Biochemical and Cytochemical Evidence Indicates That Coated Vesicles in Chick Embryo Myotubes Contain Newly Synthesized Acetylcholinesterase

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ABSTRACT We have isolated highly purified coated vesicles from 17-d-old chick embryo skeletal muscle. These isolated coated vesicles contain acetylcholinesterase (AChE) in a latent, membrane-protected form as demonstrated enzymatically and morphologically using the Karnovsky and Roots histochemical procedure (*J. Histochem. Cytochem.*, 1964, 12:219–221). By the use of appropriate inhibitors the cholinesterase activity can be shown to be specific for actylcholine. It also can be concluded that most of the AChE represents soluble enzyme since it is rendered soluble by repeated freeze-thaw cycles.

To determine the origin of the coated vesicle–associated AChE, we have isolated coated vesicles from cultured chick embryo myotubes which have been treated with diisopropylfluorophosphate, an essentially irreversible inhibitor of both intra- and extracellular AChE, and have been allowed to recover for 3 h. This time is not enough to allow any newly synthesized AChE to be secreted. These coated vesicles also contain predominantly soluble AChE. These data are compatible with the hypothesis that coated vesicles are important intermediates in the intracellular transport of newly synthesized AChE.

The pathway by which cells make and secrete proteins involves synthesis on ribosomes associated with the rough endoplasmic reticulum, modification in the Golgi apparatus, and eventual release at the plasma membrane. During the last 10 years, it has become evident that essentially all secretory proteins follow this now classic route first described by Palade (for review see reference 1). Palade also presented evidence that transitional vesicles were involved in the transport from the rough endoplasmic reticulum to the Golgi apparatus (1). Evidence has accumulated supporting a vesicular intermediate mediating the transport from the Golgi apparatus to plasma membrane in non-Ca^{++}-regulated secretory processes (for review see reference 2). Although the nature of these vesicular transport vehicles has not been determined, the capacity to recognize subcellular compartments and the ability to move through the cell would seem to be essential qualities.

Clathrin-coated vesicles have generated considerable interest as candidates for intracellular transporters. A large body of evidence now exists implicating coated vesicles in the processes of receptor-mediated endocytosis (for review see references 3 and 4) and membrane recycling in the presynaptic element of the neuromuscular junction (5, 6). Both of these processes require recognition capacities and mobility. Franke et al. (7) found that coated vesicles and large secretory vesicles that appeared to have coats were involved in the secretion of casein from lactating rat mammary epithelial cells, supporting the possibility that coated vesicles function as carriers of secretory molecules.

Recently, biochemical evidence has been presented by Rothman and Fine (8) which suggests that coated vesicles are intermediates in the transport of the newly synthesized membrane glycoprotein of vesicular stomatitis virus at two or more stages in its transit from the endoplasmic reticulum to the plasma membrane. This conclusion has been challenged on morphological grounds (9). However, work by Kinnon and Owen (10) has suggested that coated vesicles serve as intermediates at multiple stages in the transport of newly synthesized histocompatibility antigens, HLA-A,-B, and -D, three integral plasma membrane proteins, between the endoplasmic

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reticulum and the plasma membrane. Moreover, they present evidence that coated vesicles are involved in the intracellular transport of newly synthesized secretory IgM molecules to the cell membrane.

We have chosen to investigate the possible role of coated vesicles in the intracellular transport of newly synthesized acetylcholinesterase (AChE)[1] molecules by embryonic chick skeletal muscle cells. This molecule has been previously shown to follow the classic secretory pathway (11). We present biochemical and cytochemical evidence that coated vesicles within chick embryo skeletal muscle cells contain newly synthesized AChE molecules, supporting the hypothesis that these vesicles are important intermediates in the intracellular transport of this predominantly secretory glycoprotein. A preliminary report of this work has been published (12).

MATERIALS AND METHODS

Isolation of Coated Vesicles from Embryonic Chick Skeletal Muscle: 300 g of leg and breast muscle dissected out of 17- or 18-d-old chick embryos were placed in 300 ml of 0.1 M 2-(*N*-morpholino)ethane sulfonic acid, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide, pH 6.5 (MES buffer) and homogenized by three 15-s bursts in a Waring blender. The homogenate was centrifuged for 15 min at 13,000 *g*. The resulting supernatant was adjusted to contain 10 μg/ml bovine pancreatic ribonuclease A and centrifuged at 100,000 *g* for 60 min. The pellet was resuspended in a small volume of MES buffer with 10 strokes of a tight-fitting Dounce homogenizer and adjusted to 6% (wt/vol) sucrose, 6% (wt/vol) Ficoll type 70. This mixture was centrifuged for 20 min at 100,000 *g* and the supernatant decanted and set aside. The pellet was rehomogenized and again brought to 6% sucrose, 6% Ficoll type 70, and centrifugation was repeated. Supernatants were combined and diluted with MES buffer to 1% (wt/vol) sucrose, 1% (wt/vol) Ficoll type 70, and centrifuged at 100,000 *g* for 60 min. The resulting pellet was resuspended in a minimal amount of MES buffer and layered upon two discontinuous gradients of 5 ml each of 20, 30, 40, 50, 55, and 60% (wt/vol) sucrose in MES buffer. The gradients were centrifuged for 14–16 h at 55,000 *g* in an SW 27 rotor. The coated vesicle fraction, seen as an opalescent band at the 50–55% interface, was removed dropwise through a puncture at the bottom of the tube. Purified coated vesicles were obtained by diluting this fraction twofold and pelleting the material at 100,000 *g* for 60 min. The pellet was resuspended in a minimum of MES buffer and spun at 12,000 *g* for 5 min. The supernatant was collected and the pellet resuspended and respun three times. All supernatants were combined. In some experiments, to achieve separation of individual coated vesicles from aggregated coated vesicles and filaments, the supernatants were layered onto four gradients of 800 μl each of 3, 10, 16, 23, and 30% Ficoll type 70 (wt/vol) in 9, 24, 50, 70, and 90% D_2O respectively in MES buffer, pH 6.5. The gradients were spun in an SW 50.1 rotor at 170,000 *g* for 20 min. Nonaggregated coated vesicles were obtained from the first two gradient layers and either used directly or pelleted and resuspended as described above for the total coated vesicle fraction.

Protein and AChE Assays: Protein determinations were made by the method of Lowry et al. (13) or by the fluorescamine method (14). Measurements of AChE activity were made by the radiometric assay of Johnson and Russell (15) using [^{14}C]acetylcholine (New England Nuclear, Boston, MA) as a substrate, or by the colorimetric assay of Ellman et al. (16). Inhibitors of AChE (BW284C51 [Burroughs Wellcome & Co., Raleigh, NC]) and pseudocholinesterase (tetraisopropylpyrophosphoramide [iso-OMPA] [Worthington Biochemical Corp., Freehold, NJ]) were used to determine the specificity of the reaction (17). Various fractions taken throughout the isolation procedure, as well as samples of coated vesicles, were assayed for AChE activity in the presence and absence of 1% Triton X-100 to look for unmasking of activity. The enzyme is totally stable under these conditions (18). Coated vesicle samples were assayed in the absence of inhibitors, in the presence of 10^{-4} M iso-OMPA, and in the presence of 10^{-4} M BW284C51. All assays on 17-d-old embryonic muscle coated vesicles were performed in triplicate and are expressed as the mean of these determinations. Additionally, coated vesicle samples were taken through three cycles of freeze-thawing and then centrifuged at 100,000 *g* in a Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, CA) for 10 min to pellet ruptured vesicles. The pellet and supernatant were assayed for AChE activity in triplicate.

