Evidence for Both Prelysosomal and Lysosomal Intermediates in Endocytic Pathways

BRIAN STORRIE, ROBERT R. POOL, JR., MEENA SACHDEVA, KAREN M. MAUREY, and CONSTANCE OLIVER

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061; and Laboratory of Oral Biology and Physiology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT Horseradish peroxidase (HRP), an enzyme internalized by fluid phase pinocytosis, has been used to study the process by which pinosome contents are delivered to lysosomes in Chinese hamster ovary cells. Pinosome contents were labeled by allowing cells to internalize HRP for 3–5 min. Following various chase times, cells were either processed for HRP and acid phosphatase (AcPase) cytochemistry or homogenized and fractionated in Percoll gradients. In Percoll gradients, pinosomes labeled by a 3–5-min HRP pulse behaved as a vesicle population more dense than plasma membrane and less dense than lysosomes. In pulse-chase experiments, internalized HRP was chased rapidly (3–6-min chase) to a density position intermediate between the "initial" pinocytic vesicle population and lysosomes. With longer chase periods, a progressive accumulation of HRP in more dense vesicles was observed. Correspondence between the HRP distribution and lysosomal marker distribution was reached after a ~1-h chase. By electron microscope cytochemistry of intact cells, the predominant class of HRP-positive vesicles after pulse uptakes or a 3-min chase period was characterized by a peripheral rim of reaction product and was AcPase negative. After 10–120-min chase periods, the predominant class of HRP-positive vesicles was characterized by luminal deposits and HRP activity was frequently observed in multivesicular bodies. HRP-positive vesicles after a 10- or 30-min chase were AcPase-positive. No HRP activity was detected in Golgi apparatus. Together these observations indicate that progressive processing of vesicular components of the vacuolar apparatus occurs at both a prelysosomal and lysosomal stage.

Prelysosomal endocytic compartments have recently been the subject of much interest in cell biology. These compartments, referred to variously as pinosomes, endosomes, intermediate vacuoles, or receptosomes (for reviews, see 6, 16, 31), have been described predominantly by electron microscopy and recently by cell fractionation. A major physiological function of these compartments appears to be ligand-receptor dissociation in response to vesicle acidification. In the present work, we have addressed the question of whether lysosomal as well as prelysosomal endocytic intermediates exist. Horseradish peroxidase (HRP[1]), an enzyme internalized by fluid phase pinocytosis in fibroblasts (1), has been chosen as the pinocytic marker because as a solute it should be included in all pinocytic vesicles irrespective of their origin and because it can be readily assayed spectrophotometrically and localized cytochemically. A pulse-chase approach combined with cell fractionation and cytochemistry has been taken. The chief result of this work has been to demonstrate a rapid and progressive processing of HRP-positive vesicles at both the pinosomal (prelysosomal) and lysosomal level. A preliminary report of this work has been presented (18).

[1] *Abbreviations used in this paper:* AcPase, acid phosphatase; CHO, Chinese hamster ovary; DAB, diaminobenzidine; FC10, 10% fetal calf serum; HRP, horseradish peroxidase; αMEM, Eagle's minimum essential medium, alpha modification; MIT, monoiodotyrosine.

MATERIALS AND METHODS

Cell Culture: Chinese hamster ovary (CHO)-S(C2) cells were grown in suspension culture (19). Cell number was quantitated with a hematocytometer.

HRP Uptake and Chase Conditions: In the standard uptake protocol, suspension culture CHO cells (~5 × 10^6 cells/ml) in Eagle's minimal essential medium alpha modification (26) without ribonucleosides (αMEM) and with 10% fetal calf serum (FC10) were exposed to 1 mg/ml HRP (type II, Sigma Chemical Co., St. Louis, MO) for 3 or 5 min at 37°C. The internalization

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period was terminated either by pouring the culture onto crushed, frozen saline (1) equal to two-fifths of the culture volume or by diluting the culture 100-fold in 4° or 37°C αMEMFC10. Cells poured onto crushed, frozen saline were then washed three times at 2–4°C in 50-ml volumes of saline (1) and resuspended at a concentration of ~5 × 10^6 cells/ml in 4° or 37°C αMEM with or without FC10. Addition of warm αMEM was taken to be t_0 for chase periods. After various time intervals, aliquots of cells at 37°C were removed and poured onto two-fifths volume of crushed, frozen saline to terminate the chase.

Cell Fractionation: Cells were washed three times at 4°C in 50-ml volumes of saline and processed for nitrogen cavitation (19). Total postnuclear supernatants were prepared (19) and layered over 32 ml 10% or 20% Percoll (Pharmacia Inc., Piscataway, NJ) suspensions prepared in 0.25 M sucrose. Centrifugation was at 28,000 *g* at 4°C in the Dupont Sorvall SV288 rotor (DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Wilmington, DE) for either 100 or 130 min depending on the Percoll lot. The centrifugation conditions needed to generate a given gradient depended on the aggregation state of the Percoll lot and were determined empirically. Gradient fractions were collected by displacement of the gradient with 2.2 M sucrose.

Gradient fractions were frozen at −20°C and later assayed for HRP, β-hexosaminidase, and alkaline phosphodiesterase I. Previous work (19) indicated that the distribution and recoveries of marker enzyme activity is identical whether assayed immediately or after freeze-thawing of the fractions. Densities of the upper two-thirds of the gradients were determined by comparing the refractive index of the fractions with a standard curve of density versus refractive index. Densities of the remaining fractions were determined by weighing 100-μl aliquots.

