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# In Vivo Biosynthesis of Clathrin and Other Coated Vesicle Proteins From Rat Liver

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A biosynthetic study of rat liver coated vesicle (CV) proteins was undertaken by using in vivo labeling with L-[$^{35}$S]methionine. CVs were isolated and purified by using standard procedures and characterized by electron microscopy, sedimentation, and sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by fluorography, or by gel slicing and liquid scintillation counting. After 5½ min of labeling (the earliest time examined), incorporation of radioactive clathrin heavy-chain (180-kD (kilodalton)) subunits as well as a 90-kD CV-associated protein into purified CVs was demonstrated. The level of labeled 180-kD clathrin in coated vesicles increased rapidly during the first 2 hr of labeling and then continued to rise at a slower rate between 4 and 16 hr. This slow accumulation of labeled clathrin heavy chains in the CV pool may reflect early compartmental sequestration of a fraction of newly synthesized clathrin with delayed assembly into free CVs. By 16 hr of labeling, clathrin 180-kD chains and the 90-kD CV-associated protein accounted for approximately 48 and 26%, respectively, of the radioactivity in all CV proteins. Two proteins of $MW_a$ 68 kD and 53 kD showed marked declines in cpm/unit protein between 30 min and 4 hr, raising the possibility that these species may be transferred out of CVs during or after transport without loss of the other CV proteins. The possibility is also raised that clathrin heavy chains may be recycled during CV formation. Possible heterogeneity within individual CV preparations with respect to protein composition and derivation from both plasma membrane and Golgi regions are proposed.

**Key words: intracellular transport**

Coated vesicles (CVs) are subcellular organelles which display a characteristic "bristle coat" by electron microscopy and which participate in a wide variety of

Abbreviations used: CV, coated vesicle; SDS, sodium dodecylsulfate; $MW_a$, apparent molecular weight from mobility on SDS gels; EGTA, ethyleneglycol-bis-(β-amino-ethylether)N,N′ tetraacetic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PBS, (4mM phosphate) buffered saline, pH 7.4.

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transport functions, the best known being receptor-mediated endoctyosis of a variety of ligands such as lipoproteins [1], several hormones [2,3,4], galactose-terminal glycoproteins [5], and even virions [6]. These structures, which are present in nearly all tissues and cell types studied, have varying diameters between 70 and about 150 nm and demonstrate an average sedimentation constant of 210 S and an average molecular weight of 37–49 $\times$ $10^6$ daltons [7]. The vesicles possess a protein coat easily removed under mildly alkaline pH, 2 M urea, or low ionic strength buffer conditions, leaving a phospholipid "uncoated vesicle" which still has considerable associated protein. Clathrin triskelions are 640-kD pinwheellike structures having three heavy chains (180 kD) and three light chains each (32–36 kD), and function as the building blocks of the CV coat. Triskelions can polymerize to form closed basket- or cagelike structures in the presence of 100–110-kD clathrin-associated proteins, even in the absence of the lipid vesicle [8].

CVs are found most frequently near the plasma membrane, presumably derived from clathrin-coated pits, and in the region of the Golgi. These latter CVs are often of smaller diameter [9,10] and may function differently from those near the plasma membrane.

Very little is known about the biosynthesis of clathrin and its in vivo assembly into CVs. Keen et al [11] used an anticlathrin light chain antibody to immunoprecipitate a labeled 175-kD protein from a homogenate of cultured 3T3 fibroblasts exposed to L-[$^{35}$S]methionine for 48 hr at 37°C. Presumably the labeled clathrin heavy chains were bound to clathrin light chains under the conditions of immunoprecipitation employed. These authors found only trace labeling of the clathrin light chains with [$^{35}$S]methionine by this technique.

In this report we provide the first kinetic data concerning the incorporation of newly formed clathrin into CVs. Radioactivity incorporation curves obtained by using [$^{35}$S]methionine are compared for clathrin heavy- and light-chain subunits, as well as with other CV-associated proteins. The results are discussed in terms of possible heterogeneity within populations of purified CVs with respect to protein composition, function, and clathrin heavy-chain subunit turnover or recycling.

## METHODS

### In Vivo Labeling With [$^{35}$S]methionine

Male Fisher rats (290–370 g) were fasted for 6–24 hr prior to the intraperitoneal injection of 0.75–1.0 ml of a solution of L-[$^{35}$S]methionine (specific activity 1,270–1,320 Ci/mmol) in PBS, giving a dose of 0.74–1.59 mCi/animal in various experiments. Following injection of tracer, individual rats were decapitated (with of without brief antecedent exposure to $CO_2$) after varying labeling intervals ranging from 5½ min to 6½ hr. Livers were rapidly excised and weighed in a tared beaker containing ice-cold PBS, rinsed, and minced finely with scissors. Each liver was separately homogenized in a loose-fitting Dounce homongenizer (12 strokes) with sufficient buffer (0.1 M 2-(N-morpholino)-ethanesulfonic acid–NaOH pH 6.5–1 mM EGTA–0.5 mM $MgCl_2$–3 mM $NaN_3$) (referred to as homogenization buffer) at 4°C to yield 30 ml of homogenate; 5 ml of homogenate was reserved and frozen for later total protein specific-activity determinations, and the remainder was used for coated vesicle preparation, according to a modification of the method of Nandi et al [12].