Agarose and PAGE: Agarose gel electrophoresis was performed on 17-d-old embryonic muscle coated vesicles according to the method of Rubenstein et al. (19). SDS PAGE was performed by the method of Laemmli (20).

Electron Microscopy (EM) of Isolated Coated Vesicles: All coated vesicle samples were assessed for purity by EM of preparations negatively stained with 2% uranyl acetate. The purity of five preparations were also evaluated by morphometry of thin section of pelleted coated vesicles cut parallel to the axis of centrifugal force. 17-d-old embryonic muscle coated vesicles were stained by the method of Karnovsky and Roots (21) for AChE. Staining solutions contained either the substrate, acetylthiocholine iodide (ATCh), and an irreversible inhibitor of AChE, diisopropylfluorophosphate (DFP); ATCh and no DFP; or no ATCh and no DFP. Coated vesicles were reacted for 15 min and then pelleted for 15 min at 100,000 *g* in a Beckman Airfuge (Beckman Instruments, Inc.). Pellets were washed in 0.1 M maleate buffer, pH 6.0, and fixed for 1 h at 4°C in 0.1 M sodium phosphate buffer, pH 7.4, containing 3% EM grade glutaraldehyde, 0.5% paraformaldehyde, and 1.2% tannic acid. Pellets were rinsed in three 15-min changes of 0.1 M phosphate buffer, pH 7.4, and postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h at 4°C. Postfixed pellets were rinsed as above, dehydrated in graded ethanols through propylene oxide, and embedded in Araldite 502 (22). Silver sections were cut on a Porter-Blum MT-2B ultramicrotome and viewed unstained or stained with alcoholic uranyl acetate and 0.2% lead citrate with a Philips 300 or an AEI 6B electron microscope.

Culture of Chick Myotubes: Single cell suspensions of myoblasts were obtained by mincing and mechanical disruption of thigh and breast muscle from 11-d-old chick embryos. Cells were plated in 60-mm (for AChE assays) and 35-mm (for EM) tissue culture dishes (Falcon Labware, Oxnard, CA) that had been coated with 1% gelatin. The plating densities were 2.88×10^6 cells/60-mm dish and 1.2×10^6 cells/35-mm dish. Cells were plated with Eagle's minimum essential medium (MEM) containing 10% horse serum, 100 U/ml streptomycin, 100 μg/ml penicillin, and 30 μg/ml iron-conjugated conalbumin (Type II, Sigma Chemical Co., St. Louis, MO). At 29 h after plating, cells were fed with the above medium containing 10^{-5} M cytosine arabinocide (Sigma Chemical Co.). On day 4 of culture, plates contained many large, multinucleate myotubes, some smaller myotubes, and a few mononucleate cells. Large myotubes exhibited spontaneous contraction, but little or no striation when examined by phase-contrast microscopy.

AChE, Whole Cell Assay: 60-mm plates were divided into six groups of four plates each on day 4 of culture. Groups 1 and 2 were treated with 4 ml of Eagle's MEM for 10 min and rinsed in three 1-liter volumes of phosphate-buffered saline (PBS). Group 1 cells were harvested in a 50-mM Tris buffer (pH 7) containing 0.15 M NaCl, 0.25 mM EDTA, and 0.5% Triton X-100. 4 ml of Eagle's MEM were added to the group 2 plates which were placed back in the 37°C, CO_2 incubator. Plates from groups 3–6 were treated with 4 ml of Eagle's MEM containing 10^{-4} M DFP for 10 min, and rinsed sequentially in three 1-liter volumes of PBS. Cells from group 3 were harvested in the above Tris buffer; cells from groups 4 and 6 were given 4 ml/plate of Eagle's MEM; cells from group 5 were given 4 ml/plate of Eagle's MEM containing 100 μg/ml cycloheximide. Groups 4–6 were incubated as above for group 2. After a 3-h incubation, medium was collected from plates in groups 2, 4, 5, and 6 for assay of AChE activity. Cells from groups 2, 4, and 5 were harvested in the above Tris buffer. Group 6 plates were given 4 ml/plate of Eagle's MEM containing 100 μg/ml cycloheximide and returned to the 37°C incubator for 3 h. The medium from these plates was collected and the cells were harvested. Media and cells were assayed for AChE activity in the absence and presence of 10^{-4} M BW284C51 by the colorimetric assay of Ellman et al. (16).

Isolation of Coated Vesicles from Cultured Chick Myotubes: For coated vesicle isolation from myotubes, myoblasts from 9–11 dozen 11-d-old chick embryos were plated at a density of 2×10^7 cells/dish on $(245 \text{ mm})^2$ tissue culture dishes (A/S NUNC, Denmark) which were coated with 0.5% gelatin. Cells were fed once 3 d after culturing with culture medium containing 10^{-5} M cytosine arabinocide (Sigma Chemical Co.).

On day 5, plates were treated for 10 min at room temperature with Eagle's MEM containing 10^{-3} M DFP. Plates were then rinsed in three 1-liter volumes of physiological PBS. The dishes were each given 50 ml of Eagle's MEM and put back in the 37°C, CO_2 incubator. After 3 h, cells were scraped off the dishes with rubber jobbers and pelleted for 10 min at 1,000 *g*. Cells were swollen for 5 min at 0°C in an equal volume of 10 mM Tris, 1 mM EDTA, pH 7.5, and then disrupted using 20 strokes of a tight-fitting glass Dounce homogenizer. Enough five-times concentrated MES buffer was added to give a final concen-

[1] *Abbreviations used in this paper:* AChE, acetylcholinesterase; ATCh, acetylthiocholine iodide; DFP, diisopropylfluorophosphate; EM, electron microscopy; iso-OMPA, tetraisopropylpyrophosphoramide; MEM, minimum essential medium; MES buffer, 0.1 M 2-(*N*-morpholino)ethane sulfonic acid, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide, pH 6.5.

tration of 0.1 M MES and the homogenate was centrifuged at 600 *g* for 5 min. The pellet was resuspended in MES buffer and rehomogenized in a Sorvall omnimixer with three 10-s bursts. The homogenate was respun as above. The supernatants were combined, 10 μg/ml ribonuclease was added, and crude membranes were pelleted by centrifugation at 100,000 *g* for 60 min. The remainder of the preparation was identical to that described for the isolation of coated vesicles from whole muscle.

