Endocytosis and the Recycling of Plasma Membrane

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1 INTRODUCTION

The study of endocytosis has traditionally focused on the contents of endocytic vacuoles, i.e., extracellular fluid, dissolved solutes, and macromolecules or particles which specifically or nonspecifically bind to the plasma membrane (PM). Substances that are endocytosed include important nutrients, toxins, effector molecules (growth factors, hormones, antibodies), enzymes, and pathogens.

This article centers on the properties and dynamics of the endocytic vacuole membrane. In many instances we will stress observations on cultured mouse macrophages with which we are most familiar. We will emphasize four points: (*a*) Movement of vesicles is rapid such that endocytosed membrane and contents move from one cellular compartment to another in seconds to minutes. (*b*) Vesicular movement requires the interiorization and flow of large amounts of PM.[1] (*c*) In many instances, internalized PM must recycle or return intact to the cell surface. (*d*) During recycling, contents and membrane components can be sorted from one another; e.g., endocytosed contents can accumulate within the cell while the container (membrane) can move into and out of the cell after one or more fusion events with other endocytic vacuoles, lysosomes (Ly), or Golgi apparatus.

While it has been difficult to obtain direct evidence, the literature is replete with examples in which rapid membrane flow and recycling readily explains the data. Two of the more striking examples derive from studies of pinocytosis in cultured cells. Fibroblasts, for instance, interiorize the equivalent of 50% of their surface area and 5-10% of their cell volume during each hour of pinocytic activity. Yet, the overall dimensions of the cells and the vacuolar system remain constant throughout hours, even days, of endocytic activity. Since it is unlikely that internalized PM is rapidly degraded, it was proposed that

membrane continually recycles through the cells (1). Likewise, during the receptor mediated endocytosis of PM bound ligands, the amount of ligand delivered to cells per hour can be many times the binding capacity of the PM (reviewed in reference 2). Therefore, some PM receptors for ligand may repeatedly enter and recycle through the cell, delivering their ligands to Ly each time.

In addition to its rapidity and magnitude, a further characteristic of endocytosis can be described by the term "sorting." For example, internalized solutes accumulate continuously in cultured cells, while membrane flow is bidirectional. In effect, endocytosed content is sorted from membrane such that solutes and ligands can accumulate intracellularly while the membrane "container" recycles to the PM. Sorting of membrane from membrane must also occur. During endocytosis, the PM communicates extensively via membrane fusion with Ly, Golgi apparatus, or with another surface of the cell. Nevertheless, organelle and membrane diversity is maintained. A good example is endocytosis in polarized cell types such as intestinal epithelium and hepatocytés. Vacuoles continuously move from the apical (or sinusoidal) surface and deliver solutes to the basolateral (or bile cannalicular) surface. In spite of these interactions, two biochemically distinct PM domains are maintained.

2 THE VACUOLAR SYSTEM

The structures that participate in endocytosis are collectively termed the vacuolar apparatus or vacuolar system (Fig. 1). At least four organelles can be distinguished: (*a*) the cell surface or PM; (*b*) phagocytic and pinocytic vacuoles, which bud off from the cell surface and probably have a life span of seconds to minutes before undergoing further changes or fusion events; (*c*) digestive granules or secondary Ly, which participate in digestion and/or delivery of internalized molecules to the cytoplasm; and (*d*) a complex that synthesizes lysosomal hydrolases, secretory proteins, and vacuolar membrane and includes Golgi apparatus, endoplasmic reticulum, and primary lysosomes. The components of the vacuolar system rapidly and extensively communicate with one another by membrane fusion. Nevertheless, each structure can be distinguished by a combination of cytologic, biochemical, and physical criteria. In this section we will review the vacuolar system with emphasis