Enzyme Assays: HRP, alkaline phosphodiesterase I, and β-hexosaminidase were assayed as described previously (19). Recoveries of enzyme activities typically ranged from 80–120%.

Determination of Internalized [^{125}I]BSA Degradation: The initiation of [^{125}I]BSA degradation was determined by the appearance of [^{125}I] monoiodotyrosine (MIT) in the medium. [^{125}I]BSA was prepared by modification of the chloramine T procedure (10). The reaction mix of 0.12 ml (800 μg BSA, 2 mCi neutralized ^{125}I, 6 mM chloramine T) was incubated for 30 s at room temperature. The reaction was terminated by addition of excess sodium metabisulfite and the reactants separated by chromatography on a PD-10 column (Pharmacia, Inc.). The resulting preparation had a specific activity of 1–2 μCi ^{125}I/μg BSA and was >95% acid precipitable.

For [^{125}I]BSA uptake, cells were resuspended at 2 × 10^7 cells/ml in 37°C αMEM supplemented with 10 mg/ml unlabeled BSA and 20 mM HEPES buffer, pH 7.4. [^{125}I]BSA was then added to 20 μg/ml and after a 5-min internalization period, uptake was terminated as described above. Washed cells were then resuspended at 1–2 × 10^7 cells/ml in αMEM, 10 mg/ml BSA, 20 mM HEPES, pH 7.4 at 37°C. This is time t_0. After various chase times, 1 ml of cell suspension was added to 0.4 ml crushed, frozen saline (1) and pelleted at 4°C. The cell pellet was washed one time in 0.5 ml cold saline and the supernatants were combined and stored at −20°C. A column of Sephadex G-25 fine (Pharmacia, Inc.) was used to quantitate the radioactivity present in [^{125}I]MIT as described (11).

HRP and Acid Phosphatase (AcPase) Cytochemistry: For standard HRP cytochemistry, cells incubated with HRP were fixed after various chase times and processed as described previously (32) with minor modifications. Briefly, the diaminobenzidine (DAB) incubation was for 15 min at room temperature in 0.5 mg/ml DAB·4HCl, 0.01% H_2O_2, 0.1 M phosphate buffer, pH 6.0 (R. Rodewald, personal communication). Samples were embedded in agar (7) after generation of DAB reaction product, and the uranyl acetate staining step was omitted. After dehydration samples were embedded in eponaraldite (20). Sections were examined with and without lead citrate staining.

For combined HRP and AcPase cytochemistry, cells incubated with HRP were fixed after various chase times in 0.5% glutaraldehyde, 1.0% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4 for 5 min at 4°C and stored overnight in 0.1 M cacodylate buffer, pH 7.4 containing 7% sucrose (sucrose buffer). Cells were first incubated for 10 min in DAB-H_2O_2 medium as described above, rinsed, and then incubated for 60 min at 37°C in the medium (pH 5.0) described by Novikoff (15) using cytidine 5′-monophosphate as substrate. Following incubation the cells were washed twice in 0.05 M Tris-maleate buffer (pH 5.0) and three times in sucrose buffer, and rinsed in 1% sodium sulfide in sucrose buffer to visualize the reaction product for light microscopy. The tissue was postfixed for 1 h in 1% osmium tetroxide, dehydrated, and embedded in Spurr's resin (25). Thin sections were examined in a JEOL 100-C electron microscope either unstained or lightly stained with Reynold's lead citrate (21).

RESULTS

Pinosome contents were labeled by incubating CHO cells with HRP for 3 or 5 min at 37°C. HRP is ingested by CHO cells through fluid phase pinocytosis[2] (1) and hence should be present in pinocytic vesicles irrespective of the presence or absence of specific receptors. Following various chase times, cells were either processed for HRP and/or AcPase cytochemistry or homogenized and fractionated in Percoll gradients. HRP internalized by CHO cells during brief incubations (2.5–10 min) is present in a pinosome population which in 10% Percoll gradients is separated partially from plasma membrane and completely from lysosomes (19). Alkaline phosphodiesterase I and β-hexosaminidase were used as the standard marker enzymes for plasma membrane and lysosomes, respectively. The validity of these and other enzymic and nonenzymic markers for CHO cell organelles has been described previously (19).