RESULTS

Characterization of Coated Vesicles Isolated from 17-d-old Chick Embryo Myotubes

We have used a technique for coated vesicle purification from chick embryo skeletal muscle based on a modification of the original Pearse procedure (23) which successfully purified coated vesicles from brain and liver (24). Coated vesicles isolated using this method appeared qualitatively to be >90% pure by morphological criteria using negative staining (Fig. 1*A*). Coated vesicles and baskets comprise 98 ± 1.2% of the

TABLE I. *Effects of Detergent Solubilization and Trypsin Treatment on Coated Vesicle AChE Activity*

Preincubation	Incubation	Acetylcholine hydrolyzed	Retained activity
		cpm	%
—	Triton	27,912	100
—	—	8,231	29
—	Triton + 10^{-4} M Iso OMPA	24,773	89
—	Triton + 10^{-4} M BW284C51	1,313	4.7
—	10^{-4} M DFP	8	0.03
Trypsin	Triton	25,063	90
Trypsin	—	8,126	29
Trypsin + Triton	Triton	5,829	21

Purified chick embryo muscle coated vesicles (10 μg) in MES buffer were incubated with or without 0.025% trypsin in the presence or absence of 0.3% Triton X-100 for 1 h at 38°C (*Preincubation*). All samples were brought to 0.1% soybean trypsin inhibitor and 24 mM [^{14}C]ACh (100,000 cpm) again in the presence and absence of Triton X-100. 10^{-4} M Iso-OMPA, 10^{-4} M BW284C51, and 10^{-4} M DFP, specific inhibitors of pseudo- and acetylcholinesterase and both, respectively, were also included as indicated. The samples were incubated for 1 h at 22°C (*Incubation*) and acetylcholine hydrolysis determined by the method of Johnson and Russell (15). Results represent the mean of three independent experiments each carried out in duplicate. SEM was <5% in all cases.

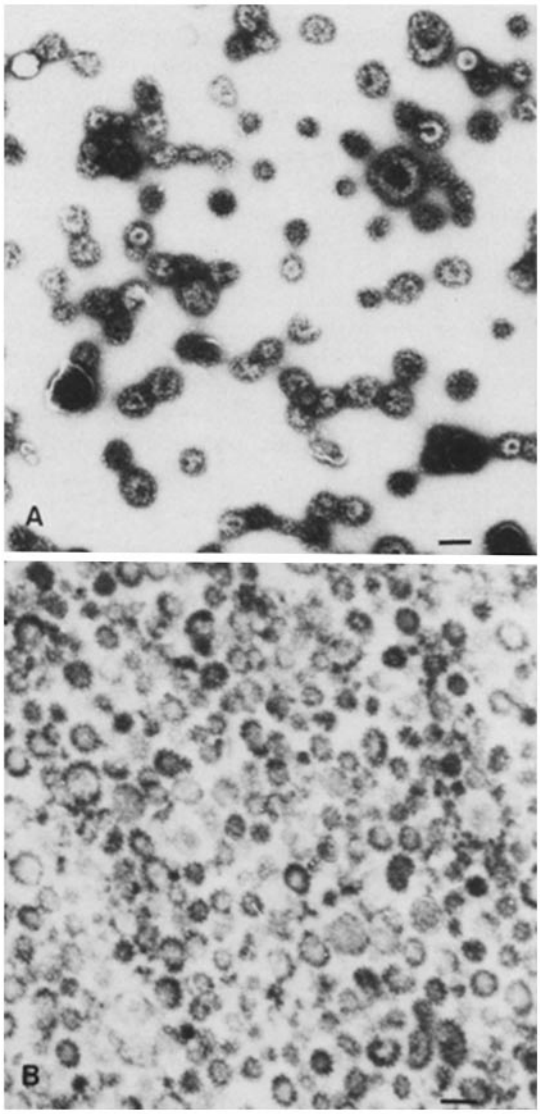


FIGURE 1 Coated vesicles isolated from the leg muscles of 17-d-old embryonic chickens. (*A*) Negatively stained coated vesicles after sucrose gradient purification. × 48,000. (*B*) Thin section of gradient-purified coated vesicles that were fixed in 1% glutaraldehyde + 4% paraformaldehyde + 1.2% tannic acid in 0.1 M phosphate buffer, pH 7.4, and viewed on an AEI 6B electron microscope. The section was cut parallel to the axis of centrifugation. Bars, 0.1 μm. × 54,000.

TABLE II. *Protein, AChE Activity, Enrichment, and Latency at Successive Stages of Coated Vesicle Purification from Chick Embryo Muscle*

	Protein		Total AChE (+ Triton)				Exposed AChE (− Triton)			
		Recovery		Recovery	Specific activity	Enrichment		Recovery	Specific activity	Enrichment
	mg	*% of LSS*	*cpm × 10^{-3}*	*% of LSS*	*cpm/mg*	*fold*	*cpm × 10^{-3}*	*% of LSS*	*cpm/mg*	*fold*
LSS‡	5,775	—	959.4	—	166.1	—	1,138	—	197.1	—
HSP§	952	16.5	609.7	63	640.4	3.86	573	50.4	602	3.05
Ficoll–Sucrose supernatant	30	0.53	132.4	13.8	430.9	2.59	48.5	4.3	159	0.808
Sucrose gradient–purified coated vesicles	3.78	0.065	0.651	0.068	172.13	1.03	0.196	0.017	51.9	0.264

Successive stages of the coated vesicle purification were monitored for protein by the fluorescamine method (14) and for AChE activity

* %Latency = (AChE masked)(AChE total) × 100.

‡ LSS, low speed supernatant.

§ HSP, high speed pellet.

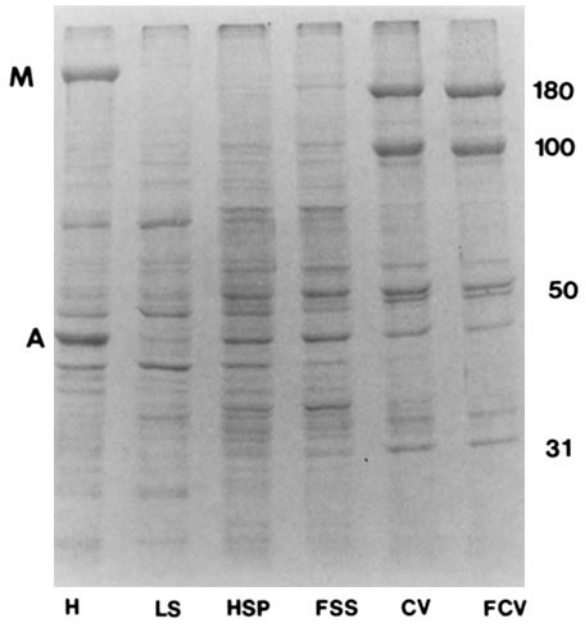


FIGURE 2 7.5% SDS PAGE demonstrating the purification of coated vesicle proteins from chick embryo skeletal muscle. 50 μg of protein from each of the stages of coated vesicle purification were dissolved in sample buffer and electrophoresis was carried out as described (22). *H*, homogenate; *LS*, low speed supernatant; *HSP*, high speed pellet; *FSS*, Ficoll–Sucrose supernatant; *CV*, coated vesicles removed from a sucrose equilibrium density gradient; *FCV*, coated vesicles removed from a Ficoll–Sucrose–D_2O gradient. The mobilities of the major brain coated vesicle polypeptides (180, 100, 50, and 31 kD, respectively) are indicated as are the mobilities of chick skeletal muscle myosin heavy chain (*H*) and actin (*A*).

samples as determined by morphometry of random fields from several preparations examined by thin section EM (Fig. 1 *B*). The sample pellets, cut parallel to the axis of centrifugation, appeared homogenous throughout. 82.5 ± 3.5% of the baskets contained vesicles and very few smooth vesicles were present. Partially coated membranous structures resembling CURL (compartment of uncoupling receptors and ligands) (25) were infrequently present and 50-nm diameter filaments having the appearance of actin were also seen. The average diameter of the coated vesicles was ~750Å, similar to that of brain coated vesicles (23).