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[1] The main abbreviations used in this paper are the following. PM, Plasma membrane. PV, Pinocytic vesicle. Ly, Lysosome. LPO, Lactoperoxidase. ASGP, Asialoglycoprotein. HRP, Horseradish peroxidase. SP, Secretory piece. PVP, Polyvinylpyrrolidone. LDL, Low density lipoprotein. Ab, Antibody. Mφ, Macrophage. CF, Cationized ferritin. SFV, Semliki Forest virus. PLV, Plasmalemmal vesicles. FcR, Receptor for the Fc region of immunoglobulin. ACh, Acetylcholine. EGF, Epidermal growth factor. RER, Rough endoplasmic reticulum. man-6-P, Mannose-6-phosphate. PMA, Phorbol myristate acetate.
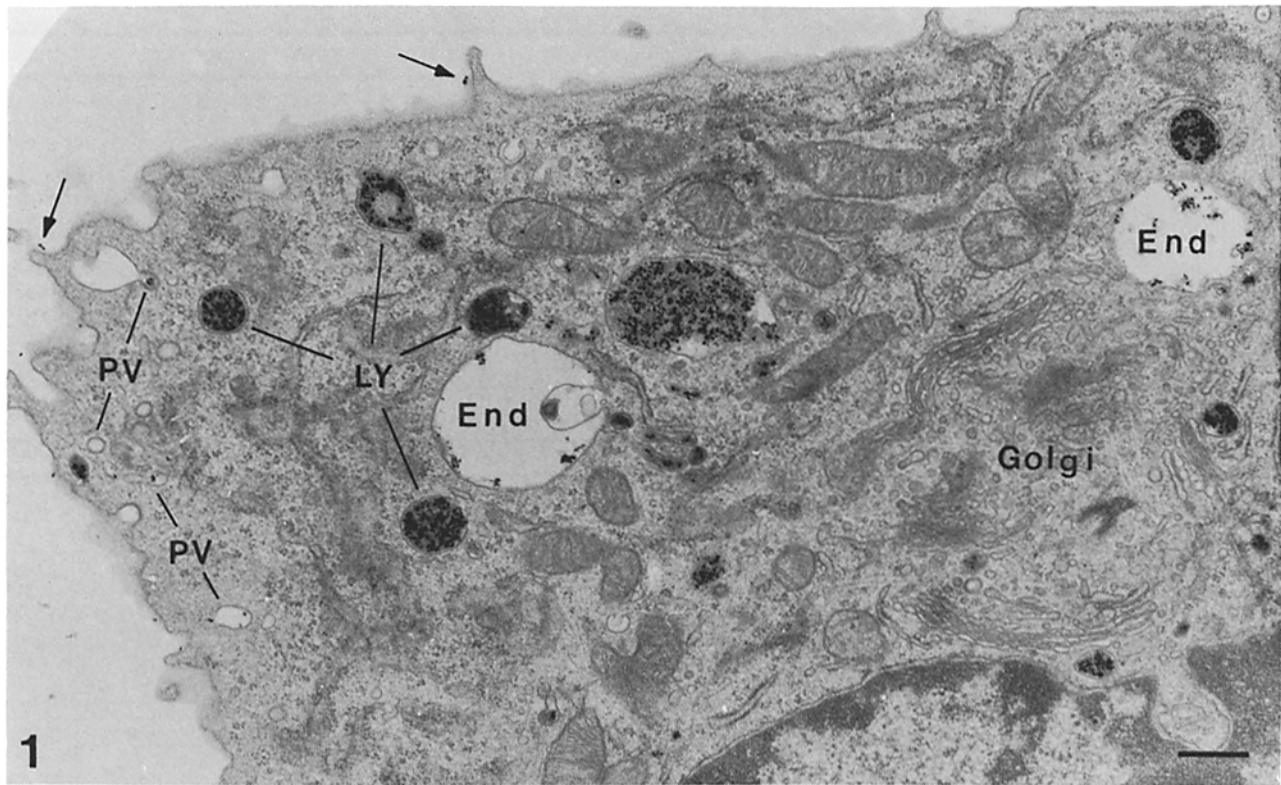
FIGURE 1 The organelles that participate in endocytosis: survey view of a mouse macrophage exposed for 2 h to colloidal thorium dioxide. The electron-dense colloid particles adsorb in small amounts to the PM (arrows). Small 0.1–0.2 μm pinocytic vesicles (PV) arise from the PM. Larger vacuoles, called endosomes (End) in this review, probably arise by the fusion of PV. The bulk of the cell-associated colloid is found in lysosomes (Ly), which also contain an endogenous amorphous content. Thorium does not enter the Golgi apparatus. The extent to which thorium is concentrated in the vacuolar system increases progressively from PM to PV to endosome to Ly. Bar, 0.5 μm. × 19,000.

on the methods used to identify individual organelles and their membrane composition.

