplings of the distributions of particle masses among (a) purified rat liver coated vesicles; (b) LCV extracted with Triton X-100; (c) uncoated vesicles derived from LCV by stripping off their clathrin coats by dialysis against 10 mM Tris-HCl (pH 8.0).

distinct subsets on morphological grounds. (In contrast, over 90% of T-LCV may be unambiguously assigned to the two categories.) Nevertheless, we may compare the quantile point masses of the global LCV and T-LCV distributions. The ratios of these quantile masses are plotted in Fig. 4a. At the low-mass end, the ratios are quite constant, with T-LCV masses exceeding those of their LCV counterparts by about 10%. Beyond this point, increasing levels of mass depletion are observed. Since clathrin lattices are not dissociated by Triton, the most straightforward explanation for the apparent mass increase of the smaller particles is that they should have retained, on average, detergent in amounts equivalent to about

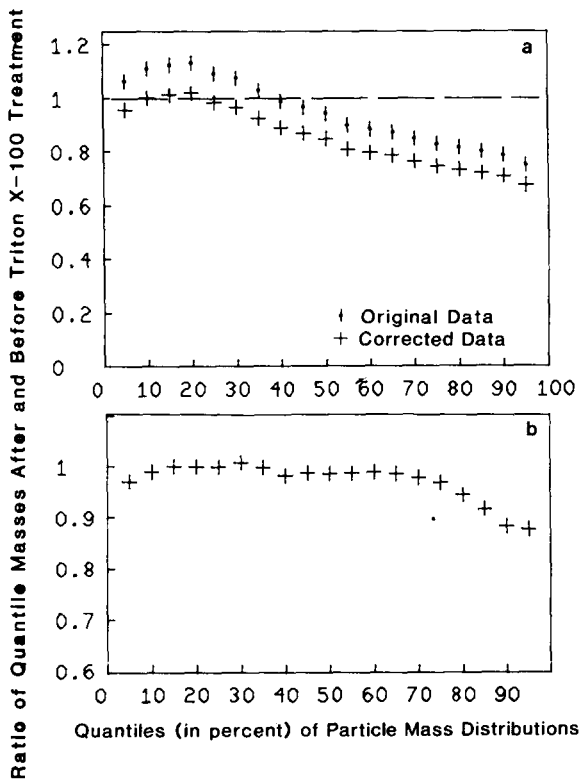

Fig. 4. (a) Fractional mass depletion of LCV upon extraction with Triton X-100 is estimated as a function of particle mass from the ratios between the corresponding quantile masses of the respective distributions. (b) Corresponding data for BCV (corrected for Triton retention).

10% of their masses. If we assume that proportional levels of detergent retention apply to particles in all mass ranges, the corrected quantile ratio curve is given in Fig. 4a (crosses). Thus, the 30% of smaller, less massive, LCV lose none of their contents upon Triton extraction, whereas the larger particles lose substantial amounts, up to about 35% of their total masses. This trend is consistent with the appearance of 'large non-dense particles', which seem to be largely devoid of contents. Overall, the effect of Triton extraction is to extract about 14 MDa of the median LCV mass of 68 MDa (cf. Table I).

Corresponding analyses of T-BCV exhibit a similar trend, except that 70–75% of all BCV exhibit no detectable mass loss (Fig. 4b). To check the validity of the correction for detergent retention, a preparation of BCV was extracted using ^{3}H-labeled Triton X-100 (New England Nuclear Research Products, Boston, MA). After the washing protocol described above (Materials and Methods, Extraction...), no detectable counts remained in the supernatant, whereas a substantial amount of radioactivity remained associated with the pellet. The amount of Triton retained was calculated to be equivalent to 7–9% of the protein mass, assuming that labeled and unlabeled detergent were bound with the same affinity.

Effect of extraction with Triton X-100 on the chemical composition of LCV

Protein composition. The protein compositions of LCV and T-LCV were compared by SDS-polyacrylamide gel electrophoresis (Fig. 5a, b). To enhance visualization of non-clathrin components which are present in relatively small amounts, the gels were heavily overloaded, so that the optical absorbance of their photographic records saturated in the vicinity of the clathrin band. Accordingly, the relative amounts of clathrin and other proteins were determined by elution of the Coomassie blue dye from appropriate segments of dissected gels [11]. Control experiments established that, despite the overloading, the experimental conditions remained within the linear region of dye-binding by clathrin. Thus, it was found that clathrin accounts for $56 \pm 2\%$ (S.D.; $N = 4$) of the dye-binding material in LCV preparations, and $63 \pm 3\%$ ($n = 6$) for T-LCV. Since the integrity of clathrin surface lattices is not affected by Triton X-100, the amount of other proteins solubilized by the detergent was calculated relative to a fixed amount of clathrin, giving 11% of the total protein, or 25% of the non-clathrin proteins.