## Preparation of Rat Liver Coated Vesicles

All procedures were carried out at 4°C unless otherwise stated.

Individual labeled rat liver homogenates were centrifuged at 13,200g × 50 min in a Sorvall SS 34 rotor. The pellets were discarded and the supernatants were diluted approximately threefold with homogenization buffer prior to centrifuging at 36,000 rpm (105,000g) × 60 min in a Ti 45 (Beckman) rotor. The supernatant was removed and the pellet was gently rinsed twice with 1 ml buffer and then suspended in 8 ml homogenization buffer in a Dounce homogenizer. The suspended 105,000g pellet was then clarified at 10,000 rpm × 10 min in the SS 34 rotor and the resulting opalescent supernatant was centrifuged at 39,000 rpm (109,000g) × 60 min in the Ti 70.1 rotor. The supernatant was aspirated and discarded, and the pellet was rinsed with 0.5 ml buffer. The pellet was next overlaid with 3.5 ml homogenization buffer and left undisturbed for 1 or more hr to promote resuspension. The sample was resuspended and again clarified at 10,000 rpm × 10 min. The resulting low-speed pellet was reextracted in 2.5 ml buffer and centrifuged at 10,000 rpm as above. The combined 10,000-rpm supernatants were layered onto 6 ml of a 17% sucrose solution in homogenization buffer made up by using $D_2O$ and spun at 30,000 rpm × 100 min at 20° C in an SW 40 rotor. The turbid $H_2O/D_2O$ interface and supernatant layers were discarded and the pellet was dissolved in either 1% Triton X-100/50 mM Tris-HCl, pH 7.5/0.5% Trasylol (v/v) (and stored at 4°C until subjected to further studies including SDS-PAGE) or in homogenization buffer for sucrose density-gradient centrifugation.

In some experiments, labeled rat livers were homogenized in 0.25 M STM buffer (0.2 M sucrose–5mM Tris·Cl–0.5 mM $MgCl_2$–3 mM $NaN_3$) at pH 7.4 or pH 7.2, allowing preparation of plasma membrane according to the method of Hubbard et al [13] as well as permitting isolation of coated vesicles from the 1,500g supernatant. In this case, the above supernatant was adjusted to 0.1 M MES and 1 mM EGTA by the slow addition with stirring of a 10× concentrated pH 6.4 stock solution of these reagents followed by the coated vesicle purification scheme outlined above. Labeling patterns and coated vesicle protein content as judged on SDS gels did not differ appreciably regardless of which buffer was used for liver homogenization.

## Sucrose Density Gradient Centrifugation of In Vivo [$^{35}$S]Methionine-Labeled Rat Liver Coated Vesicles

Two rats, each labeled with 1.25 mCi of [$^{35}$S]methionine for 16 hr, were used to prepare coated vesicles from the pooled livers as noted above. The pellet from the $H_2O/D_2O$ centrifugation was suspended in 1,000 $\mu$l of homogenization buffer and clarified at 10,000 rpm (SS 34 rotor) × 10 min. An aliquot of the supernatant (750 $\mu$l) was combined with 8 × $10^{-4}$ ml of $10^{-3}$ M diphenylhexatriene (DPH) in tetrahydrofuran as an extrinsic phospholipid fluorescent probe, incubated at 23°C × 30 min, and applied to 11.2 ml of a 10–30% (w/v) linear sucrose gradient prepared in homogenization buffer. After centrifugation at 27,000 rpm at 20°C × 70 min, 9-drop fractions were collected by pumping from the bottom of the tube with an LKB positive displacement pump. Protein content of each fraction was estimated by tryptophan fluorescence on a Perkin-Elmer Model MPF3 spectrofluorometer ($\lambda$ excitation = 290, $\lambda$ emission = 340). Phospholipid content was quantitated with DPH fluorescence ($\lambda$ excitation = 366, $\lambda$ emission = 430). Fractions were then

counted in 10 ml Ultrafluor with a Packard Tricarb Model 3390 liquid scintillation counter. Counting efficiency was 92%.