2.1 Plasma Membrane

To understand how endocytic vacuoles are generated, many groups are comparing the biochemical properties of PM with those of nascent and recently interiorized endocytic vacuoles. It is still difficult to purify PM free of other vacuolar elements. Thus, selective labeling of the PM before study of whole cells or cell fractions has been the mainstay of current work (reviewed in reference 3). The PM can be radio-labeled with [125]I-(lactoperoxidase-glucose oxidase), $NaB[^3H]_4$ (neuraminidase-galactose oxidase), or radioactive amino acids (followed by external derivatization with trinitrobenzene sulfonate). The cell surface can also be probed noncovalently with lectins, receptor-bound ligands, and specific antisera. An extremely useful tool has been provided by the advent of monoclonal antibodies (Ab) directed to individual cell surface proteins. These Ab can be employed to quantify specific PM antigens on intact cells and can be used to purify even minor PM proteins from cell lysates. Reagents can be produced against cell surface receptors that both block receptor activity and provide a means to visualize receptor distribution independent of ligand. Finally, by adding Ab to intact cells before homogenization or lysis, one can distinguish surface and intracellular pools of antigen (4).

With each of the above techniques, it is necessary to inhibit endocytosis to restrict labeling to the cell surface. This is usually accomplished by using cells that are chilled (0–4°C) or alde-

hyde-fixed. Low temperature is the most effective noninvasive inhibitor of pinocytosis. Uptake of an endocytic tracer like horseradish peroxidase (HRP) is undetectable at 4°C by both quantitative or cytochemical assays (5, 6). Similarly the interiorization of bound ligands does not occur in chilled or fixed cells (7–16). In contrast, endocytosis of HRP or adsorbed ligands is relatively easy to detect at temperatures >10°C (6, 17, 18).

The comparative study of PM and endocytic vacuole membrane must also take into account that the cell surface can be divided into distinct domains. Differentiated macrodomains are well known, particularly in polarized epithelial cells where they can be identified by morphological criteria. The apical and basolateral surfaces of renal and intestinal epithelia, for example, can also be differentiated biochemically; PM enzymes such as aminopeptidase are found only on the apical surface, while others such as Na^+-K^+ ATPase are restricted to the basolateral surface (19, 20). Virus budding can also occur selectively at basal or apical surfaces of cultured cells (21). Specialized pseudopods of motile cells and cleavage furrows of dividing cells represent macrodomains that are specialized for increased and decreased endocytic capacities respectively (22, 23). Differentiated microdomains, such as junctional complexes and coated pits represent examples of further biochemical heterogeneity within a continuous membrane bilayer.

2.2 Endocytic Vacuoles

Endocytic vacuoles are usually divided operationally into pinocytic and phagocytic vacuoles. The latter internalize large

(>0.5 μm) particles or molecular aggregates immediately following attachment to the PM. Phagocytic vacuoles usually are studied in specialized cell types such as leukocytes and certain protozoa. Pinocytic vesicles (PV) contain extracellular fluid and dissolved solutes (fluid phase pinocytosis) as well as molecules that are bound to the PM (adsorptive and receptor-mediated pinocytosis). PV formation occurs in most cells and is usually constitutive, i.e., it need not be induced by the contents being internalized (see section 8.1).

2.2 a PHAGOCYTIC VACUOLES: "Phagosomes" are easy to identify because the particles they contain can be seen by both light and electron microscopy. In mammalian cells and many amoeba, the vesicle membrane is closely apposed to the particle, while in other protozoa, large food vacuoles (>1 μm in diameter) contain both particles and fluid. Beneath the forming phagosome, there is usually an organelle-free filamentous network that contains actin and probably other contractile proteins (24–26). Phagocytic vacuoles are the one type of endocytic vacuole that can be isolated in a high degree of purity and yield. This can be accomplished using low density latex beads or oil emulsions as the phagocytic stimulus (27, 28). However, the incoming latex phagosome fuses with Ly within minutes of entry.

Biochemical evidence that phagocytosed membrane is a representative sample of PM has been obtained in several cells (reviewed in reference 29). Ulsamer et al. (30) found that the lipids of amoeba phagocytic vacuoles were similar to PM. In macrophages (Mφ) and L-cells, purified latex-phagosomes contain the PM ectoenzymes, 5'-nucleotidase and 3'-phosphodiesterase, in amounts proportional to the area of membrane internalized (31–33). If the PM is radio-iodinated before phagocytosis, more than 20 radio-labeled species can be identified on one dimensional SDS PAGE, and these are similar in both phagocytic vacuole membrane and PM. This observation has

been made both in leukocytes and fibroblasts (32, 34). On the other hand, Willinger et al. (35) have reported that the ^{125}I-polypeptides of neutrophil phagosomes exhibit small differences from the PM, but these could be due to secondary alterations such as proteolysis. Nevertheless, it is likely that specific PM components can be enriched or depleted in phagocytic vacuoles. A direct example is the Fc receptor of cultured macrophages which is selectively enriched in the membrane of phagosomes formed during the internalization of IgG-coated erythrocytes (36). Apparently, this is the consequence of ligand-induced movement of PM Fc receptors before or during endocytosis.