To examine the effect of Triton extraction on individual bands, the photographic records of gels were scanned by two-dimensional densitometry, and these data were then re-scaled to compensate for non-linearity of the photographic response [11] (although for clathrin, this was not fully possible, see above). After effecting a background subtraction, the resulting matrices were then integrated laterally to obtain traces such as those shown in Fig. 5. It should be noted that Coomassie blue has markedly different affinities for different proteins [32], and so caution should be exercised in relating the amounts of bound dye to the stoichiometry of

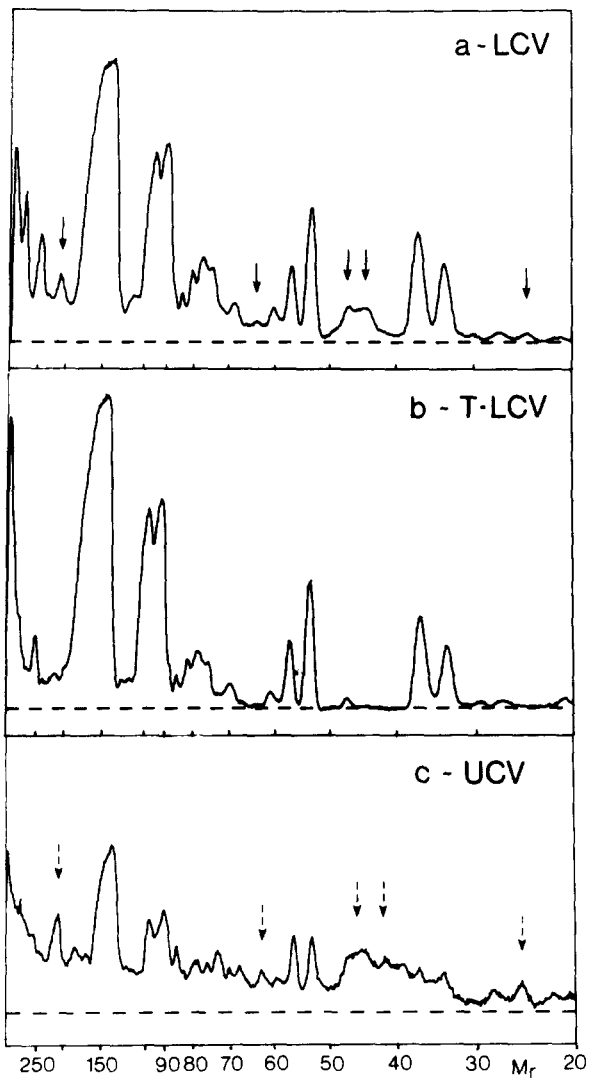

Fig. 5. The protein compositions of LCV(a), T-LCV(b), and UCV produced at 10 mM Tris-HCl (pH 8.2) (c) as represented by density profiles of SDS-polyacrylamide gel electrophoregrams stained with Coomassie brilliant blue. To obtain these profiles, photographic records of the gels were digitized and analyzed by computer image processing (cf. Materials and Methods). The arrows (a) indicate the locations of discrete LCV bands that are missing or heavily depleted in T-LCV: these bands are strongly represented in UCV (dashed arrows in (c)), whereas coat protein bands are greatly reduced relative to LCV(a).

the various proteins. Nevertheless, the relative amounts of the same protein in the LCV and T-LCV traces should be represented in the correct proportions.

The gel profiles of LCV and T-LCV are generally similar. On close inspection, however, it is apparent that they differ in two respects. First, the background level is appreciably higher for LCV, particularly in the relatively high-M_r range (over 50 kDa). This background is presumably contributed by a spectrum of proteins, each present in an amount too small to contribute a discrete band. Second, there are several minor bands which are present in T-LCV in substantially reduced amounts: a broad doublet at about 45 kDa that is virtually eliminated, bands of about 210 kDa and about 24 kDa, and a very weak, but nevertheless reproducibly eliminated, band at about 63 kDa. Thus, the effect of Triton extraction on the protein contents of LCV is to reduce the background level and to extract several discrete but minor components.

Lipid quantitation. We have previously determined the lipid content of similar LCV preparations to be 130 μg (0.18 μmol) phospholipid per mg protein [18]. These data were confirmed in the present study. The cholesterol content was determined to be 23 μg/mg protein. Thus, the combined mass-fraction of phospholipid and cholesterol was 0.13. The effect of Triton extraction was to reduce the contents of phospholipid and cholesterol to less than 10% of the original level, a more precise figure being precluded by the limitations of the assays used.