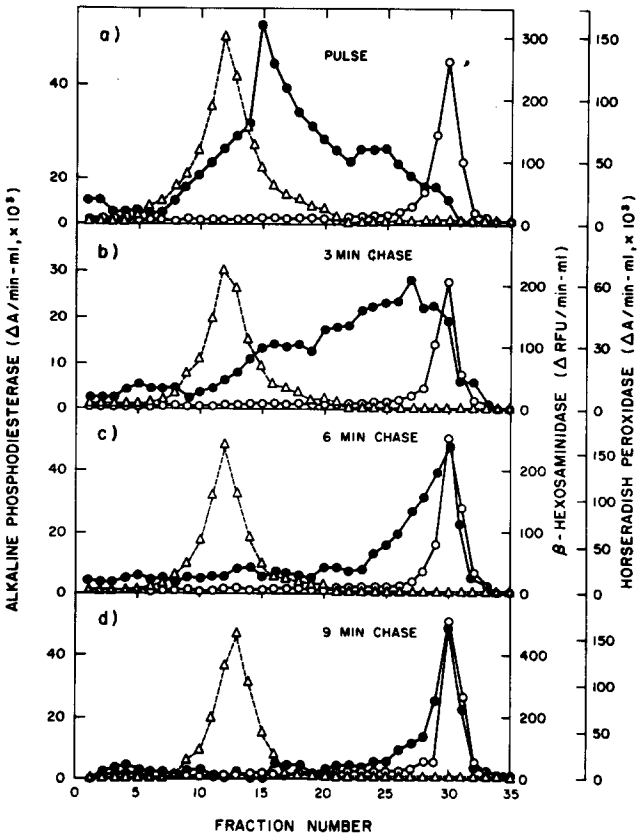

FIGURE 1 Distribution of pinocytized HRP activity in 10% Percoll gradients after a pulse (3 min) or a 3–9-min chase. Cells were exposed to 1 mg/ml HRP for 3 min in αMEM, poured onto one-fifth volume of crushed frozen saline, washed, and chased in αMEM. Total postnuclear supernatants were applied to 10% Percoll gradients. Fractions were collected and analyzed for HRP (●), β-hexosaminidase (○), and alkaline phosphodiesterase I (△) activities.

Rapid and Progressive Change in the Density of Peroxidase-positive Vesicles

In initial experiments to assess the transfer of HRP into lysosomes, CHO cells were incubated with HRP for 3 min at 37°C, rapidly chilled, washed, and homogenized after various chase times in warm HRP-free medium. Total postnuclear supernatants were fractionated in 10% Percoll density gradients. After a 3-min pulse, HRP was found associated with

[2] HRP is a mannose rich glycoprotein. Mannose concentrations as high as 150 mM have no effect on HRP uptake by CHO-S cells (Storrie and Nelson, unpublished observations).

a pinosome population (peak density = 1.041 g/ml) that was slightly more dense than plasma membrane (peak density = 1.040 g/ml) and distinctly less dense than lysosomes (peak density = 1.065 g/ml) (Fig. 1*a*). During a chase HRP was found in a continuous series of progressively more dense compartments (Fig. 1). After a 3-min chase a pronounced shift in the HRP distribution was observed (Fig. 1*b*). After a chase period of 6 min, considerable overlap of the distribution of HRP activity and the distribution of β-hexosaminidase activity was observed (Fig. 1*c*). By the end of a 9–10-min chase the distributions in a 10% Percoll gradient of HRP and β-hexosaminidase, the standard lysosomal marker, were found to be almost coincident (Fig. 1*d*).

To detect further changes in the distribution of HRP during longer chase times, total postnuclear supernatants were fractionated in 20% Percoll gradients. In such a gradient, after a 3-min pulse the distribution of HRP was intermediate between that of the plasma membrane and the lysosomal marker (Fig. 2*b*). After a 10-min chase, the distribution of HRP had shifted towards the lysosomal marker by a density change consistent with that observed in 10% Percoll gradients (Δ = 0.006–0.008 g/ml). With longer chase periods, a further progressive shift in the density of HRP positive vesicles was observed in 20% Percoll gradients (Fig. 3). After a 60-min chase, the HRP and β-hexosaminidase distributions were nearly identical (Fig. 3*c*). Little, if any, further changes in HRP distribution were observed upon lengthening the chase period to 120 min (data not shown). Similar if not identical HRP distributions in 20% Percoll gradients were obtained in experiments in which chase conditions were achieved by diluting the culture 100-fold with 37°C αMEMFC10 to terminate uptake and initiate the chase period. The total increase in the modal density of HRP-positive vesicles over a 1–2-h chase period was 0.018 g/ml.

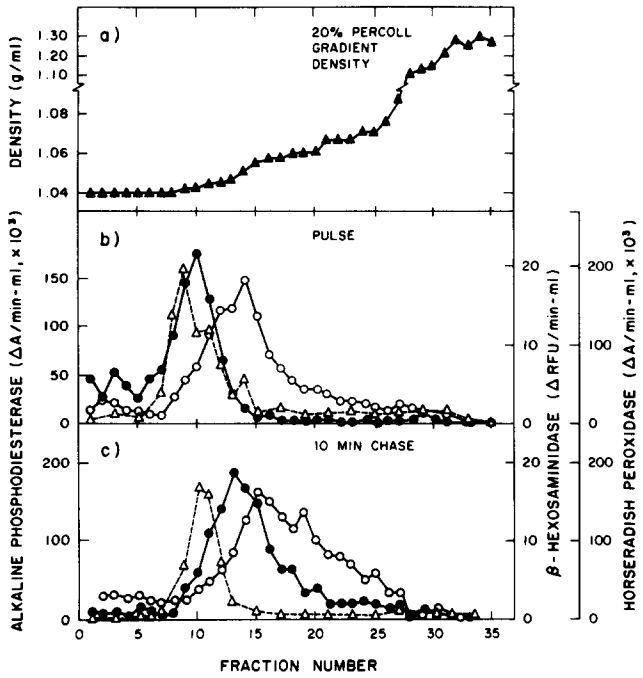

FIGURE 2 Distribution of HRP activity in 20% Percoll gradients after a pulse (3 min) or 10-min chase. Cells were exposed to 1 mg/ml HRP for 3 min in αMEMFC10, poured onto two-fifths volumes of crushed frozen saline, washed, and chased in αMEMFC10. Total postnuclear supernatants were applied to 20% Percoll gradients. Fractions were collected and analyzed for HRP (●), β-hexosaminidase (○), and alkaline phosphodiesterase I (△) activities. Density values (▲) were determined by measuring the refractive index and direct weighing of fractions of a parallel gradient.