2.2 b FLUID PHASE PINOCYTIC VESICLES VISUALIZED USING FLUID PHASE MARKERS: By definition, fluid phase PV are vesicles which are labeled after a brief exposure to an impermeable solute that does not bind to the PM. Useful cytologic tracers include: electron-dense materials like ferritin (37, 38) and some colloidal gold preparations (39); enzymes that can be detected cytochemically, like HRP (5, 6); and fluoresceinated dextrans (22). HRP has been used extensively because it both allows a sensitive, electron microscope (EM) cytochemical visualization of all PV (with H_2O_2 and diaminobenzidine) and because the enzyme can be readily quantified biochemically (1, 5, 6).

When Mφ or L-cell fibroblasts were exposed to HRP for 30–300 s at 37°C, the labeled PV were usually spherical in shape with a diameter of 0.1 to 0.3 μm (Fig. 2) and were devoid of associated coats or filaments (1). The labeled PV were fully internalized because reaction product was not seen on the PM, or in cells exposed to HRP at 4°C to block pinocytosis. The average volume of HRP-containing PV increased progressively from 0.0143 to 0.0356 μm^3 between 30 and 300 s of exposure, suggesting that PV were fusing shortly after uptake. However, fusion with Ly was not detectable (see Section 4.3). Cultured
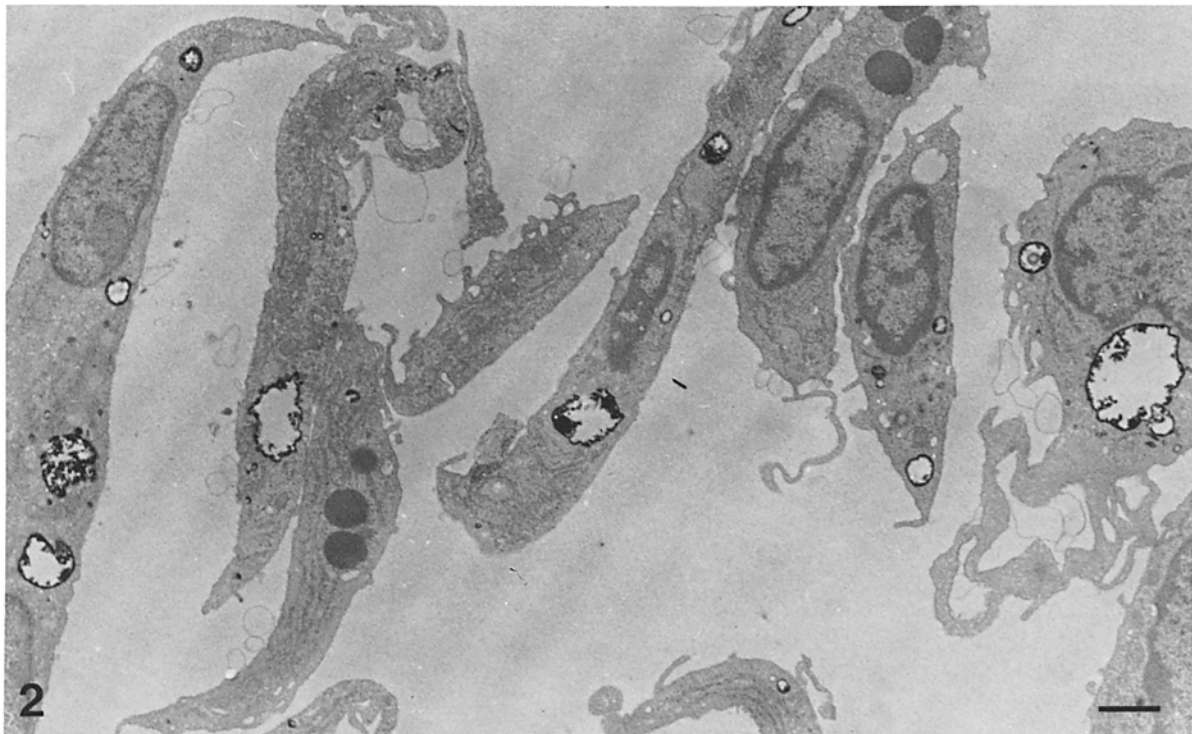