STEM mass analysis and protein composition of liver uncoated vesicles

Uncoated vesicles were prepared by exhaustive dialysis of purified LCV against 10 mM Tris (pH 8.2), a treatment that dissociates clathrin coats [33]. As estimated by the gel dissection/dye elution method, the clathrin band accounted for 17% of the total dye-binding material, corresponding to dissociation of 84% of clathrin, assuming that clathrin is indeed the only protein in this region of the gel. Other coat proteins, i.e., the 110–100 kDa and approx. 55 kDa 'assembly factors' [33,9] and the light chains, were also much reduced (Fig. 5c). In contrast, the Triton-extractable bands are relatively prominent in UCV, consistent with their being vesicle proteins.

When examined by STEM microscopy (Fig. 6a), two types of UCV were reproducibly observed, although with substantial variability in

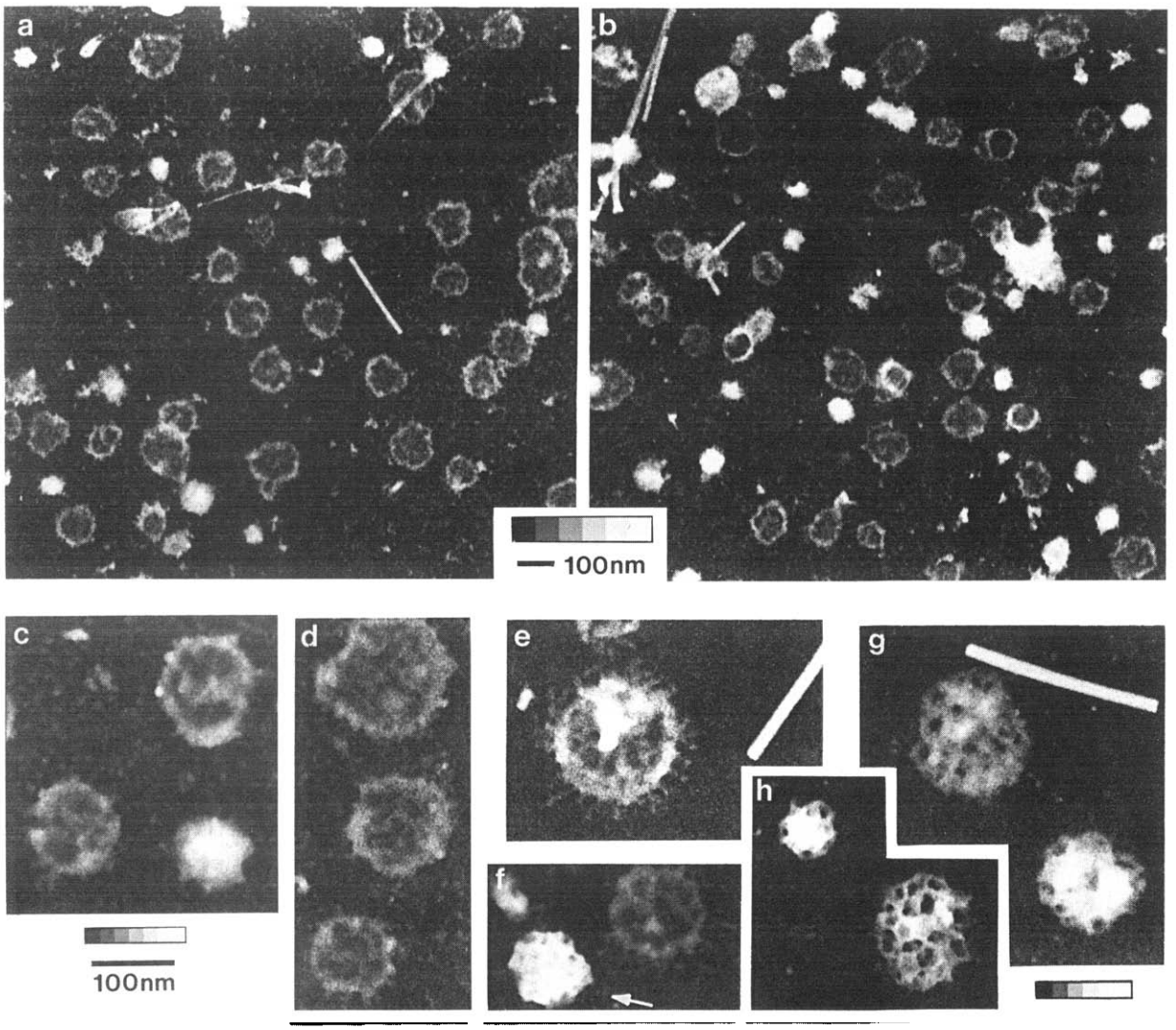

Fig. 6. Uncoated vesicles derived from purified LCV by dialysis against (a) 10 mM Tris-HCl (pH 8.0) and (b) 100 mM Tris-HCl (pH 8.0). The specimens were imaged without staining by dark-field scanning transmission electron microscopy after preparation by freeze-drying. Both preparations contain low-density, rimmed particles ('floppy disks') as well as relatively compact, distinctly denser particles ('nuggets'). Their morphologies are compared at higher magnification in (c) and (d). For comparison, the two particle-types found in Triton extracted LCV, 'small dense particles' and 'large non-dense particles' respectively, are shown in (g) and (h): in contrast to UCV, their predominant features are their clathrin surface lattices. UCV produced at 100 mM ionic strength (b) contain a significantly higher incidence of 'nuggets' than UCV