### SDS Polyacrylamide Gel Electrophoresis, Fluorography, and Determination of Specific Activity of [$^{35}$S]-Labeled Coated Vesicle Proteins

Linear acrylamide gradient (5–15%) slab gels containing 0.1% SDS were used according to a modification of Laemmlli [14]. Gels were stained and destained with Coomassie Brilliant Blue R250. A mixture of [$^{14}$C]methylated (Amersham Corp, Arlington Hts, IL) and unlabeled (Bio Rad, Cambridge, MA) protein standards was used for molecular weight standards. [$^{3}$H]-methylated 8 S clathrin (generously provided by Dr. Clifford Steer) from bovine brain was also used as a MW standard for electrophoresis and fluorography. Coomassie-stained gels were scanned at 590 nm on a Beckman Model DU-8 Spectrophotometer equipped with a slab gel scanner to obtain numeric quantitation of areas of individual coated vesicle protein peaks. Gels were then treated with PPO-DMSO, dried, and exposed usually for 4–12 days at −80°C to Kodak XAR-2 x-ray film. Fluorograms were scanned at 500 nm on the above scanner to obtain radioactivity quantitation in the various $^{35}$S-labeled rat liver CV protein peaks. Radioactivity incorporation at the various labeling intervals was compared by calculating the areas of the individual proteins' radioactive peak on the fluorogram divided by the area under the Coomassie-stained (180 kD) clathrin peak in the corresponding samples.[1] By this method, the absolute values of specific activity of the various coated vesicle proteins cannot be compared, but a comparative analysis of the *kinetics* of radioactivity incorporation into CV proteins is permitted; cpm/unit protein values were normalized to the animal's weight by dividing by the radionucleotide dose per gram body weight in experiments where rat weights varied by more than ± 4%.

## RESULTS

With the aid of a modification of the procedure of Nandi et al [12] for the purification of coated vesicles from bovine brain, CVs were prepared from individual livers from rats labeled in vivo with [$^{35}$S]methionine. The electron microscopic appearance of the purified rat liver CVs was similar to that previously reported [15] and was essentially free of empty clathrin cages and membrane fragments.[2]

When CVs purified from the livers of two rats (each labeled in vivo for 16 hr with [$^{35}$S]methionine) were applied to a sucrose density gradient, the sedimentation

[1]Errors in the determination of the total clathrin content of each sample by this method were estimated to be less than approximately 15%, as judged by preparation of a standard curve of area under the densitometrically scanned clathrin peak as a function of clathrin quantity applied to the gel (using purified bovine brain clathrin of known concentration).

[2]Other workers, using a similar method for isolating CVs [16], have noted the presence of contaminating smooth membranes in their preparations. While it may be advisable to perform an additional purification step involving gel filtration of CVs with Sephacryl S1000 or controlled-pore glass beads for most studies [16], the simplified procedure herein described resulted in a coated vesicle fraction with minimal smooth membrane contamination, as judged by sucrose density-gradient centrifugation (Fig. 1) and EM (data not shown).

profile seen in Figure 1 was obtained. Relative tryptophan fluorescence intensity superimposed rather closely with diphenylhexatriene (DPH) fluorescence, the latter being an extrinsic phospholipid probe. Both trytophan and DPH fluorescent peaks were centered in the region expected for CVs. The radioactivity profile was quite similar, indicating significant incorporation of label into CVs. A smaller radioactive peak was seen around fraction 10, the nature of which is unknown. A similar secondary peak has been observed in sucrose gradient profiles of bovine thyroid CVs prepared from thyroid slices labeled in vitro for 25 min with [$^{35}$S]methionine [17].

The overall rate of [$^{35}$S]methionine incorporation into trichloroacetic acid (TCA)-precipitable protein present in the whole cell homogenate is plotted in Figure 2. After a rapid rise in the first 2 hr the curve flattens, with no further increase by 16 hr.

When rat liver CVs were subjected to polyacrylamide gel electrophoresis under reducing conditions in SDS, the protein patterns shown in Figures 3A and 4A were obtained. The overloaded Coomassie-stained gel patterns of rat liver CVs were generally similar to that of brain CVs; some differences were apparent. Liver CVs had much less associated tubulin (53–55 kD) and the apparent MWs of the clathrin light-chain subunits were slightly lower (data not shown); the latter difference has been noted by others [18].

Experiments were performed in which the labeling interval between intraperitoneal (ip) injection of isotope and death and harvesting of the livers was varied from as little as 5½ min to up to 16 hr. Studies by Itoh and co-workers [19] demonstrated that peak plasma concentrations of a radioactive amino acid were obtained ~ 15 min after ip injection of rats followed by rapid disappearance. Barton [20] demonstrated that the concentration of free labeled amino acid in liver peaked at ~ 30 min with subsequent rapid decline similar to that observed in plasma. In view of the fact that unlabeled methionine arising from protein turnover continues to be added to the