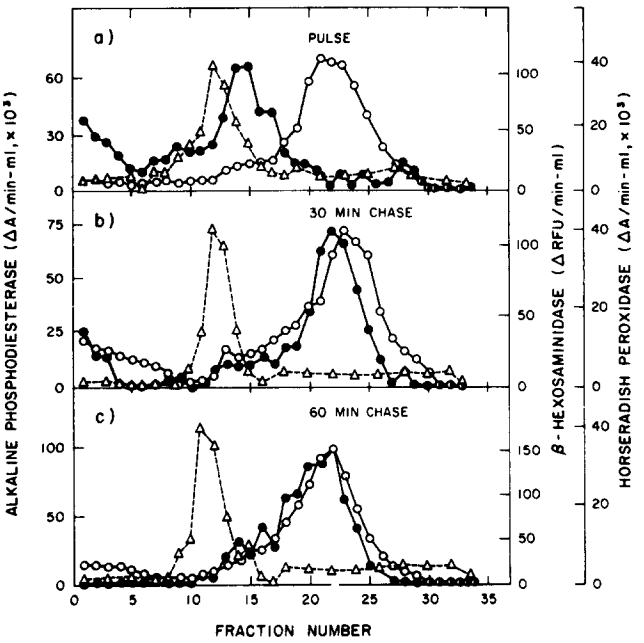

FIGURE 3 Distribution of pinocytized HRP activity in 20% Percoll gradients after a pulse (5 min) or a 30- or 60-min chase. Cells were exposed to 1 mg/ml HRP for 5 min in αMEMFC10, poured onto two-fifths volume of crused frozen saline, washed, and chased in αMEMFC10. Total postnuclear supernatants were applied to 20% Percoll gradients and analyzed for HRP (●), β-hexosaminidase (○), and alkaline phosphodiesterase I (△) activities.

TABLE I

Appearance of [¹²⁵I]MIT in Culture Medium

Treatment	[¹²⁵I]MIT
min	*cpm*
Pulse (5)	0
Chase	
5	0
10	455
20	1,420

CHO cells were incubated with [¹²⁵I]BSA for 5 min at 37°C, chilled, washed free of exogenous [¹²⁵I]BSA, and resuspended in warm medium. At various chase times cells were harvested and [¹²⁵I]MIT levels in the medium were determined by column chromatography.

Transfer of Pinosome Contents into a Degradative Compartment

As an initial step in determining when during a chase period pinosome contents have been transferred to lysosomes, a compartment rich in hydrolytic enzymes, the onset of [¹²⁵I]BSA degradation by CHO cells was assayed by the release of [¹²⁵I]MIT into the culture medium[3]. After a 5-min [¹²⁵I]BSA internalization period followed by a 5-min chase, no [¹²⁵I]MIT was found in the medium. After a 10-min chase, [¹²⁵I]MIT was readily detected in the medium (Table I). These data indicate that after a 10-min chase endocytized material is present in a degradative compartment, presumably lysosomal.

[3] [¹²⁵I]HRP is degraded too slowly for such experiments (27). The kinetics of [¹²⁵I]BSA accumulation in prelysosomal vesicles appear similar to HRP.