produced at 10 mM ionic strength (a), since they also include a fraction of relatively small, coat-retaining, particles (e.g., arrow in (f)). In (e), a partially-stripped LCV particle is shown: its filamentous protuberances probably represent clathrin molecules which have retained contact with the vesicle.

both size and mass in each case. Particles of the first class are relatively large, 80–180 nm in diameter, averaging 125 nm, and project very low densities except for a characteristic rim of higher density. We interpret these particles to be vesicles that have completely flattened on adsorption to the carbon film substrate. Accordingly, their unflattened diameters should be less by a factor of $1/\sqrt{2}$, i.e., 55–125 nm, with an average of 88 nm. We refer to them as 'floppy disks'. Particles of the second type are smaller (35–80 nm, averaging 60 nm), and are much denser, implying that they are spread/flattened to a lesser degree. We refer to them as 'nuggets'. The relative proportions of

these two classes, 61% and 39% resspectively, were strictly reproducible. The distributions of masses within these two classes are quite similar (Fig. 7a, b). Although substantial heterogeneity is apparent, the distributions are approximately unimodal. Their average masses, 38.5 MDa and 41.5 MDa (Table I), indicate that the 'nuggets' tend to be slightly more massive.

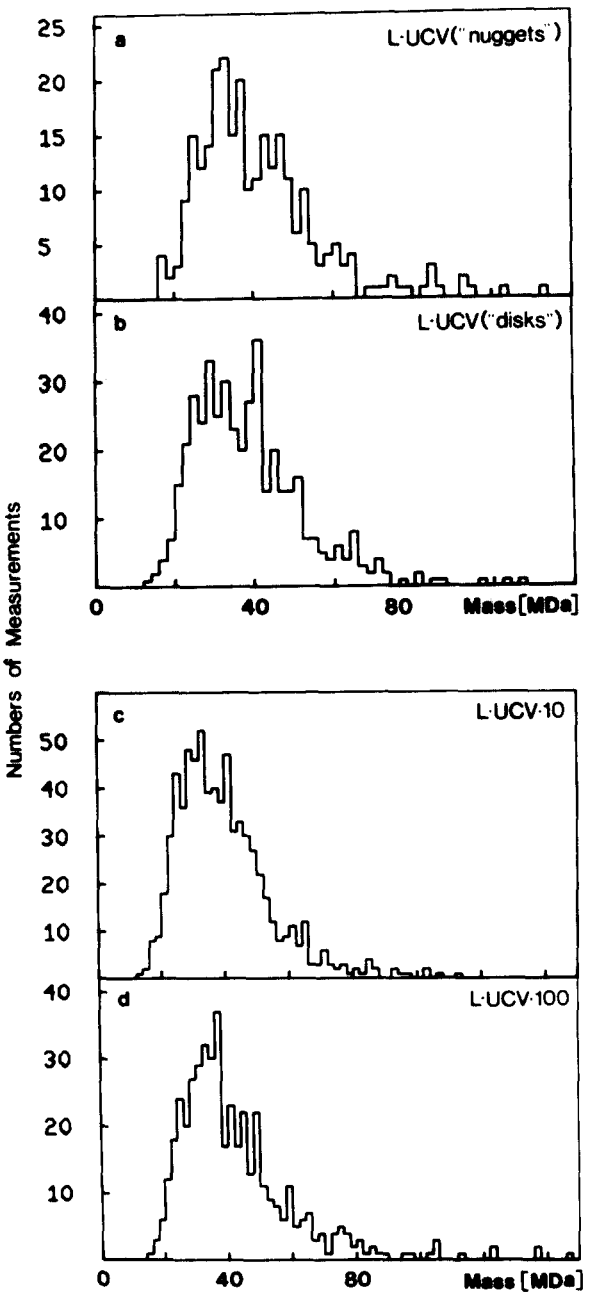

Fig. 7. Histograms comparing the respective distributions of masses of (a) 'nuggets', and (b) 'floppy disks', morphologically defined subclasses of UCV. Particle masses were determined by dark-field STEM microscopy. Also compared are the overall mass distributions of UCV prepared at 10 mM ionic strength (c) and at 100 mM (d).