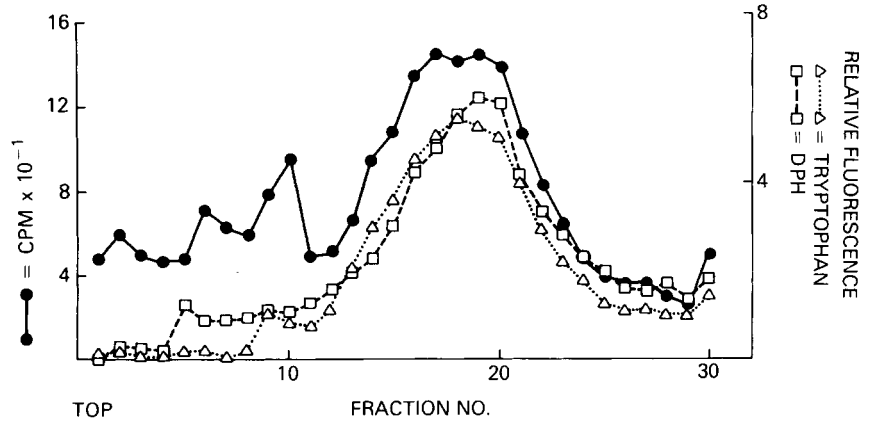


Fig. 1. Sucrose density-gradient profile of CVs purified from in vivo-labeled rat livers. Two rats received intraperitoneal injections of L-[$^{35}$S]methionine (1.25 mCi each, specific activity 1300 Ci/mmol) and their livers were harvested after a 16-hr labeling interval. CVs, made $10^{-6}$ M in diphenylhexatriene (DPH), were prepared from liver homogenates as described and three-fourths of the preparation was applied to a 10–30% (w/w) linear sucrose gradient in homogenization buffer and centrifuged at 27 K rpm, 70 min, 20° C in an SW40 rotor. Fractions were collected and examined for relative tryptophan fluorescence ($\lambda_{excitation}$ = 290, $\lambda_{emission}$ = 340 nm) and relative DPH fluorescence ($\lambda_{excitation}$ = 366, $\lambda^{emission}$ = 430), as well as for radioactivity by counting for 10 min in 10 ml Ultrafluor.

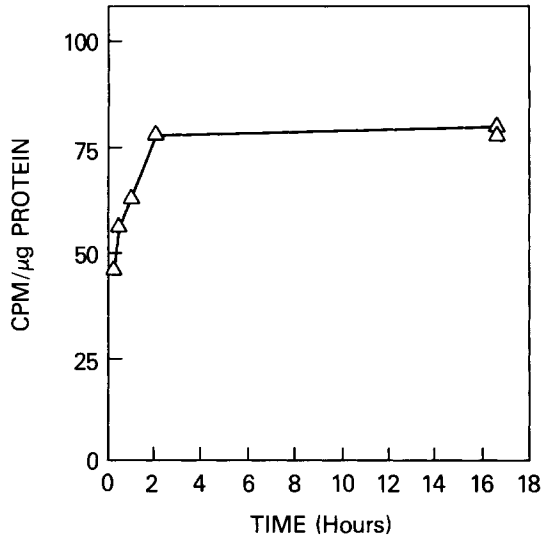


Fig. 2. The time course of incorporation of labeled methionine into the whole cell homogenate trichloroacetic-acid-precipitable protein is illustrated. Here, aliquots of the homogenates obtained during the above experiments were analyzed in triplicate for radioactivity and in duplicate for protein [Lowry method, 24] with the results plotted as cpm/g protein vs time.

intracellular methionine pool, the in vivo pulse labeling experiments herein described are expected to give results resembling those obtained by "pulse-chase"experiments [21]. Illustrated in Figures 3 and 4 are the protein (A) and radioactivity (B) patterns obtained by Coomassie brilliant blue staining and fluorography of gels on which were run purified CVs derived from rat livers labeled in vivo for intervals ranging from 5½ min to 20 min (Fig. 3) and from 1 hr to 16 hr (Fig. 4).

Even after only 5½ min of labeling (Fig. 3B, lane 4), one sees the association of a strongly labeled 68-kD protein with the purified CVs, as well as the appearance of a trace of labeled 180-K clathrin heavy-chain subunit. Incorporation into clathrin can be seen to increase through 16 hr of labeling.

Radioactivity incorporation into CV proteins was measured both by gel slicing and counting as well as by fluorography with densitometric scanning of the resultant fluorogram. Excellent agreement between the methods was obtained. Figure 5A and B illustrate the scans of the Coomassie-stained gel and of the corresponding fluorogram of CV proteins after 2 hr of labeling. In Figure 6 are seen the radioactivity profiles of CV proteins labeled in vivo for 1, 2, 4, and 16 hr, as determined by liquid scintillation counting of gel slices. Total radioactivity in each individual protein band was divided by the amount of protein in the 180-K clathrin band (determined by scanning the Coomassie-stained gel) in order to correct for variations in the amounts of total protein applied to the gel lane. The clathrin heavy-chain subunit represented about 51% of the total stainable CV protein mass as determined by gel scanning.

In Figure 7 is shown a composite of results of three separate experiments covering different labeling intervals. This allows the general trend of label incorporation into each of several CV-associated proteins to be compared over the entire range of labeling times studied.