FIGURE 2 Macrophage pinocytic vesicles labeled with soluble HRP. Survey view of a mouse Mφ population exposed 3 min to HRP (1 mg/ml), washed in the cold, fixed, and processed for peroxidase cytochemistry (diaminobenzidine, H_2O_2). The section is otherwise unstained. HRP reaction product rims the periphery of vacuoles of various sizes. Bar, 1 μm. × 7,840.

cells formed large numbers (100 or more) of PV per minute. The dimensions of the PV compartment usually did not exceed 3–5% of cell volume or 10–15% of cell surface area (see Section 4.3).

PV containing latent HRP, but lacking in acid hydrolases, could be retrieved as low density, sedimentable organelles in high yield from cell homogenates (4, 40). However, these PV have not been purified free from PM so that alternative approaches are required to characterize their membrane. Mellman et al. (4) allowed cells to pinocytose lactoperoxidase (LPO) and glucose oxidase. LPO was taken up in the fluid phase of PV, so that the internalized enzymes could be used to selectively radio-iodinate PV membrane. The labeled membrane was compared to iodinated PM by one dimensional SDS PAGE and by immunoprecipitation of surface antigens with monoclonal Ab. For the most part, the iodinated polypeptides were identical to those found in PM, both in molecular weight and intensity of labeling (see Section 4.4). An interesting molecule was a 21,000-dalton species immunoprecipitated by a monoclonal Ab designated 2.6. The amount of labeled 2.6 was increased two- to fourfold in PV membrane relative to PM suggesting that this molecule was enriched in internalized membrane. Similar observations have now been made in latex phagolysosomes labeled before, or after, a latex meal (41).

2.2c PV MEDIATING ADSORPTIVE PINOCYTOSIS: These PV can be visualized using specifically bound virions (13, 42, 43, 44) or ligands coupled to ferritin, gold, HRP or tyrosinase (16, 38, 45–51). This approach has the advantage over soluble tracers in that the initial events of pinocytosis can be studied, i.e., before the closure of the forming PV. In most instances, uptake occurs at coated regions of PM which transform from coated pits to coated PV ~0.1–0.2 μm in diameter (13, 44, 45). Within seconds to minutes, the coat is lost and larger smooth-surfaced vacuoles with ligand are found. The latter can be spherical or irregularly shaped (13, 45, 46, 50) and seem to form by fusion of smaller PV, as occurs in fluid phase pinocytosis (see section immediately above). The larger vacuoles, which have been termed endosomes (52) or receptosomes (50, 51), are not just PV that form more slowly than coated PV, since the density of ligand in endosomes is greater than on the total PM.

The intense current interest in the coated vesicle pathway was stimulated by the work of Brown, Goldstein, and Anderson (45) on the uptake of low density lipoproteins (LDL). LDL, as well as asialoglycoproteins (ASGP), bind primarily to coated pits, even if the cells are chilled to 4°C or fixed with formaldehyde before addition of ligand (16, 45). Other ligands, like Semliki Forest virus (SFV), bind primarily to noncoated regions (microvilli) and then seem to move in the plane of the PM to coated areas at the microvillar base before uptake (13). Discussion of the preferential binding to coated regions is given in recent papers by Wall and Hubbard (16) and Anderson et al. (53).

Coated vesicles were first noted by Roth and Porter (54) and by Droller and Roth (55) in oocytes, and by Fawcett (56) in reticulocytes. Coats were postulated to have a role in adsorptive uptake. The oocyte is unusual since most of the PM can be coated (58), whereas the fibroblast is only 2% coated (59). Similar coats are seen on smaller (<0.1 μm diameter) vesicles and budding membrane in rough endoplasmic reticulum (RER) and Golgi apparatus (60). Perinuclear coated vesicles do not label with endocytic tracers, but often contain acid hydrolases suggesting they are newly synthesized, or primary

Ly (60). Kanaseki and Kadota (61) established that the bristle coats seen in thin sections correspond to a basket composed of hexagonal and pentagonal arrays, similar to a soccer ball. These polygonal baskets or cages have been dramatically displayed on the cytoplasmic aspect of the PM by Heuser and Evans (62) using the deep-etch, rotary-shadowing technique.