Fig. 2 shows a 9% SDS gel which monitors the successive stages of the coated vesicle purification. The initial homogenate contained principally actin and myosin, most of which was removed by low speed centrifugation as seen by the reduced amounts of these proteins in the low speed supernatant. The clathrin band can first be distinguished in the low speed supernatant. Removal of large membrane aggregates and filaments by centrifugation through Ficoll–Sucrose achieved a further small enrichment of clathrin, and sucrose density gradient centrifugation resulted in a great enrichment of clathrin and the associated 100-, 50–55-, and 30–35-kD groups of peptides characteristic of coated vesicles purified from many different tissues (see references 19 and 24, for example). A further sedimentation through Ficoll-D_2O resulted in the removal of all myosin, and most of the residual actin, from the preparation.

Masked AChE (Total–Exposed)				Latency*
	Recovery	Specific activity	Enrichment	
cpm × 10^{-3}	*% of HSP*	*cpm/mg*	*fold*	%
0	—	0	—	0
36	—	37.9	—	5.9
83	237	272	7.18	63.1
0.455	1.3	120	3.17	69.9

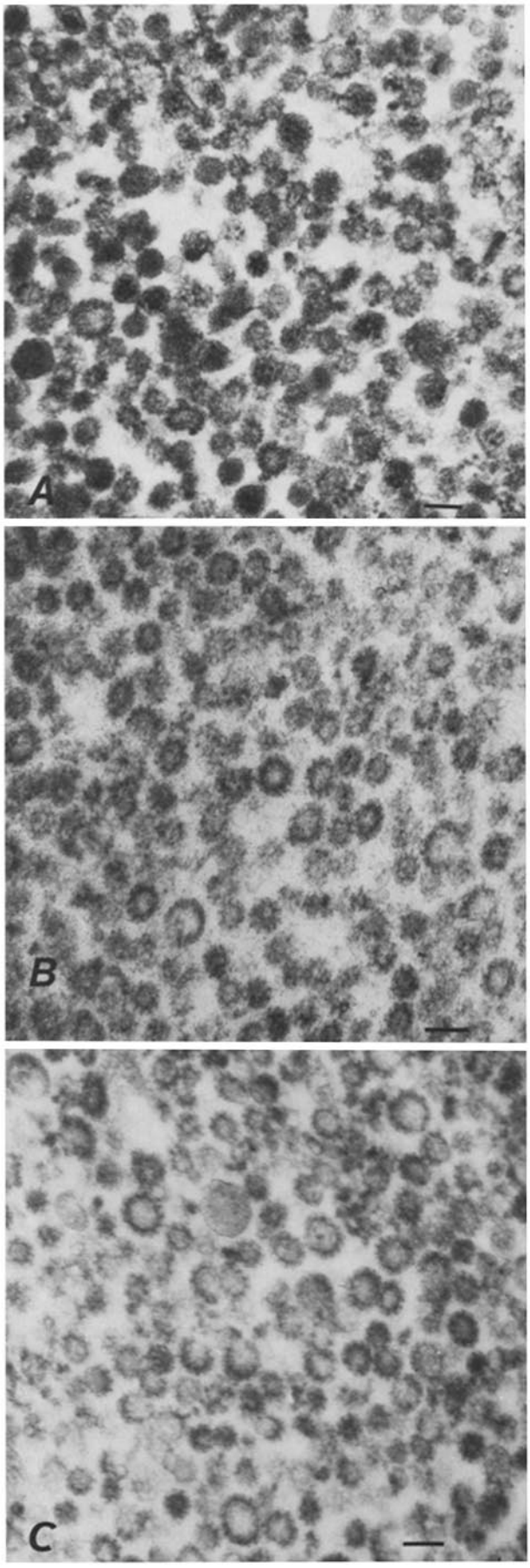
by the method of Johnson and Russell (15).

AChE Activity Is Associated with Highly Purified Coated Vesicles in a Latent Form

Table I demonstrates that highly purified chick muscle coated vesicles contained readily detectable AChE activity when assayed by the technique of Johnson and Russell (15). This assay directly measures the hydrolysis of acetylcholine to acetate and choline in the presence of 1% Triton X-100 which has no effect on the rate of acetylcholine hydrolysis (18). In our hands, with this assay, detergent has very little effect on the measurement of soluble esterase, although the detergent-treated samples may show up to a 15% decrease in activity, possibly due to decreased partitioning of the acetate into the organic phase (e.g., the measurement of LSS activity, Table II). The latency of the AChE (70%) was shown by performing the assay in the absence of detergent resulting in a much lower activity. Pretreatment of coated vesicles with trypsin in the presence and absence of 1% Triton X-100 before assaying activity demonstrated that most of the AChE is protected from trypsin degradation unless detergent was also present. This finding also indicates the AChE is contained in a membrane-enclosed compartment. Table I also demonstrates that the AChE activity in the coated vesicles was completely inhibited by 10^{-4} M BW284C51, a known inhibitor of AChE or by DFP, a potent irreversible inhibitor of AChE which can freely penetrate the intact membrane. The activity was only slightly inhibited by 10^{-4} M iso-OMPA, a selective inhibitor of pseudo- or butyrylcholinesterase. These data indicate that AChE is the major cholinesterase activity found in the coated vesicles. This is not surprising since at least 95% of the cholinesterase activity present in skeletal muscle is AChE (11).

The AChE Associated with Coated Vesicles Is the Secretory Form

Chick embryonic skeletal muscle contains two classes of AChE, a soluble "secretory" form and a hydrophobic integral membrane-associated form (11). To distinguish between these two forms, we subjected the coated vesicles to three cycles of freezing and thawing in isolation buffer and separated the membrane-containing pellet and supernatant by centrifugation. Over 80% of the activity was solubilized by this procedure. Over 90% of the total activity could be solubilized if 1 M NaCl was added to the coated vesicle-containing solution before freezing and thawing. These data indicate that most, if not all, of the AChE is the secretory form as would be expected since it is known that most of the intracellular AChE in skeletal muscle is also the secretory form (11).



The Karnovsky–Roots Reaction Can Be Used to Localize AChE within Coated Vesicles

We used the histochemical technique of Karnovsky and Roots (21) to confirm the presence of AChE within coated vesicles isolated from chick embryo skeletal muscle. Coated vesicles shown in Fig. 3*A* were incubated for 15 min in ATCh, then fixed, sectioned, and examined by EM. Many of the vesicles showed dark reaction product within them. No reaction product was seen outside of the confines of the clathrin basket of the coated vesicles, which would be expected if the enzyme was nonspecifically sticking to the coat. The vesicles in Fig. 3*B* were incubated in the presence of ATCh and 10^{-5} M DFP. The coated vesicles pictured in Fig. 3*C* were incubated in the absence of ATCh. Neither the coated vesicles incubated without ATCh nor those incubated with ATCh and DFP showed appreciable staining.

Agarose Gel Electrophoresis

As further evidence that AChE is a true constituent of isolated coated vesicles and not a contaminant which copurifies with them through the sucrose gradient purification, we subjected sucrose gradient–purified coated vesicles to agarose gel electrophoresis, which removes smooth membranes and filaments and renders the coated vesicles nearly homogeneous by morphological criteria (19). Fig. 4*A* shows a micrograph of agarose gel–purified chick muscle coated vesicles negatively stained with 1% uranyl acetate. Only coated vesicles are seen.