In earlier experiments, we noted that UCV prepared at 10 mM ionic strength have a tendency to osmotic instability [18]. To explore the possibility that this property may lead to disruption of some UCV – in which case, the observed population would not represent an unbiased sampling of the vesicles initially contained within coats – they were also prepared by dialysis against pH 8.2 at moderate ionic strength (100 mM Tris-HCl). Under these conditions, a smaller fraction of clathrin was released (about 51%). STEM microscopy yielded results similar to those obtained when 10 mM ionic strength was used, except that a smaller proportion of 'floppy disks' was observed (49% compared with 61%) (cf. Figs. 6a and 6b). However, their masses were indistinguishable from those of 'floppy disks' produced at 10 mM ionic strength (Table I). On close examination of the micrographs, we found that the 'nuggets' produced at 100 mM ionic strength included some particles that retained their clathrin coats intact (e.g., Fig. 6f). Because these particles are relatively small, inclusion of them in the overall mass distribution does not displace it to any significant extent relative to that of UCV produced at 10 mM Tris-HCl (Table I and Fig. 7c,d). We conclude that at least some of the smaller LCV retain their coats at 100 mM ionic strength, but lose them at 10 mM and the same pH. It is the presence of these particles that accounts for the enhanced retention of clathrin at 100 mM ionic strength, rather than a uniformly less efficient shedding of clathrin by all LCV. The apparent paradox of a markedly higher protein content of UCV produced at 100 mM ionic strength without a corresponding increase in particle mass is resolved by the finding that clathrin retention is confined to this subset of smaller and less massive particles.

In experiments in which LCV were incompletely stripped by less protracted dialysis against 10 mM Tris, we observed an additional type of particle, e.g., Fig. 6e. (Because maximal clathrin removal was not achieved in these experiments, mass measurements from them were not included

in the distributions presented in Figs. 3c and 7c.) These particles resemble 'floppy disks', but, in contrast to the smooth periphery of the archetypal 'floppy disk', they have 'hairy' borders with characteristic filamentous protrusions. These protrusions are about 20 nm in length and have blobs of density at their distal tips. Our tentative interpretation is that they represent clathrin molecules whose interactions in the surface lattice have been disrupted, but which are still vesicle-associated.

Discussion

One difficulty in relating experiments on isolated CV to membrane transport phenomena in vivo is that intracellular CV are transient structures [34], whereas in vitro experiments necessarily relate to particles with long-term stability. However, conditions under which clathrin surface lattices remain intact also promote assembly of soluble clathrin into cages [35]. Since recent studies have detected intracellular pools of unassembled clathrin [36], it is distinctly possible that CV isolates may include particles generated artifactually during tissue homogenization. The major goals of the present study have been to characterize CV polymorphism and to measure their membrane contents by employing a technique that is capable of quantitating – and correcting for – the incidence of artifactual vesicle-free particles.

Previously, we have performed STEM mass analyses to compare the distinctly different populations of LCV and BCV [11]. Of these, BCV were found to contain a much higher level of smaller (50–80 nm), less massive (20–40 MDa), particles. On the basis of thin sectioning studies, Merisko et al. [37] have reported that only 25% of purified BCV exhibited well-defined vesicle membranes, a number consistent with our finding that 70–75% of BCV exhibit essentially no mass depletion when extracted with Triton X-100. In contrast, most LCV appear to contain membrane vesicles, as evidenced by thin section electron microscopy [18]. For this reason, we have focused on a more detailed characterization of LCV polymorphism. Specifically, we have performed STEM microscopy and correlative biochemical analyses on LCV perturbed either by extraction with Triton X-100, or by dissociation of their coats under appropriate ionic conditions.