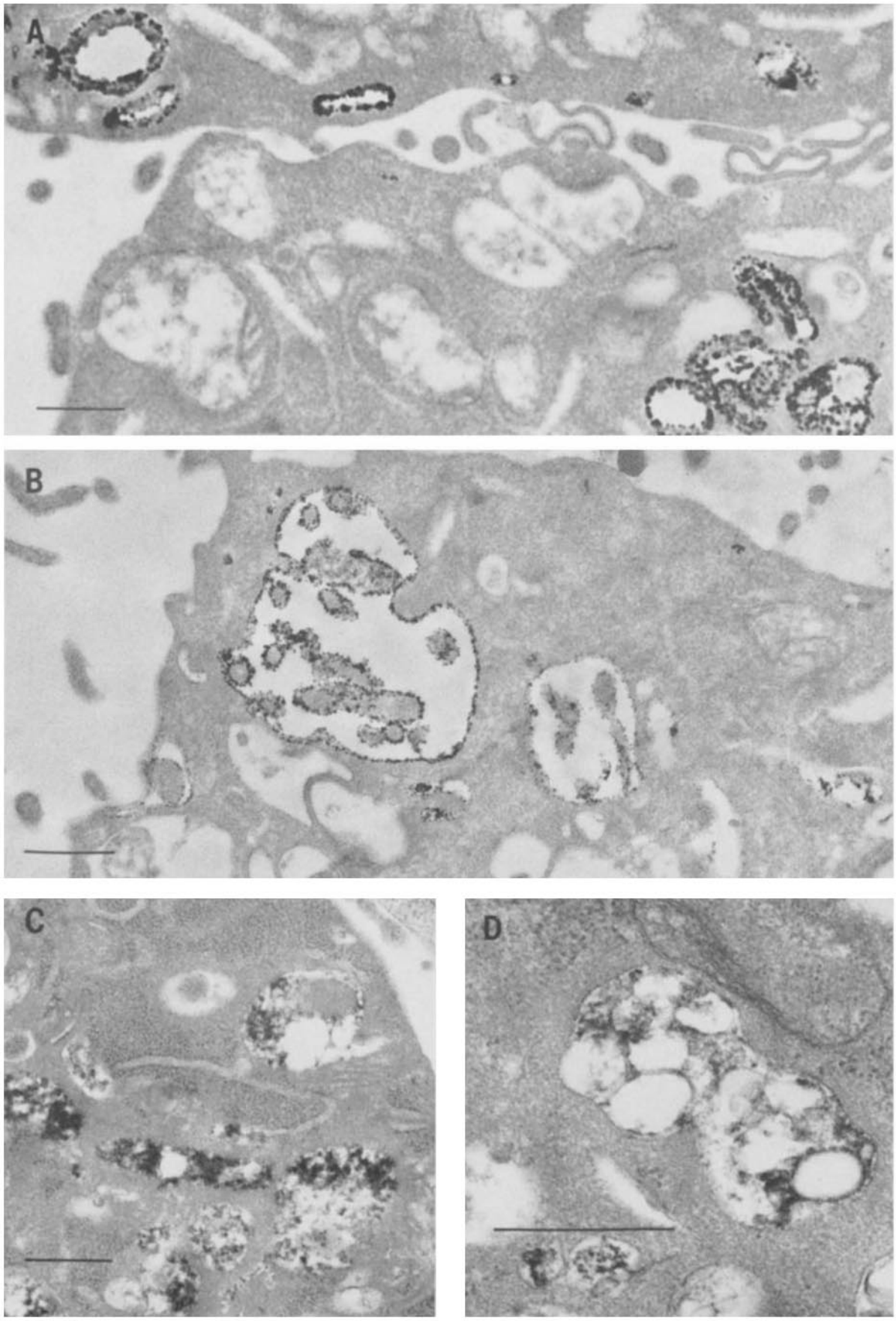

FIGURE 4 Cytochemical localization of HRP in CHO cells after a pulse (3 min, *A*) or a 3- (*B*) or 10-min chase (*C* and *D*). Cells were exposed to 1 mg/ml HRP for 3 min in αMEMFC10, poured onto two-fifths volume of crushed frozen saline, washed, and chased in αMEMFC10. The black deposits indicate the sites of HRP activity. *A* and *B* are shown without lead staining. *C* and *D* are shown with lead staining. Bars, 0.5 μm. (*A*, *B*, and C) × 30,600; (*D*) × 63,750.

Identification of Peroxidase-Positive Subcellular Compartments by Electron Microscope Cytochemistry

By electron microscope cytochemistry of intact cells, HRP activity after a 3-min pulse was localized in round and elongate vesicles with the DAB reaction product rimming the periphery of the vesicles (Fig. 4*a*) with no cell surface reaction product being detectable. Vesicles labeled by a 30-s HRP pulse showed similar morphology (not shown). After a 3-min chase the reaction product was again found to rim the periphery of the vesicles (Fig. 4*b*). The reaction product was less intense and often found in vesicles containing cytoplasmic protrusions rimmed with reaction product. Striking changes in peroxidase staining patterns were apparent after a 10-min chase; the DAB reaction product was now found to be luminal