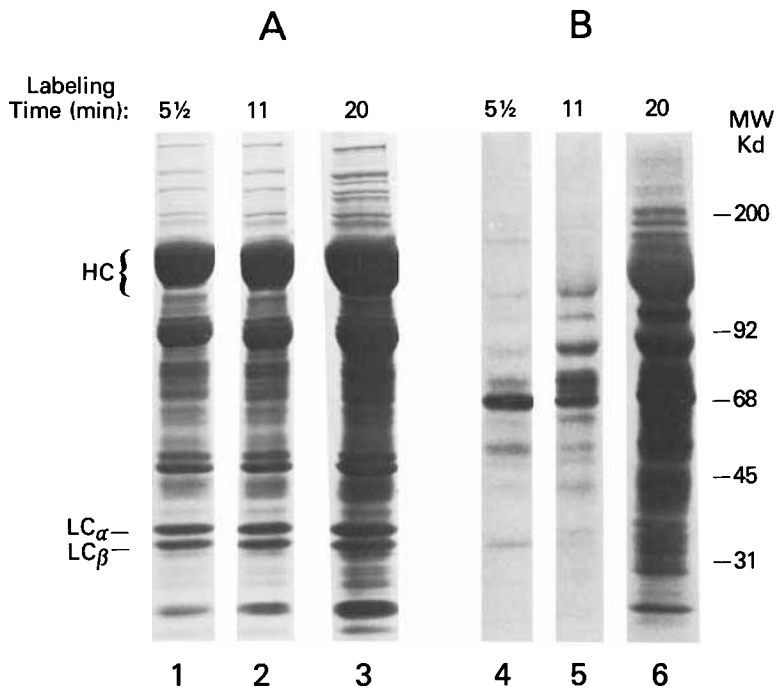


Fig. 3. Incorporation of newly synthesized proteins into rat liver CVs at early labeling times. Fasting rats were labeled in vivo as described for 5½, 11, and 20 min, followed by isolation of CVs from individual livers from single animals, corresponding to each of the three labeling intervals. SDS-PAGE (employing a 5–15% linear gradient of acrylamide) was performed on the entire CV pellet from the sucrose-$D_2O$ step for each sample. The overloaded gels were stained with Coomassie brilliant blue, followed by gel drying and fluorography (16 days exposure) employing 2,5-diphenyloxazole (PPO) dimethylsulfoxide. (A) Coomassie-stained gel pattern of CVs corresponding to [$^{35}$S]methionine labeling intervals of 5.5 (lane 1), 11 (lane 2), and 20 min (lane 3). (B) Radioactivity as revealed by fluorography of gel shown in (A) at the same time intervals. HC = clathrin heavy-chain subunits; $LC_\alpha$ and $LC_\beta$ = 34 and 32−kD clathrin light chain subunits, respectively.

Incorporation of [$^{35}$S]methionine into the heavy-chain clathrin subunit (Fig. 7A) proceeded at a rapid rate through 2 hr of in vivo labeling and then tapered off but continued to increase (another 48% of the 4-hr value) between 4 and 16 hr. The rate of incorporation of 180-kD clathrin closely paralleled that of a 90 kD protein early on, with radioactivity in the latter protein tapering off between 4 and 16 hr. Several less-abundant proteins were seen to roughly parallel the initial incorporation rate seen with 180-kD clathrin, but some, such as the clathrin light chains, then appeared to reach a plateau by 2–4 hr. The incorporation of radioactivity into 258; 190; 108; and 46-kD proteins increased up to at least 2 or 4 hr of labeling (Fig. 4).

The data from Figures 6 and 7 (experiment 3) suggest that the incorporation of light chain subunits reached a plateau by 4 hr, while heavy-chain clathrin subunits continued to be incorporated through at least 16 hr of labeling. Because of the relatively low level of radioactive methionine incorporation into 34-kD and 32-kD clathrin light-chain subunits, the conclusion that the kinetics of incorporation into CVs differ for light and heavy chains must be regarded as only tentative.