In this way, three major subclasses of LCV have been identified. Within each subclass, the particles are markedly heterogeneous, but nevertheless share distinctive structural properties. Ap-

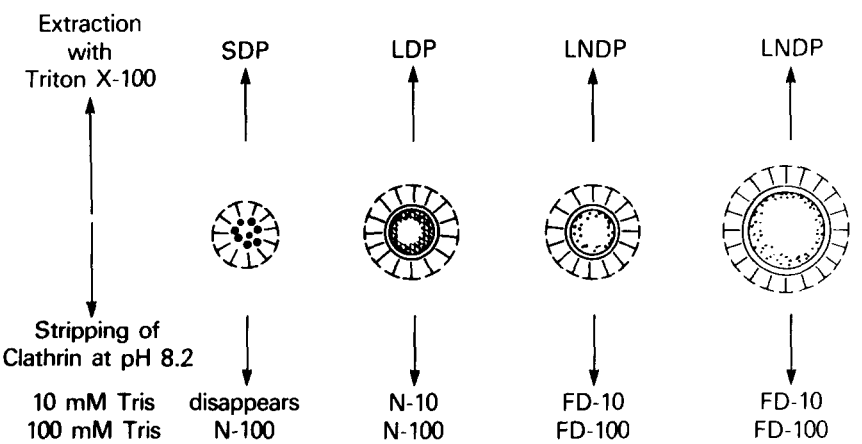

Fig. 8. Schematic diagram indicating the different particle types present in preparations of purified LCV, and how they generate the respective subpopulations of particles observed when LCV structure is perturbed either by extraction with Triton X-100, or by dissociation of their clathrin coats. The SDP represents a clathrin-shelled particle with internal proteins, but no membrane vesicle. The other three particles shown contain vesicles, particles of the second type containing systematically greater amounts of material within their vesicle lumens (shown as cross-hatching) as inferred to account for the populations of dense 'nugget' (N) particles observed in preparations of UCV. Considerable heterogeneity in both mass and size exists within each class represented by these prototypic models. FD, 'floppy disk', LNDP, 'large non-dense particle'.

prox. 30% of 'LCV' are vesicle-free (but not necessarily empty) and some, at least, are likely to have assembled subsequent to tissue homogenization from soluble clathrin. Consistent with this inference, in vitro assembly products from liver clathrin have masses and sizes very similar to those of SDP (unpublished results). On the other hand, small coated particles might also be assembled in vivo under some circumstances, possibly as a convenient storage form of clathrin. For instance, Larkin et al. [38] have observed small coated particles in cultured fibroblasts in which endocytosis has been inhibited by K^+-depletion.

The remaining 70% of LCV contain bona fide vesicles of two different kinds. The first (about 40%) tend to be smaller, although both classes are polymorphic and their respective size distributions overlap. Moreover, vesicles of the first kind contain relatively large amounts of internal (luminal) material. This classification is summarized in Fig. 8, and we proceed to discuss the evidence on which this scheme is based.

Effects on LCV of extraction with Triton X-100

Structural effects. T-LCV are of two distinct morphological types – 'small dense particles', and 'large non-dense particles'. The pronounced difference between their average masses (Table I) rules out the possibility that the respective morphologies simply represent qualitatively different responses to preparation for electron microscopy. If this explanation were to hold, their mass distributions should be the same.

The integrity of clathrin coats is unaffected by exposure to this detergent, and so the observed population of T-LCV should correspond directly and without bias to the untreated LCV. When the respective mass distributions are compared (Figs. 3 and 4), it is apparent that all LCV do not lose the same fractions of their masses. There is a subpopulation of smaller LCV – about 30% of the total – that loses essentially no mass. These 'small dense particles' represent a vesicle-free form of clathrin-coated particle. The possibility that they may contain some form of Triton-resistant membrane is ruled out by the observation that our biochemical assays detect no appreciable content of phospholipid and cholesterol in T-LCV.

Quantitative correlation of mass analyses with chemical composition. According to the STEM mass measurements, an average 21% of the contents of LCV are extracted by Triton X-100. As determined biochemically, phospholipid and cholesterol are reduced by more than 90% on exposure to the detergent, and about 11% of the total protein is extracted as well, amounting to an overall mass loss of 20–22%. Thus, the mass loss values given by these two entirely different methods are in close agreement. The concurrence of the biochemical determination with the outcome of the STEM mass analyses – which are unaffected by the presence of membranous contaminants – confirms that these LCV preparations are indeed highly purified [18].

Effects on protein composition. According to SDS-PAGE (Fig. 5), most of the protein loss encurred by LCV on extraction with Triton X-100 takes the form of a reduction in the background. That the few discrete bands extracted should be vesicle proteins rather than coat-associated proteins is supported by the enhancement of their bands in the UCV gel profile (Fig. 5c).

The electrophoretic mobility of the 45 kDa doublet is somewhat similar to that of the 38 kDa BCV protein that is also Triton-extractable [14]. By immunoelectron microscopy, this protein was estimated to be a component of about 20% of BCV [14], a figure consistent with its being present in all or nearly all vesicle-containing BCV, in view of the evidence (discussed above) that these particles constitute no more than 25–30% of the total.