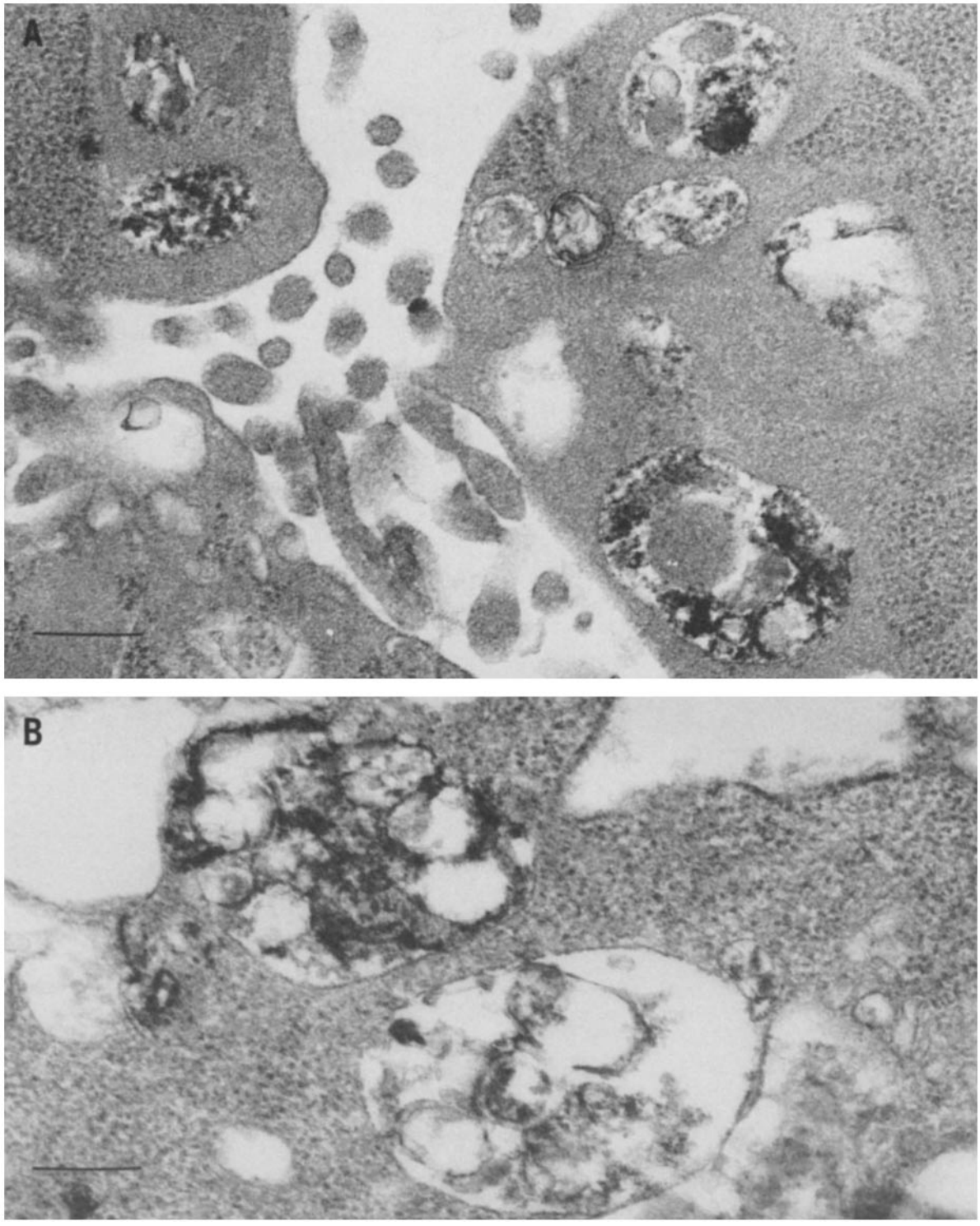

FIGURE 5 Cytochemical localization of HRP in CHO cells after a 30-min (*A*) or 120-min (*B*) chase. Aliquots of the cells described in Fig. 4 were processed for HRP cytochemistry. The black deposits indicate the sites of HRP activity; lead stained. Bars, 0.25 μm. × 72,500.

TABLE II

Changes in Staining Pattern of HRP-positive Vesicles during Pulse-Chase Experiments

Treatment	HRP staining pattern		
	Peripheral	Luminal	Unassignable
min	%	%	%
Pulse (3)	84	5	11
Chase			
3	75	15	10
10	8	88	4
30	6	89	5
60	0	96	4
120	2	96	2

For each treatment between 40 and 121 vesicles were scored from a minimum of nine different cell profiles.

TABLE III

Fraction of HRP-positive Vesicles That Can Be Identified as Multivesicular Bodies

Treatment	Multivesicular bodies: fraction
min	
Pulse (3)	0.00
Chase	
3	0.05
10	0.56
30	0.55
60	0.44
120	0.66

For each treatment between 40 to 121 vesicles were scored from a minimum of nine different cell profiles.

rather than peripheral and reaction product was observed within multivesicular bodies (Fig. 4, *c* and *d*). Morphometric measurements indicated little or no change in the cross-sectional area of HRP-positive vesicles during a 10-min chase (data not shown). Little, if any, further change in vesicle class or the character of the DAB deposits could be observed with chase times as long as 120 min (Fig. 5). The incidence of peripherally and luminally stained HRP-positive vesicles (Table II) and the fraction of HRP-positive vesicles that could be identified as multivesicular bodies (Table III) were scored. This quantitation indicated a pronounced change in the staining properties and nature of the HRP-positive vesicles over a 10-min chase. Peripherally stained and luminally stained vesicles have been previously described by Steinman et al. (28, 29) as pinosomes and secondary lysosomes, respectively[4]. Multivesicular bodies are generally considered to be lysosomes (for review, see reference 8). These data strongly suggest that, after chase periods of a few min, HRP is located in a pinosome compartment and, after a 10-min chase, it is found in secondary lysosomes. No peroxidase activity was observed at any chase time in Golgi apparatus.

The relationship of internalized HRP to lysosomes marked by hydrolase activity after both a 3-min pulse and a 3-min pulse followed by a 3-, 10-, or 30-min chase was examined by first incubating the cells to detect HRP activity and subsequently incubating them to localize AcPase activity. After a 3-min pulse, the HRP was restricted to elongate and round vesicles, and no HRP reaction product was detected in any lysosome marked by AcPase activity (Fig. 6*a*). The predominant class of HRP-positive vesicles after a 3-min chase was AcPase negative (Fig. 6*b*) with <1% of the HRP-positive vesicles positive for AcPase activity. After a 10- or 30-min chase, >90% of all HRP-positive vesicles were also positive for AcPase activity (Fig. 6, *c* and *d*) and most lysosomes marked by AcPase activity also contained HRP reaction product. The AcPase cytochemistry thus confirms the biochemical results and shows that initially HRP is indeed in a nonlysosomal compartment and after a ~10-min chase accumulates in a lysosomal compartment. The correlation between the appearance of HRP in AcPase positive vesicles and multivesicular bodies should be noted.

DISCUSSION

These experiments were done to characterize the process by which pinocytic contents are transferred into lysosomes in CHO cells, a cell line of fibroblastic origin. Our results support three major conclusions. First, rapid and progressive vesicle processing occurs in which internalized material is immediately found in increasingly dense vesicles. Second, processing events can be divided into a prelysosomal stage that is complete within a 6–10-min period and a lysosomal stage that is complete over a period of ~1 h. Third, pinocytic contents in CHO cells do not appear to transit through Golgi apparatus. Experiments using membrane markers will be necessary to establish whether endocytic membrane passes through Golgi apparatus in these cells.