Biochemically, the coat consists primarily of clathrin (63), a 180-kdalton protein which has been extensively purified and reassembled into cages in vitro (64–69). Other coat-associated proteins have been identified, the "light chains" (with molecular weights of 30,000 and 32,000) as well as an additional polypeptide of 100,000 (66). The light chains are believed to attach in vitro to the arms of clathrin trimers (known as triskelions) (66–68), while the 100-kdalton protein may be important in mediating clathrin attachment to membranes (70). Anticlathrin Ab can be made and attached to solid phase supports to immunoadsorb coated vesicles from cell homogenates (69). Because clathrin can form spherical or hemispherical cages in vitro, its function may be to drive the endocytosis or budding of the segment of PM on which clathrin assembles. Pearse and Bretscher (71) have postulated that clathrin acts as a molecular sieve that selects specific proteins to be interiorized.

Evidence is available that phagocytosis and fluid phase pinocytosis can also be initiated at coated regions. Using Heuser's technique, Aggeler et al. (72) have shown that the cytoplasmic surface of some latex phagosomes may be partially coated. Marsh and Helenius (17, 42) have used quantitative data to argue that coated PV interiorize most of the solute measured with fluid phase tracers. SFV entered cultured baby hamster kidney (BHK) cells mostly via coated PV, with an average of 1.3 virions per vesicle. Because the rate of entry of SFV was known, they could calculate the volume of fluid internalized. The latter volume was identical to that measured using a fluid phase solute (sucrose). Helenius et al. (13) and Marsh and Helenius (42) emphasized that coats were not found on PV shortly (<1 min) after uptake. Thus, to see material predominantly in coated vesicles, one must administer the tracer, wash, and fix all in a matter of seconds. In the case of HRP uptake, Steinman et al. (1, 6) probably did not wash and fix the cells rapidly enough to detect this fluid phase marker in coated structures. HRP has been detected in coated pits when enzyme fills the extracellular space during fixation (60) or when HRP is attached covalently to an adsorbed ligand (16, 45, 50). In addition, a fraction (perhaps 50%) of the structures seen in thin section as coated vesicles may still be in continuity with the cell surface (46). This would further diminish the likelihood of visualizing fluid phase markers in these coated vesicles.

The most important datum on the composition of coated membrane is that the binding sites for certain ligands are enriched there (reviewed in references 71, 73). To date, localization has most often been achieved with ligands coupled to electron-dense tracers. It remains possible that binding of a multivalent ligand induces receptor clustering and even clathrin attachment; or the coated pit is a region in which receptors are suitably disposed for multivalent binding, while noncoated regions contain receptors that bind ligand in a monovalent, reversible fashion. A more direct approach for visualizing the topographical distribution of surface receptors will be to use high affinity Fab fragments of antireceptor Ab, which are now being prepared in several systems (e.g., references 53, 74–77). Recently, Anderson et al. (53) have employed ferritin conjugates of a monoclonal Ab against the low density lipoprotein

receptor to look for the selective localization of receptor to coated pits, even in the absence of added ligand.

Little is known about the lipid composition of coated PV. Montesano et al. (78) found that filipin did not bind to coated pits in fixed cells. If filipin bound preferentially to sterols, this observation would indicate that cholesterol is excluded from forming PV. On the other hand, the intracellular endosome compartment tagged by ASGP (internalized via coated vesicles in hepatocytes) is apparently rich in cholesterol, i.e., these PV bind significant amounts of digitonin in subcellular fractions (79).

No one has analyzed the composition of coated PV membrane directly. The analysis is especially complicated since coated vesicle fractions may consist primarily of coated Golgi apparatus- or RER-derived vesicles rather than nascent pinocytic vesicles. Assuming that most fluid phase PV are internalized as coated vesicles (17; see above), then the LPO-labeling data of Mellman et al. (4) show that a representative sample of PM polypeptides are included (see section immediately above). This applies to the Mϕ Fc receptor, which was internalized in the absence of ligand. Likewise, latex phagosome membrane may be coated (72) and this membrane is well known to contain typical PM polypeptides (32, 34). However, the absolute concentration or density or these proteins on internalized membrane is unknown.