The graph in Fig. 4 shows the results obtained when sequential segments of an agarose gel through which 20 μg coated vesicles had been electrophoresed were excised and assayed for AChE activity in the presence of 1% Triton X-100 by the method of Johnson and Russell (15). Over 90% of the total AChE activity added to the gel could be recovered in a region of the gel containing coated vesicles as determined by the presence of clathrin in these fractions by SDS PAGE (*A*). The latency of the AChE was shown by the much lowered activity seen when Triton X-100 was omitted (*B*). No increase in latency was seen, presumably because the aggregated coated vesicles, filaments, and smooth vesicles separated out by this technique have a roughly equivalent latency to that of the pure coated vesicles. All activity was abolished when the incubation was carried out in the presence of 10^{-4} M BW284C51 (*C*), confirming that the activity was that of true AChE.

As a further criterion for the association of the AChE with coated vesicles rather than with contaminating structures, we incubated aliquots of coated vesicles with either 4 μl of anti-clathrin serum (26) or with preimmune serum, and then subjected them to agarose gel electrophoresis as described above. The results shown in the inset of Fig. 5 reveal that incubation with anti-clathrin serum retards the major protein band significantly when compared with the coated vesicles incubated either with preimmune serum or with no serum. Fig. 5 demonstrates that the mobility of AChE is also de-

FIGURE 3 Thin sections of embryonic chick muscle coated vesicles stained for AChE by the method of Karnovsky and Roots (21), fixed, and pelleted. ATCh was used as a substrate and 10^{-4} M DFP as an inhibitor. (*A*) + ATCh, − DFP. × 57,000. (*B*) + ATCh, + DFP. × 66,000. (*C*) − ATCh, − DFP. × 57,000. Bars, 0.1 μm.

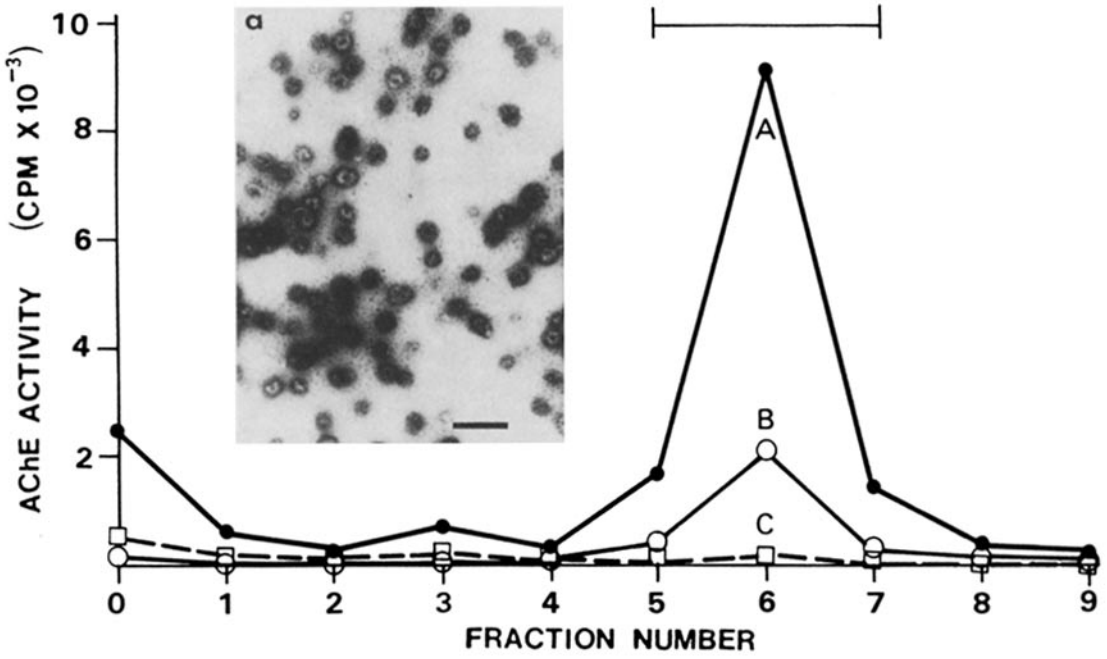


FIGURE 4 Latent AChE activity is associated with chick embryo skeletal muscle coated vesicles purified to near homogeneity by agarose gel electrophoresis. Two adjacent lanes each containing 25 μg of sucrose gradient-purified coated vesicles were subjected to agarose gel electrophoresis as described (19). The coated vesicle-containing portion of one lane identified as a turbid zone was excised from the gel, homogenized briefly, and the agarose removed by a 10-s spin in an Eppendorf centrifuge. A negatively stained preparation from the supernatant is shown in inset a (bar, 0.25 μm; × 29,000). Ten consecutive equal 0.5-cm slices of the second lane were excised and the agarose removed as described above. The supernatants were adjusted to 100 μl and three equal 25-μl aliquots assayed for AChE by the Johnson and Russell assay (15). *A* shows the result when the assay was performed in the presence of 1% Triton X-100; *B*, in the absence of detergent; *C*, in the presence of 1% Triton X-100 and 10^{-4} M BW284 C51. The other 25 μl from each sample was subjected to SDS PAGE as described above. The bar shows the fractions which contained clathrin detectable by Coomassie Blue staining. These fractions correspond to the positions of AChE activity, and coated vesicles shown in *a*.

creased in parallel with that of the clathrin by incubation with anti-clathrin serum. These data further support the contention that the AChE is an authentic coated vesicle constituent.

Enrichment of AChE in Coated Vesicles during Coated Vesicle Purification

Table II demonstrates the enrichment and recovery of total and latent AChE at successive stages of coated vesicle purification as well as the protein recovery. Several points are evident. One is that our yield of coated vesicles (1 mg/100 g of tissue) was considerably lower than that achieved with other tissues (~3–10 mg/100 g of tissue) (23, 27). One likely explanation is that a lower percentage of the total protein of skeletal muscle is coated vesicle associated than in other tissues because of the great amount of actomyosin present.

A second important point is that while we did not achieve an overall enrichment of AChE activity in purified coated vesicles we did achieve a highly significant 3.2-fold enrichment of latent AChE activity in coated vesicles which was reflected in the great percentage increase in latency achieved in going from the high speed pellet to purified coated vesicles. Since at least 60% of the total protein in coated vesicles represents clathrin and the other associated coat proteins (27), our calculated enrichment is certainly a considerable underestimate of the true value.

Since most of the AChE in our purified coated vesicles is soluble, one possible source of it is the trapping of soluble AChE within artifactually formed coated vesicles resulting from homogenization and/or subsequent manipulation during coated vesicle purification. To investigate this possibility, we incubated isolated 17-d-old chick embryo skeletal muscle with 10^{-3} M DFP. A 10-min incubation with 10^{-3} M DFP reduced the assayable AChE to an undetectable level. Coated vesicles isolated from this DFP-treated preparation contained no detectable AChE as well, demonstrating the irreversibility of DFP (data not shown).