The pulse-chase approach that used the solute marker HRP as an endocytic tracer permitted the unequivocal ordering of compartments with respect to endocytic content transport; however, it should be noted that this approach gave little, if any, information regarding relative compartment size. In cell fractionation experiments, total postnuclear supernatants were centrifuged in Percoll density gradients. Total postnuclear supernatants from CHO cells homogenized by nitrogen cavitation have been shown previously (19) to yield a preparation containing in a latent form 70–80% of total pinosomal and lysosomal marker activities. For CHO cells the preparation of either M or L fractions by differential centrifugation as described by de Duve et al. (2) does not enrich either pinosomes or lysosomes and there is a considerable loss in yield (19). 10% Percoll gradients that are very shallow over the density range of 1.04 to 1.05 g/ml proved particularly useful in resolving early pinosomal processing events. 20% Percoll gradients that are approximately linear over the density range of 1.04 to 1.08 g/ml were particularly useful in resolving later lysosomal processing events. A dual Percoll gradient system has been also found useful by others in resolving early processing events (13, 14).

While the present work was being completed, a cell fractionation approach was taken by other laboratories to identify prelysosomal intermediates in receptor mediated endocytosis. The ligands studied include epidermal growth factor (14), low density lipoprotein (14), β-hexosaminidase (14), and asialoglycoprotein (9). All these ligands are internalized into prelysosomal intermediates that contain little, if any, lysosomal

[4] The molecular basis of this difference in HRP staining pattern between pinosomes and secondary lysosomes is unknown. It may reflect, for example, differences either in the nucleation of DAB precipitation onto membranes or in the intravesicular distribution of HRP after fixation.

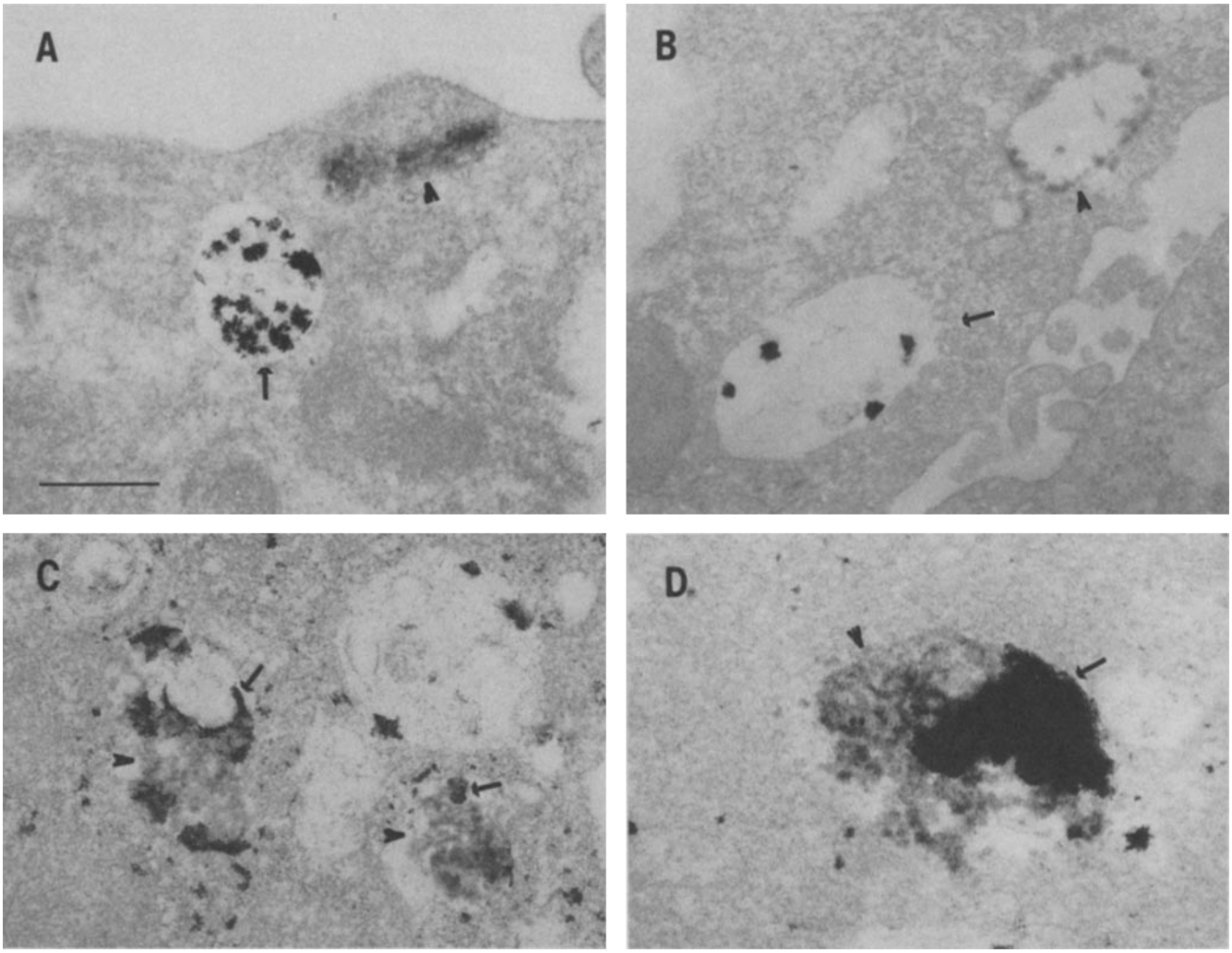

FIGURE 6 Cytochemical localization of HRP and AcPase in CHO cells after a pulse (3 min, *A*) or chase (3 min, *B*; 10 min, *C*; 30 min, *D*). Cells were exposed to 1 mg/ml HRP for 3 min in αMEMFC10, poured onto two-fifths volume of crushed frozen saline, washed, and chased in αMEMFC10. Lightly fixed cells were incubated sequentially with substates for HRP cytochemistry and AcPase cytochemistry. The light black deposits (arrowheads) indicate the sites of HRP activity. The black, crystalline deposits (arrows) indicate the sites of AcPase activity. Bar, 0.25 μm. (*A*) × 68,000; (*B*) × 42,000; (*C*) × 63,000; (*D*) × 80,000.