In summary, the membrane of different types of endocytic vacuoles is similar to PM by many criteria. A possible corollary is that proteins are free to diffuse into the domain of PM to be internalized. In addition, some selectivity can be superimposed on the nascent endocytic vacuole membrane: e.g., enrichment of ligand-binding sites, clathrin, and reduced cholesterol. Still at issue are the PM specializations required for the internalization event (see section 8.1)

2.3 Lysosomes

By definition, Ly are membrane bounded vacuoles rich in acid hydrolases. A primary Ly is the package of acid hydrolases that is formed by the concerted action of RER and Golgi apparatus, whereas a secondary Ly is an acid hydrolase rich vacuole that has acquired substrates by endocytosis or by autophagy. While the early events in primary and secondary Ly formation are difficult to study, a number of coordinate criteria can be used to identify "mature" secondary Ly. By phase-contrast microscopy, Ly granules are phase-dense and usually readily distinguished from mitochondria and lipid droplets. In thin sections, Ly usually contain an amorphous electron-dense content and, occasionally, membranes and particulates. Ly can be labeled with endocytic tracers and with acid hydrolase cytochemistry. The new technology for tagging tracers with colloidal gold should be especially useful in labeling endocytosed content and hydrolases simultaneously. Because of their low pH (see below), Ly can be stained with fluorescent weak bases like acridine orange. Intracellular lysosomal granules with all the above properties are concentrated in the perinuclear region (Fig. 3). However the size of the Ly compartment varies enormously with cell type, environment, and endocytic loads. The volume and area of Ly can be gauged by stereologic techniques (1). Mϕ under routine culture conditions contain more than 1,000 Ly which occupy ~2–5% of the cell volume and have a surface area that is 10–20% of PM area. In L-cell, the dimensions of the Ly compartment relative to total cell volume and area are ~20% of the Mϕ (1).

The pH of Ly in Mϕ is 4.5–5.0. The most elegant data are those of Ohkuma and Poole (80) who studied the pH-dependent shift in the fluorescence spectrum of fluorescein-labeled dextrans after pinocytosis. It is because of this low pH that Ly accumulate any weak base that permeates the PM (81–83), e.g., chloroquine, ammonium chloride, and acridine orange. Once in the Ly, these compounds become protonated and most are rendered impermeable. Protonophores (83) and other ionophores (80) also elevate Ly pH in Mϕ. Most observers believe that Ly are acidified by an energy-dependent proton pump (80–85), although there is evidence that impermeable anions contribute to low pH via a Donnan equilibrium (86).

Cell fractionation has been a classical procedure for isolating Ly. The density of most Ly is sufficiently high to distinguish them from other organelles on isopycnic gradients. Considerable purification has also been obtained by modifying Ly density following uptake of latex (27) or Triton X-100-serum protein complexes (87), and recently by free-flow electrophoresis (88). The membranes of purified Ly have not been studied extensively, and one still needs certain basic control information before such analyses. The membranes should be free of constituents adsorbed to the lumenal and cytoplasmic aspects; lysosomal hydrolases may induce alterations from the native state during purification; and for studies of the cytoplasmic face, the Ly membrane should be intact. The approach of intravacuolar radio-iodination was originally devised to selectively study the lumenal aspect of the Ly membrane in intact cells. Muller et al. (34) fed cells LPO covalently coupled to latex. The LPO-latex particles were localized in acid phosphatase-positive phagolysosomes which could then be selectively iodinated. Incorporated ^{125}I was covalently bound to membrane proteins, and by several criteria, the labeled polypeptides were similar to iodinated PM. The criteria included one and two dimensional SDS PAGE and immunoprecipitation with a group of monoclonal Ab (34, 41). Clearly typical PM was the principal membrane that could be labeled by this approach. Other proteins unique to the Ly membrane remain to be identified, possibly by using other radio-labels or antibodies raised to purified Ly membranes.

2.4 Golgi Apparatus, Endoplasmic Reticulum, and Primary Lysosomes

The RER is the site of synthesis for Ly hydrolases (39, 90) as well as for PM and secretory proteins. Erickson et al. (91, 92) and Rosenfeld et al. (93) have recently reported the first studies of acid hydrolase biosynthesis in cell-free systems. The hydrolases contained a typical signal sequence and became segregated (becoming fully resistant to exogenous protease) within microsomal vesicles. Thus, some mechanism must exist to sort Ly hydrolases from secretory proteins, which may be synthesized in the same cisternae of the RER.