Effect of uncoating LCV at 10 mM and 100 mM ionic strength, pH 8.2

Dialyzing LCV under conditions conducive to dissociation of their clathrin coats produces two types of particle – dense, compact 'nuggets', and larger, fully flattened vesicles ('floppy disks'). Despite their very different morphologies, these two particle species have rather similar mass distributions (Fig. 7 and Table I). Nevertheless, we consider it likely that the distinction between them is genuine, because (a) their relative proportions are strictly reproducible, and (b) 'nuggets' are systematically more massive, albeit by a rather slight margin. Thus, 'nuggets' appear to represent a type of vesicle that contains a relatively large amount of luminal material, whereas 'floppy disk' vesicles

are generally larger but have less luminal contents. Overall, their masses are rather similar because the smaller dimensions of 'nuggets' are offset by their greater density.

On comparing UCV produced at 100 mM and 10 mM ionic strength, their respective levels of clathrin retention are 48% and 16%, and their proportions of 'nuggets' are 51% and 39%. However, the masses of 'floppy disks' do not depend on the ionic strength at which they were produced (Table I), implying that clathrin stripping from this subclass of LCV is equally efficient in both cases. On the other hand, the 'nuggets' produced at 100 mM Tris-HCl have a slightly higher average mass than those produced at 10 mM, and include some particles with intact coats. It follows that there is a subpopulation of smaller LCV whose coats are removed at 10 mM but not at 100 mM Tris-HCl, pH 8.2. Thus, their clathrin surface lattices appear to be secured differently from those of other LCV.

Membrane contents of LCV – a final accounting

Since 'small dense particles' average 38 MDa in mass, the remaining 70% of (vesicle-containing) LCV should average about 80 MDa, and this figure is indeed close to the mode of the LCV distribution (Fig. 3a), when the low-mass component is disregarded. To give an overall mass-fraction of 0.13 for phospholipid and cholesterol, this average 80 MDa particle should contain about 12.5 MDa of these constituents.

An independent estimate of membrane content is given by the analysis of UCV. Incubating LCV in 10 mM Tris (pH 8.2) reduces their clathrin content by 84% and increases their mass-fraction of phospholipid and cholesterol to 0.25. The average UCV mass is 40 MDa, implying a quota of about 10 MDa of phospholipid and cholesterol per particle, in reasonable agreement with the value calculated above. Residual coat proteins are estimated to contribute 8 MDa, leaving 20–22 MDa of vesicle proteins (we stress that the given quotas per particle are average figures for decidedly heterogeneous populations).

To reconsider previous measurements of the lipid contents of CV (see Introduction), we conclude that the higher values obtained were most likely caused by the presence of contaminating (non-coated) membrane fragments. We have determined the mass-fraction phospholipid and cholesterol in vesicle-containing LCV to be 0.16, whereas an overall figure of 0.13 applies to all coated particles in these preparations. The corresponding mass-fraction may be expected to vary for CV isolated from different sources, depending primarily on the incidence of vesicle-free particles. (A secondary dependence – on diameter – may be anticipated, larger CV tending to have greater amounts of membrane per fixed amount of clathrin.) For instance, 70% of BCV are vesicle-free, and if the protein : lipid ratio of the remainder is taken to be approximately the same as for vesicle-containing LCV, an overall mass-fraction of 0.05 is to be expected for BCV. This figure is consistent with our data for Triton-extraction of BCV (Fig. 4), and is also close to the value determined by Weidenmann et al. [14].

Possible implications for subpopulations of CV within the cell

Recently, Kedersha et al. have succeeded in fractionating two major classes of LCV by agarose gel electrophoresis [12]. One class tended to have larger particles and a higher incidence of endocytic CV, whereas the generally smaller particles of the second class were enriched for enzymatic activity that suggested involvement in secretory processes. This distinction fits with earlier in situ observations of smaller CV in the vicinity of rodent (mouse) hepatocyte Golgi than around their plasma membranes [39]. It is tempting to equate the two kinds of vesicle-containing LCV found in the present study with the classes distinguished by Kedersha et al. [12]. In this case, it would follow that, although smaller than endocytic CV, secretory CV have much higher concentrations of material within their lumens. Whether this may simply result from a generally higher density of macromolecules in the Golgi lumen than in the extracellular milieu is not immediately apparent. The differing sizes of CV assembled in two parts of the cytoplasm is an intriguing phenomenon: although several possible factors suggest themselves – e.g., differing local concentrations of clathrin, or resistance to deformation of the respective membranes – its underlying mechanism remains to be solved.

Acknowledgements

We thank Ms. Kristin Elmore for assistance with specimen preparation for STEM, Mr. Frank Kito for electron microscopy, and Ms. Avis Hutchings for preparing the manuscript. The Brookhaven STEM is supported by NIH Biotechnology Resource Grant R-01777. J.S.W. is supported by the Office of Health and Environmental Research of the Department of Energy.