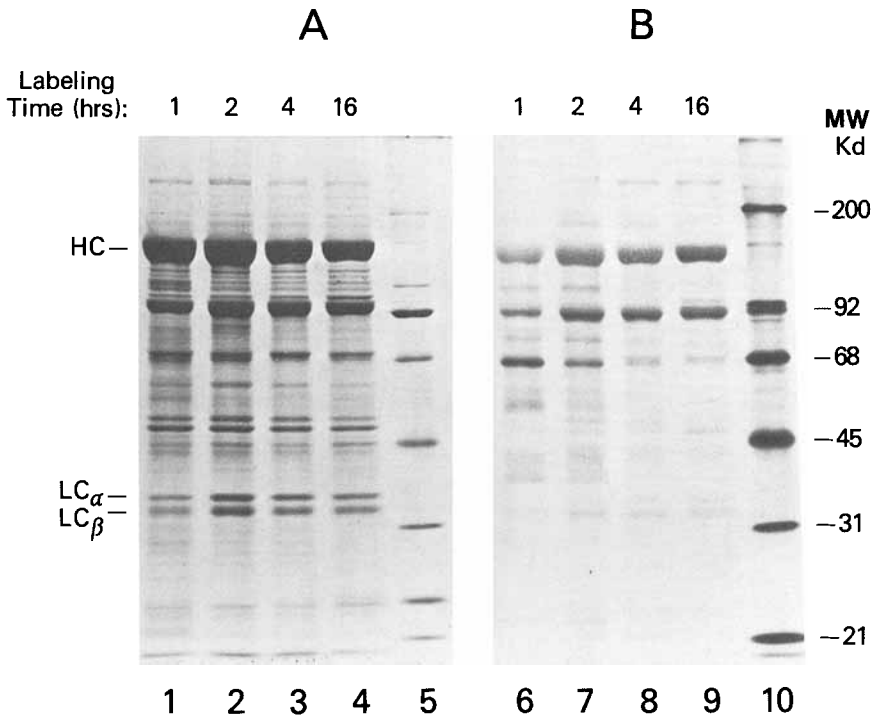


Fig. 4. Time course of in vivo incorporation of newly formed proteins into purified rat liver CVs. (A) Coomassie-stained 5–15% acrylamide gradient gel of CVs purified from single rat livers labeled for the indicated times with [$^{35}$S]methionine as described. (B) Fluorogram of gel shown in (A). HC denotes heavy-chain clathrin (~ 180 kD). $LC_\alpha$ and $LC_\beta$ represent the 34- and 32-kD clathrin light chains.

Figures 4B and 7B demonstrate that, in sharp contrast to the labeling kinetics observed for the above-mentioned proteins, the incorporated radioactivity of the 53-kD and 68-kD species fell off rapidly between 30 min and 4 hr (Fig. 7B), after an initial rise between 5½ and 20–30 min. A similar initial (20–30 min) rise in radioactivity was observed for 108-kD protein, which was followed by a modest decrease between 30 and 90 min (Fig. 7B) and a substantial decline between 2 and 4 hr (Fig. 4B).

## DISCUSSION

Electron microscopy of our purified rat liver CVs demonstrated a variety of sizes present (data not shown). Size variation within preparations of CVs has been observed for a variety of tissues [9,10]. Whether this implies heterogeneity with respect to area of subcellular origin, protein composition, and/or function or directionality of transport has not been established. Morphologic studies have suggested that CVs in the Golgi region tend to be somewhat smaller than their counterparts nearer the plasma membrane [9,10].

Nothing is known as to the mode of transport of newly synthesized clathrin to sites of coated pit and CV formation. The existence of a substantial pool of free, soluble, non-membrane-associated clathrin has been doubted by Willingham and coworkers [22], who found very little evidence for free cytoplasmic clathrin by ultra-

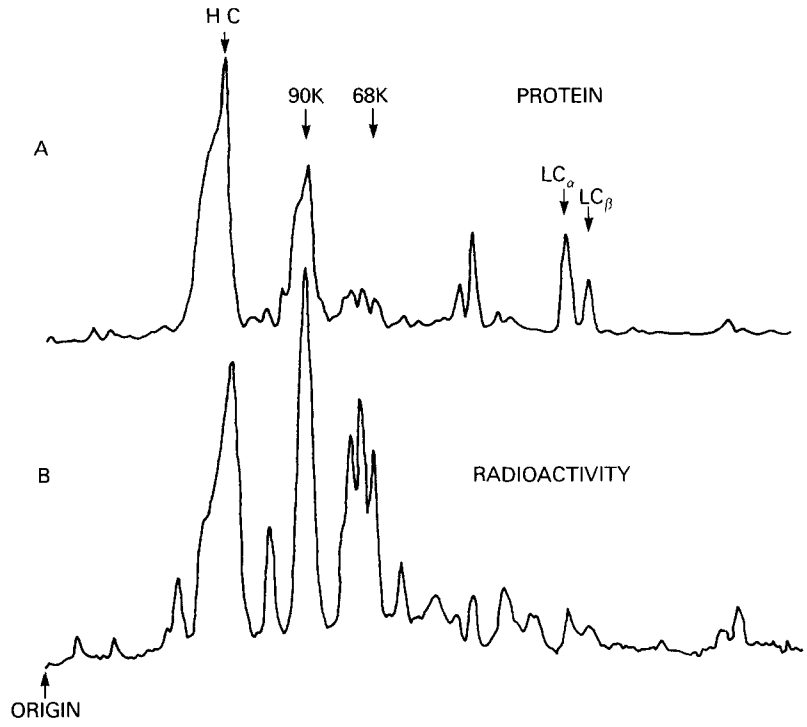


Fig. 5. Densitometric scans of 5–15% acrylamide SDS-PAGE of purified CVs derived from rat liver labeled in vivo for 2 hr with [$^{35}$S]methionine. (A) Scan of Coomassie-stained gel at 590 nm. (B) Radioactivity profile obtained by scanning fluorogram of dried gel in (A) at 550 nm. Component bands are numbered. HC denotes heavy-chain (~ 180 kD) clathrin subunit. LC$_\alpha$ and LC$_\beta$ refer to the 34-kD and 32-kD MW light-chain clathrin subunits.

structural cytochemical techniques. In contrast, Louvard et al employed a radioimmunoassay to quantify clathrin in the cytosol and found a significant level of disassembled clathrin in the 100,000g supernatant of the total immunoreactive cellular clathrin [23].