We then divided a dissected preparation of 17-d-old chicken embryo skeletal muscle into two equal aliquots. To one we added 10^{-3} M DFP as described above. We then washed the muscle three times with MES buffer to remove any residual DFP. We added soluble eel electric organ AChE (Worthington Biochemical Corp.) to the DFP-treated preparation and isolated the coated vesicles from both preparations. We assayed aliquots of successive steps of the preparation for protein and AChE as in Table II. The main conclusions of the experiment are summarized in Table III. As can be seen, only 0.02% of the initial AChE was recovered in the final coated vesicle preparation from DFP-treated muscle to which eel AChE was added, while 0.33% was recovered in the control preparation. Even more importantly, the AChE recovered in the DFP plus exogenous AChE preparation showed no latency, indicating that it was probably sticking to the outside surface of the coated vesicle rather than being contained within an intact vesicle.

These data indicate that artifactually trapped AChE probably accounts for much less than 10% of the activity associated with purified coated vesicles, and hence suggests that the

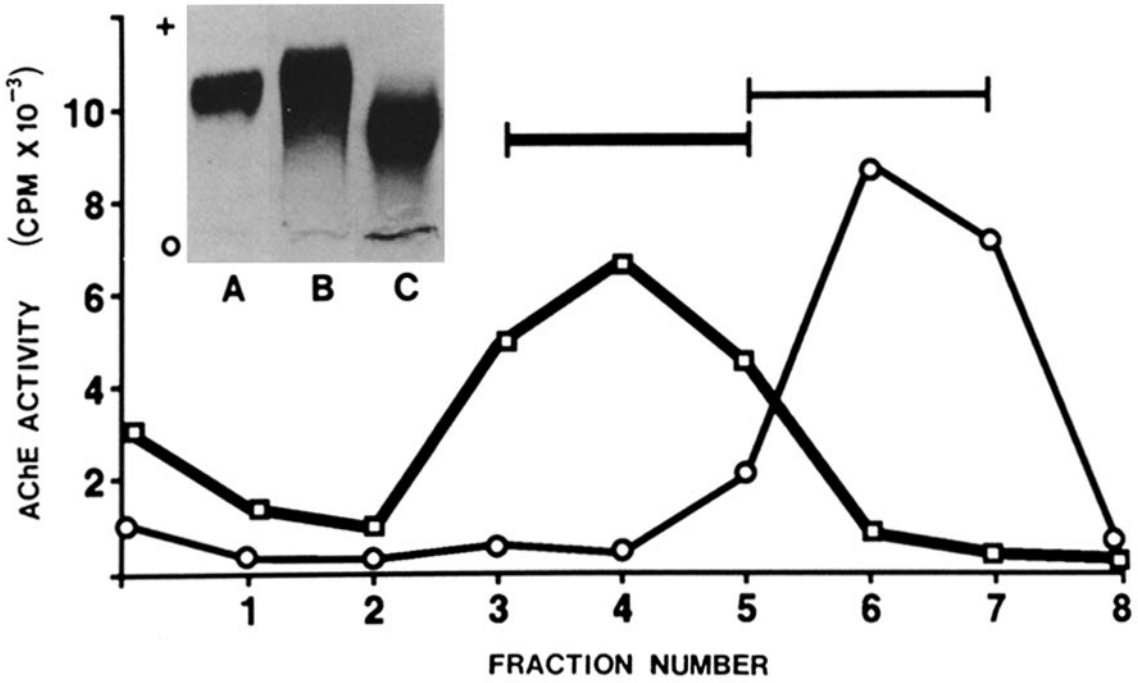


FIGURE 5 The mobility of chick muscle coated vesicles and AChE is reduced by incubation with anti-clathrin serum. To three identical 50-μg aliquots of coated vesicles in MES buffer were added (*A*) 4 μl of PBS; (*B*) 4 μl of preimmune serum; (*C*) 4 μl of monospecific anti-bovine brain clathrin serum (26). After incubation at 4°C for 1 h, each sample was divided into two equal portions and subjected to agarose gel electrophoresis (19). The inset shows the results obtained after partially drying down three adjacent gel lanes and staining for protein with Coomassie Blue. The main figure shows the results obtained when AChE and clathrin determinations were carried out on the samples incubated with preimmune (*B*) and anticlathrin (*C*) serum, respectively, using the methodology described in Fig. 4. □, amount of AChE activity in the gel slices from the anticlathrin treated samples (*C*). ○, amount of AChE activity in the gel slices from the preimmune-treated samples (*B*). The thick bar shows the fractions containing clathrin in the sample incubated with preimmune serum.

TABLE III. *Recovery of Exogenous AChE in Coated Vesicles**

	Protein	Specific AChE	Recovery	Latency‡
	mg	*cpm*	*% of LSS*	*%*
Control	4.6	4,574	0.33	69.8
DFP + eel AChE	3.1	39.3	0.02	0

* Comparison of protein, AChE activity, enrichment, and latency in purified coated vesicles isolated from DFP-treated muscle to which exogenous AChE was added and from a control generation.

‡ $\text{Latency} = \frac{\text{cpm (+ Triton)} - \text{cpm (− Triton)}}{\text{cpm (total)}} \times 100.$

AChE is an authentic constituent of chick muscle coated vesicles.

It should be noted here that our percentage recovery of AChE in this experiment was five times greater than in the experiment shown in Table II. We find considerable variation in our percentage recovery of AChE, coated vesicles, and other proteins from preparation to preparation. This may be due to the fact that the embryos used in diferent preparations unavoidably vary in age by as much as 20 h. Since the muscle cells are rapidly synthesizing and organizing myofibrils at this stage of development, the amount of protein in the low speed supernatants may vary significantly depending on the degree of myofibrillar organization.

Association of Newly Synthesized AChE with Coated Vesicles

The finding that AChE exists within coated vesicles leads to the question of the significance of this fact relative to the synthesis, transport, and degradation of the enzyme. Intracellular AChE may be either newly synthesized enzyme on its way to the cell surface, or extracellular enzyme that has been internalized by the cell. Approximately one-third of the cell-associated AChE in cultured myotubes is intrinsic to the plasma membrane (4) and could be internalized. Soluble AChE might also be taken up from the extracellular milieu. Since coated vesicles have been found to participate in both exocytotic and endocytotic processes, it is necessary to determine in which pathway coated vesicles containing AChE are involved. To this end, cultured chick muscle cells were treated with 10^{-3} M DFP on day 5 of culture, and allowed to recover for 3 h, a time period comparable to that required for reappearance of AChE activity at the cell surface or in the medium (11, 28). DFP inactivates cell surface AChE for at least 3 h after treatment with this inhibitor (11). Therefore any cell surface AChE internalized before this time would have been inactivated and not detectable by enzymatic methods. The half-time for the turnover of plasma membrane-associated AChE is on the order of 50 h (11), so very little would be expected to be internalized in 3 h in any case. Assays of whole cell homogenates and the medium for AChE activity (Table IV) confirmed that treatment of cells with 10^{-4} M DFP for 10 min (Group 3) abolished 97% of the total cellular AChE activity (Group 1). After the removal of DFP from the medium, cells again synthesized active AChE and eventually secreted this enzyme (11, 29). After a 3-h incubation in Eagle's MEM, no activity was found in the medium of cells treated with DFP (Group 4), while the medium of control cells (Group 2) contained 49% of the total cell-associated AChE.