References

1 Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Nature (Lond.) 279, 679–685.
2 Fine, R.E. and Ockleford, C.D. (1984) Int. Rev. Cytol. 91, 1–44.
3 Roth, T.F. and Porter, K.R. (1964) J. Cell Biol. 20, 313–332.
4 Heuser, J. (1980) J. Cell. Biol. 84, 560–583.
5 Pearse, B.M.F. (1976) Proc. Natl. Acad. Sci. USA 73, 1255–1259.
6 Ungewickell, E. and Branton, D. (1981) Nature (Lond.) 289, 420–422.
7 Crowther, R.A. and Pearse, B.M.F. (1981) J. Cell Biol. 91, 790–797.
8 Zaremba, S. and Keen, J.H. (1983) J. Cell Biol. 97, 1339–1347.
9 Pearse, B.M.F. and Robinson, M.S. (1984) EMBO J. 3, 1951–1957.
10 Pfeffer, S.P. and Kelly, R.B. (1985) Cell 40, 949–957.
11 Steven, A.C., Hainfeld, J.F., Wall, J.S. and Steer, C.J. (1983) J. Cell Biol. 97, 1714–1723.
12 Kedersha, N.L., Hill, D.F., Kronquist, K.E. and Rome, L.H. (1986) J. Cell Biol. 103, 287–297.
13 Bomsel, M., De Paillerets, C., Weintraub, H. and Alfsen, A. (1986) Biochim. Biophys. Acta 859, 15–25.
14 Weidenmann, B., Lawley, K., Grund, C. and Branton, D. (1985) J. Cell Biol. 101, 12–18.
15 Simion, F.A., Winek, D., Brandan, E., Fleischer, B. and Fleischer, S. (1983) Methods Enzymol. 98, 326–336.
16 Wall, J.S. and Hainfeld, J.F. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 355–376.
17 Nandi, P.K., Irace, G., Van Jaarsveld, P.P., Lippoldt, R.E. and Edelhoch, H. (1982) Proc. Natl. Acad. Sci. USA 79; 5881–5885.
18 Steer, C.J., Bisher, M., Blumenthal, R. and Steven, A.C. (1984) J. Cell Biol. 99, 315–319.
19 Lowry, O.H., Rosebrough, A.L., Farr, N.J. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
20 Bradford, M. (1976) Anal. Biochem. 72, 248–254.
21 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.
22 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
23 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
24 Eschenbruch, M. and Burk, R.R. (1982) Anal. Biochem. 125, 96–99.
25 Fenner, C., Traut, R.R., Mason, D.T. and Wikman-Coffelt, J. (1975) Anal. Biochem. 63, 595–602.
26 McGee, P.A., Trus, B.L. and Steven, A.C. (1982) Micron 13, 221–228.
27 Trus, B.L. and Steven, A.C. (1981) Ultramicroscopy 6, 383–386.
28 Wall, J. (1978) in Introduction to Analytical Electron Microscopy, (Hren, J.J., Goldstein, J.I. and Joy, D.C., eds.), pp. 333–342, Plenum, New York.
29 Mosesson, M.W., Hainfeld, J., Haschemeyer, R.H. and Wall, J. (1981) J. Mol. Biol. 153, 699–718.
30 Hainfeld, J., Wall, J.S. and Desmond, E.J. (1982) Ultramicroscopy 8, 263–270.
31 Knott, G.D. and Reece, D.K. (1972) Proc. ONLINE '72 Intl. Conf., Vol. 1, pp. 497–526, Brunel University, U.K.
32 Tal, M., Silberstein, A. and Nusser, E. (1985) J. Biol. Chem. 260, 9976–9980.
33 Keen, J.H. (1985) in Endocytosis (Willingham, M.C. and Pastan, I., eds.), pp. 85–130, Plenum, New York.
34 Goldstein, B., Wofsy, C. and Bell, G. (1981) Proc. Natl. Acad. Sci. USA 78, 5695–5698.
35 Woodward, M.P. and Roth, T.F. (1978) Proc. Natl. Acad. Sci. USA 75, 4394–4398.
36 Goud, B., Huet, C. and Louvard, D. (1985) J. Cell Biol. 100, 521–527.
37 Merisko, E.M., Farquhar, M.G. and Palade, G.E. (1982) J. Cell Biol. 92, 846–857.
38 Larkin, J.M., Donzell, W.C. and Anderson, R.G.W. (1986) J. Cell Biol. 103, 2619–2627.
39 Croze, E.M., Morre, D.M., Kartenbeck, J., Franke, W.W. and Morre, D.J. (1982) Eur. J. Cell Biol. 28, 130–138.