If the CVs that are isolated by methods such as those herein described derive exclusively from a pool of free CVs, then one could expect that a delay between incorporation of clathrin into coated pits and transformation of a coated pit into a CV (such as during receptor-mediated endocytosis) would contribute to a delay in radioactivity incorporation into free coated vesicles.

Depending on rates of coated pit turnover, the observation of a small amount of labeled heavy-chain clathrin within the preparation of purified CVs after only 5½ min of in vivo labeling is compatible with a portion of the CVs isolated not deriving exclusively from receptor-mediated endocytosis from plasma membrane coated pits, but rather with some being derived from the cytosol, or from the Golgi region (which is in close proximity to rough endoplasmic reticulum). Nevertheless, because the period of most rapid incorporation of [$^{35}$S]clathrin into purified CVs is during the initial 2 hr of labeling, it is possible that the bulk of purified CVs could be derived from plasma membrane coated pits.

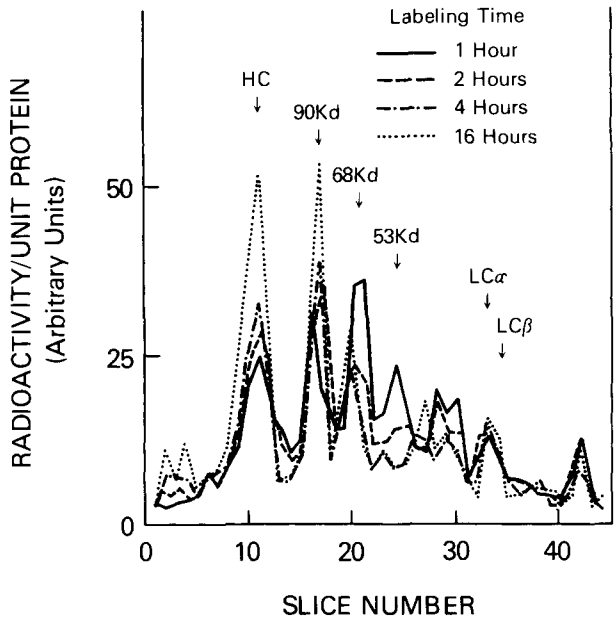


Fig. 6. Radioactivity profiles of CVs from rat liver labeled in vivo with [$^{35}$S]methionine for 1, 2, 4, and 16 hr as analyzed by liquid scintillation counting of slices of a 5–15% acrylamide SDS gel. MWs of major peaks are as indicated. HC refers to the (180-kD) heavy-chain clathrin subunit. $LC_\alpha$ and $LC_\beta$ refer to the 34-kD and 32-kD MW clathrin light chains, and were contained in slices 33 and 34, respectively. To correct for variations in the quantity of protein applied per gel lane, the cpm in each slice were divided by the area under the 180-kD clathrin peak of the densitometric scan of the corresponding Coomassie-stained lane.

The fact that groups of $^{35}$S-labeled CV proteins show divergent kinetic behavior for in vivo incorporation into purified CVs is compatible with subpopulations of CVs having overlapping, but nonidentical protein compositions. The observation that the radioactivity of two proteins of MW 68 kD (mobility on SDS gels identical to bovine serum albumin) and 53 kD decreases beyond 30 min of in vivo labeling, while the labeling of heavy-chain clathrin in CVs continues to increase, raises the possibility that the former proteins are selectively lost from (at least a subpopulation of) CVs.[3] Perhaps these are secretory proteins being transiently carried by CVs. It is interesting that the peak of radioactivity for the 68- and 53-kD proteins associated with CVs is between 20 and 30 min, roughly paralleling the previously observed kinetics for the passage of newly synthesized albumin through the Golgi [25].

Clathrin heavy chains, unlike any other CV proteins studied, demonstrated substantial further increases in radioactivity incorporated into CVs between 4 and 16

[3]Conclusive identification of the 68kD protein as rat serum albumin has not been attempted. To implicate CVs in the pathway of albumin secretion would require not only further characterization of this radioactive 68-kD protein with 2-D gels and/or immunoprecipitation but would also require an examination of the resistance of the band to coat removal and/or proteolytic digestion of the isolated CVs in order to be certain that the labeled protein was contained within the lipid vesicle and not merely surface-associated with the coat.