TABLE IV. *Treatments and Resulting AChE Activities of Myotube Cultures Expressed as Percent of Group 1 (Control) Cell-associated Activity*

Group	Treatment			AChE activity	
	DFP	3-h Recovery	Cycloheximide	Cells	Medium
				%	%
1	−	−	−	100	—
2	−	+	−	100	50
3	+	−	−	3	—
4	+	+	−	74	0
5	+	−	+	5.5	0
6	+	+	+	31	26

The activity within DFP-treated cells returned to 74% of control values after 3 h (Group 4). Since serum was absent from the recovery medium, none of this AChE activity could be derived from this source.

In cultures that were treated with DFP and then incubated in medium containing cycloheximide for 3 h (Group 5), a small increase in AChE activity was noted in the cells, and no AChE activity was found in the medium. Group 6 cultures which had been treated with DFP, incubated in Eagle's MEM for 3 h, and then incubated in Eagle's MEM containing cycloheximide for an additional 3 h exhibited a 23% drop in total AChE activity relative to Group 4 cultures (i.e., those treated with DFP and allowed to recover in Eagle's MEM for 3 h). Of the total AChE activity of the Group 6 cultures, 45% was in the medium. This figure is similar to the fraction of cell-associated AChE activity secreted into the medium by control (Group 2) cultures. In contrast, the cells lost a large portion of their activity and it is only in this group that the medium-associated activity makes up such a large portion of the total AChE activity.

Purification of Coated Vesicles from DFP-treated and Recovered Cells

We purified coated vesicles from cultured myotubes treated with DFP for 10 min and then allowed them to recover for 3 h. Negatively stained preparation of these coated vesicles revealed that the gradient-purified coated vesicles were of comparable purity to those from whole muscle except that more filaments were present (Fig. 6). The pattern of peptide bands seen in SDS gels was also identical to that of coated vesicles prepared from whole muscle except that the band at 42 kD, probably actin, was more prominent (data not shown).

The purification and latency of AChE activity contained in the coated vesicles purified from DFP-treated and recovered cells is shown in Table V, allowing comparison to the results obtained from whole muscle (Table II). As can be seen, the enrichment of AChE and the percentage of latency of the AChE in both preparations are comparable. One major difference is apparent, however. While the latency of the AChE in the high speed pellet from whole skeletal muscle is only 6%, it is 64% in the same fraction from DFP-treated and recovered myotubes. The most probable explanation for this difference is that whole muscle contains a large amount of plasma membrane–associated AChE which will be present in the high speed pellet and would likely be in an unsealed compartment. There is no plasma membrane–associated AChE present on the DFP-treated and recovered myotubes, however, since the enzyme has not reached the surface. Therefore, all the AChE is inside the cell in sealed vesicular compartments which should have significant latency.

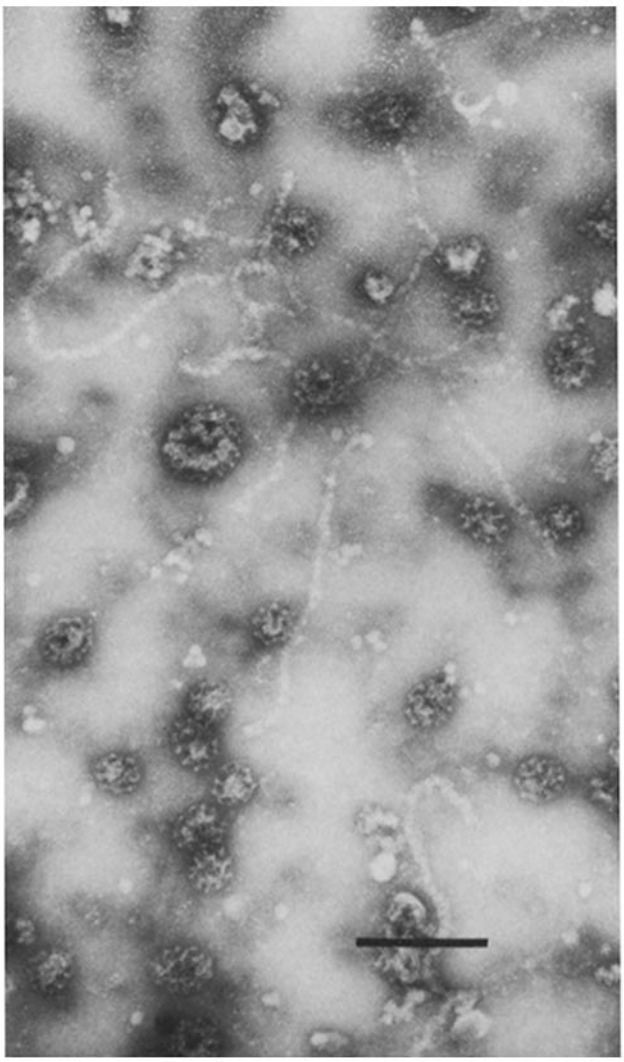


FIGURE 6 Negative stained preparation of coated vesicles purified from cultured chick embryo myotubes. Coated vesicles were purified from cultured chick embryo myotubes which had been treated with DFP and allowed to recover for 3 h as described in Materials and Methods. Bar, 0.25 μm. × 68,400.

The AChE contained in the coated vesicles is again mainly the soluble "secretory" form, since over 80% of the AChE was rendered soluble by repeated cycles of freezing and thawing (data not shown).

Agarose Gel Purification of Myotube Coated Vesicles

As further evidence that AChE is associated with the myotube coated vesicles and not with some other contaminating structure, we subjected sucrose gradient–purified coated vesicles from DFP-treated and recovered 12-d-old chick embryo myotube cultures to agarose gel electrophoresis. This gel was electrophoresed at a slightly higher voltage for a shorter time than the gel shown in Fig. 5, leading to a broader band of coated vesicles. In Fig. 7, the inset shows that the coated vesicles excised from the agarose gel were highly purified and no longer contaminated by filaments. The use of the Johnson

TABLE V. *Protein, AChE Activity, Enrichment, and Latency at Successive Stages of Coated Vesicle Purification from Cultured Chick Embryo*

	Protein		Total AChE (+ Triton)				Exposed AChE (− Triton)			
		Recovery		Recovery	Specific activity	Enrichment		Recovery	Specific activity	Enrichment
	mg	*% of LSS*	*cpm × 10⁻³*	*% of LSS*	*cpm/mg*	*fold*	*cpm × 10⁻³*	*% of LSS*	*cpm/mg*	*fold*
LSS‡	240.5	—	18,736	—	77.9	—	12,108	—	50.3	—
HSP§	151.2	62.9	9,688	51.7	64.1	0.82	3,509	29	23.2	0.46
Ficoll-Sucrose supernatant	15.8	6.6	1,108	5.9	70.2	0.90	306.5	2.5	19.4	0.38
Sucrose gradient-purified coated vesicles	1.12	0.47	103.1	0.55	92.1	1.28	27.9	0.23	24.9	0.49

Successive stages of the coated vesicle purification were monitored for protein by the fluorescamine method (14) and for AChE activity

* % Latency = $\frac{\text{(AChE masked)}}{\text{(AChE total)}} \times 100$.

‡ LSS, low speed supernatant.

§ HSP, high speed pellet.