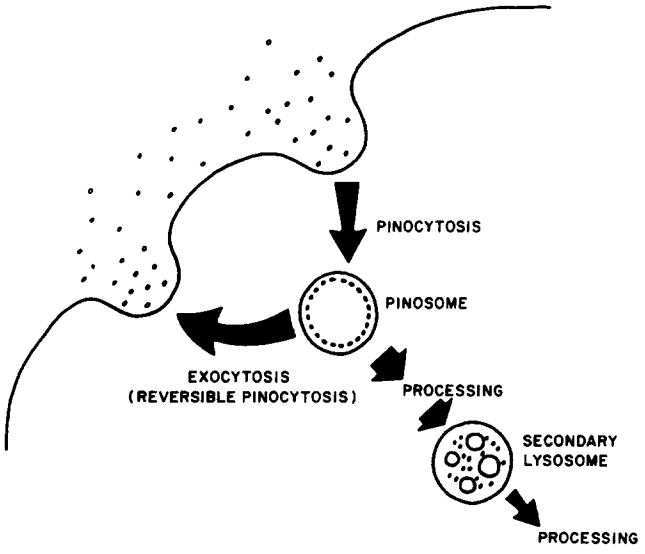

FIGURE 7 Working model for prelysosomal and lysosomal processing of vesicular components of the vacuolar apparatus.

marker and/or plasma membrane marker. The similarity of these results for receptor mediated endocytosis and our results for fluid phase pinocytosis point to the possible existence of a common pathway for endocytic content transport into lysosomes.

The processing of HRP-positive vesicular components of the vacuolar apparatus can be divided into a prelysosomal stage that is short (~6–10 min) and a lysosomal stage that continues for ~1 h. The identification of HRP-positive vesicles as prelysosomal and lysosomal is based on several criteria: centrifugation properties, onset of endocytized protein degradation, distribution (peripheral versus luminal) of the DAB deposits generated by peroxidase cytochemistry, morphology, and co-localization of HRP and AcPase activity by cytochemistry. Together these criteria permit the unequivocal assignment of a vesicle to a prelysosomal (pinosomal) compartment or a lysosomal compartment. The rapid and progressive prelysosomal increase in pinosome density reported here suggests that a vesicle maturation process may be a normal step in the transfer of endocytic contents into lysosomes. The observation that transfer of pinocytic contents into secondary lyso-

somes takes 6–10 min is consistent with observations of other investigators (for review, see 31) using many cell systems.

Centrifugation of total postnuclear supernatants in 20% Percoll gradients indicates continued processing of HRP-positive vesicles occurring at the level of secondary lysosomes. HRP was found to accumulate first in low density secondary lysosomes and then over a 1-h period in higher density secondary lysosomes to give an end-HRP-distribution characteristic of lysosomes in general. These data provide evidence for a novel form of lysosomal heterogeneity in CHO cells that could not be detected by electron microscopy. These observations may have a parallel in previously reported work of others (13, 17, 22). Rome et al. (22) have described a receptor mediated process in which initial ligand accumulation appears to be in a low density lysosomal population, which may be GERL, and later in a higher density lysosomal population. For other cell types, between 30–60 min appear to be required to transfer an endocytic pulse into dense lysosomal vesicles (28, 30).

The existence of a low density, "early" lysosomal component of endocytic pathways suggests the occurrence of a possible functional compartmentation of lysosomes. The one organelle in cells for which a clear functional compartmentation has been described is the Golgi apparatus (for reviews, see 5, 24). In Golgi apparatus, various steps in glycoprotein processing are compartmentalized to different portions of the organelle. By cell fractionation, distinct glycosidase and glycosyl transferace activities (see e.g., 3, 4) have been attributed to different Golgi vesicles. By electron microscopy immunocytochemistry, galactosyl transferase activity has been localized to *trans* elements of the Golgi apparatus (23). The "early" lysosomal compartment described here may be preferentially enriched in "early" degradative enzymes. This possibility is currently under investigation.

Fig. 7 presents a working model designed to summarize our current understanding of pinocytic vesicle processing. Exocytosis (reversible pinocytosis) is depicted as restricted to a pinosomal compartment. The actual mechanisms by which endocytic content transfer into lysosomes occurs are unknown. The point at which segregation of endocytic membrane components and endocytic contents occurs is left open. The kinetics of cell surface appearance of in situ radioiodinated endocytic membrane proteins in CHO cells (33) suggest that segregation occurs early and before the secondary lysosome stage. If segregation of endocytic membrane proteins and contents occurs sufficiently early, then the early changes in vesicle density observed may correspond to the transfer of endocytic contents into prelysosomal compartments differing in membrane composition from pinosomes. This possibility is currently under investigation. Alternatively the density changes may reflect the molecular events by which pinosome maturation occurs. Recent work (12, 35) indicates that at least one molecular feature of vesicle processing is acidification. It should be noted that this model bears similarities to a model previously presented by Tietze, Schlesinger, and Stahl (34) to summarize their work on the recycling of the mannose-specific receptor in macrophages.

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