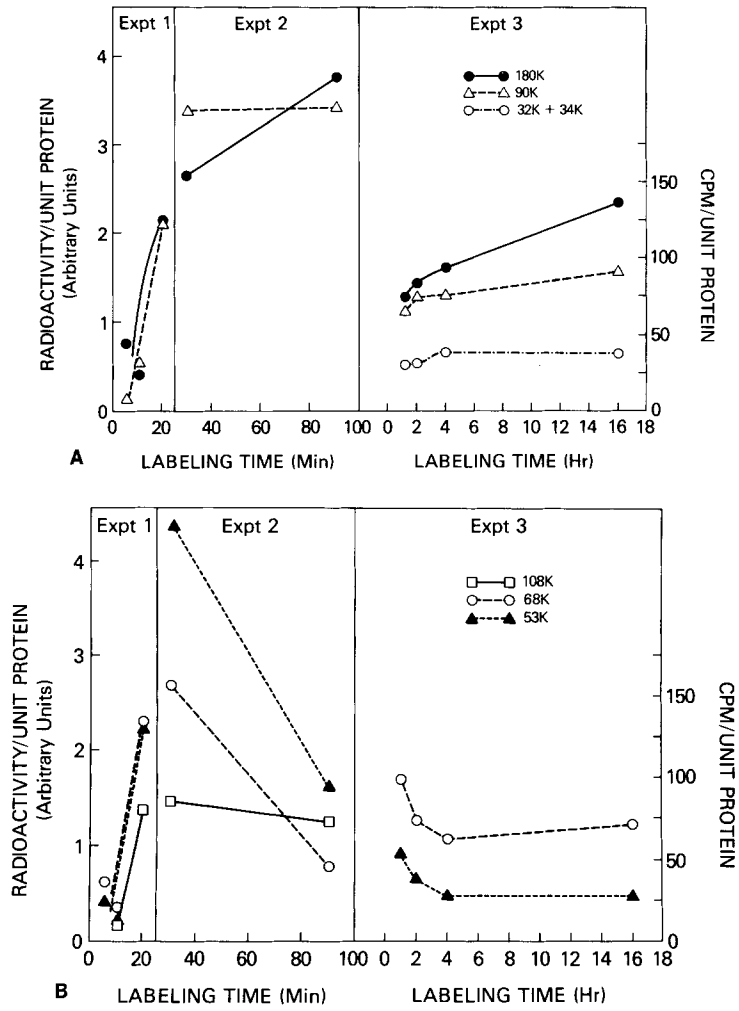


Fig. 7. Time course of incorporation of newly synthesized proteins into purified CVs derived from in vivo labeled rat livers. Radioactivity per unit protein is plotted as a function of labeling interval. The results shown are a composite of three separate experiments performed with labeling intervals of 5.5, 11, and 20 min (left panels), 30 and 90 min (center panels), and 1.08, 2, 4, and 16 hr (right panels). Purified CVs were run on 5–15% acrylamide SDS gels which were stained with Coomassie Brilliant Blue and scanned as described. For the left and center panels, radioactivity was determined by scanning a fluorogram of the gel and automatic integration of the areas under each radioactive protein peak. For the right panel, the radioactivity in each band at each labeling time was determined by couting 2-mm gel slices in Ultrafluor as described. Ordinate values were obtained by dividing the radioactivity in each peak by the area under the 180-kD clathrin peak as given by the automatic integration during scanning of the Coomassie-stained gels. This latter value was proportional to the total protein applied to the gel. It should be noted that, while the vertical scale within each column of figures corresponding to one experiment is the same, different scales are used for each experiment in different panels (left, center, and right). In experiments where rat body weights varied by $> \pm 4\%$, the results were normalized to a standard dose of 1.58 mCi [$^{35}$S]methionine per 300g rat body weight.

hr of labeling—a period during which the radioactivity in other CV-associated proteins had either reached a plateau level or decreased. This suggests that a fraction of newly synthesized clathrin may be temporarily sequestered in a form or compartment unable to contribute to CV formation. Perhaps some posttranslational modification of clathrin occurs as a preresquisite to polymerization into the coat structure. It is conceivable that coated regions of the plasma membrane might be functionally separated into classes of fast and slow turnover with correspondingly different rates of CV formation. It also appears possible that recycling of clathrin heavy chains occurs after CV uncoating. The data presented are compatible with CV-associated clathrin turnover occurring at a slower rate than that seen with other CV proteins. In order to more precisely elucidate steps leading from clathrin biosynthesis to incorporation of clathrin into CVs, future studies may combine the methodology used in the present report with determinations of total cellular clathrin pool size and the relative specific activities of total clathrin, membrane-associated clathrin, and free cytoplasmic clathrin employing immunoprecipitation techniques. Such studies have been hampered in the past by lack of availability of suffucient quantities of anti-clathrin-heavy-chain antibodies.

In summary, the kinetics of radioactivity incorporation into CVs displayed divergent behavior among several groups of CV proteins. In particular, 53-kD and 68-kD proteins showed a rise and fall during the course of experiments, whereas other proteins reached a plateau or exhibited a prolonged rise in radioactivity in CVs over time. It is hypothesized that these 53- and 68-kD proteins may be contained in a subpopulation of CVs involved in transport of secretory proteins, rather than in receptor-mediated endocytosis. The possibility is also raised that a subfraction of newly synthesized clathrin molecules might undergo some type of processing or compartmental transfer step resulting in delayed incorporation into CVs. Further studies may directly assess questions raised herein regarding functional heterogeneity within purified CV preparations.

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