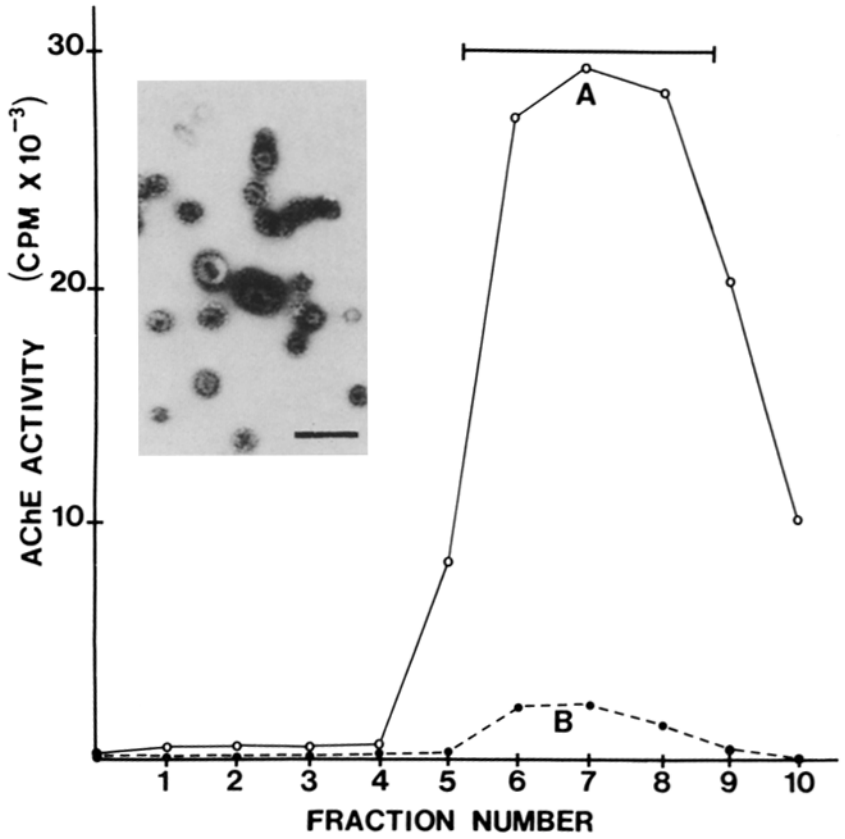


FIGURE 7 AChE activity is associated with chick embryo myotube coated vesicles purified to near homogeneity by agarose gel electrophoresis. Two adjacent lanes, each containing 10 μg of gradient-purified coated vesicles, were subjected to agarose gel electrophoresis. (*Inset*) Coated vesicles were excised and agarose removed as described in Fig. 4. A negatively stained preparation is shown. Small grains seen in the background are residual agarose. Bar, 0.25 μm. × 34,000. Eleven consecutive 0.5-cm slices were excised from the other gel lane, the agarose was removed, and the supernatants were adjusted to 50 μl. Two 15-μl aliquots were assayed for AChE by the method of Johnson and Russell (15). (*A*) Results when the assay was performed in the presence of 1% Triton X-100. (*B*) Results when the assay included both 1% Triton X-100 and 10^{-4} M BW284C51. The other 20-μl aliquots were subjected to SDS PAGE and stained for protein by the silver staining method described by the manufacturer (Bio-Rad Laboratories, Richmond, CA). The bar shows the fractions that contain clathrin. These correspond to the position of coated vesicles shown in the inset.

and Russell assay (15) demonstrates that essentially all of the AChE activity was associated with the clathrin-containing region of the gel (Fig. 7). This figure also confirms that the cholinesterase is indeed a true AChE as indicated by its almost total inhibition by 10^{-4} M BW284C51, a specific AChE inhibitor.

DISCUSSION

The data presented in this paper are consistent with the hypothesis that AChE is present within coated vesicles isolated from 17-d-old chicken embryo skeletal muscles. The AChE can be detected by both biochemical and morphological tech-

Muscle

Masked AChE (Total–Exposed)				Latency*
	Recovery	Specific activity	Enrich-ment	
cpm × 10⁻³	*% of LSS*	*cpm/mg*	*fold*	%
6,627	—	27.5	—	35.4
6,180	93	40.9	1.48	63.9
802.3	12	50.8	1.84	72.4
75.2	1.13	67.2	2.44	72.9

by the method of Johnson and Russell (15).

niques and appears to be mostly the soluble "secretory" form. The demonstrations that the AChE co-migrates with coated vesicles purified to near homogeneity by agarose gel electrophoresis and that its mobility can be altered concomitantly with that of the coated vesicles by incubation with anti-clathrin serum strongly support the conclusion that the enzyme is an authentic coated vesicle constituent.

The percent latency of the AChE in our purified coated vesicle preparations from both embryonic muscle (Table II) and from cultured myotubes (Table V) is 70% using [^{14}C]-ACh as the substrate. Much higher latency (>90%) is found for acetylcholine receptors present in identical coated vesicle preparations using [^{125}I]α-bungarotoxin as the ligand (30). We feel that the most reasonable explanation for the relatively low latency of the AChE in coated vesicles is due to the use of an extremely small molecule, [^{14}C]ACh, as the substrate. Even a tiny channel in the vesicle membrane will allow ACh to diffuse into the vesicle, while [^{125}I]α-bungarotoxin will likely be excluded due to its much larger size. Another measure of the percentage of AChE within an enclosed vesicle is its inaccessibility to degradation by trypsin which is also a much larger molecule than ACh. As shown in Table I, 90% of the AChE in the coated vesicle preparation is protected from degradation unless detergent is present, in which case only 20% of the activity is retained. We also present biochemical data (Table III) that indicates that most of the AChE found within coated vesicles is not artificially trapped within the vesicle during homogenization of the muscle but is present within intact coated vesicles. Again this is demonstrated morphologically as well (Fig. 4).

A major point addressed in this paper is the origin of the AChE found in muscle coated vesicles. Using muscle cells which have been treated with DFP and allowed to recover for 3 h (at which time no detectable AChE is secreted), we again find AChE within coated vesicles reacted with the Karnovsky and Roots reagents in situ (12) and in highly enriched populations of coated vesicles isolated from these cells (see Table V and Fig. 7). That at least the majority of the AChE is destined for secretion can be inferred from the results obtained when cells are allowed to synthesize AChE for 3 h and then further protein synthesis is prevented by addition of cycloheximide. We now detect much of the AChE made during the 3-h recovery period in the medium and no staining of coated vesicles or other organelles in cells is seen (12).

Even though the data presented in this paper indicate that coated vesicles are likely to be involved in at least one stage of newly synthesized AChE transport, we do not know at which stage(s) this involvement occurs. The work of Rothman and Fine (8), and more recently that of Kinnon and Owen (10), is consistent with the involvement of coated vesicles in at least two steps in the transport of newly synthesized integral plasma membrane glycoproteins. The steps defined operationally are an early step, involving endoglycosidase H–sensitive molecules and a late step involving endoglycosidase H–resistant molecules. Kinnon and Owen also have evidence consistent with coated vesicles mediating similar endoglycosidase H–sensitive and –resistant transport steps in the secretory pathway of newly synthesized IgM molecules (10). We are presently collaborating with Dr. Richard Rotundo to elucidate whether or not the transport of AChE involves multiple coated vesicle–mediated steps as well.

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