Evidence has been obtained to suggest that this sorting can be the consequence of the mannose-6-phosphate (man-6-P) residues which are found on the carbohydrate moieties of many Ly enzymes (reviewed in reference 94). Sly and others (57, 95–97) have demonstrated the existence of a receptor for man-6-P on fibroblasts which mediates the adsorptive pinocytosis of Ly hydrolases. Sly has proposed that the uptake function of this receptor is secondary and that its "true" function is to segregate hydrolases from other RER products during Ly biogenesis (94). Indeed acid hydrolase from patients with I-cell disease lack man-6-P residues and are secreted into the culture medium
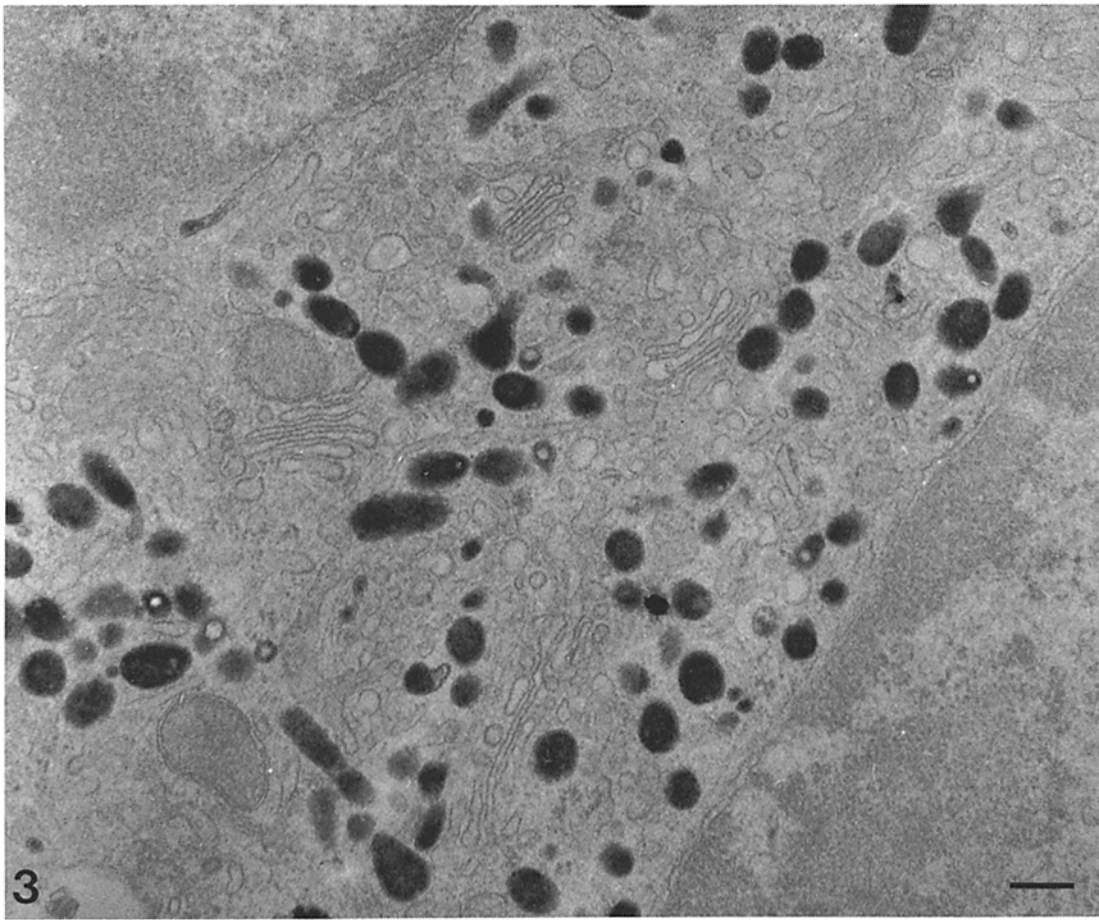
FIGURE 3 Macrophage lysosomes labeled with soluble HRP. Macrophages were exposed to HRP (1 mg/ml) for 1 h, washed, fixed, and processed for peroxidase cytochemistry. Enzyme reaction product fills numerous small lysosomes. The latter are similar in size to the "dense granules" that are normally found in the perinuclear region of cultured mouse Mφ. HRP does not enter the Golgi apparatus stacks. Bar, 0.5 μm. × 35,000.

(94). Cell lines which lack a mannose-phosphotransferase activity are deficient in this sorting function (98), and tunicamycin-treated cells secrete rather than store newly synthesized hydrolases (93). The man-6-P receptor is abundant on intracellular membranes (99). There is an extensive current effort to elucidate the enzymes and compartments responsible for Ly hydrolase biosynthesis (89). However, the man-6-P pathway may not be the only one, since in I-cell disease, some hydrolases like acid phosphatase are not secreted (94).

Newly formed or primary Ly must move out of the Golgi apparatus to interact with endocytic vacuoles, other Ly, or the PM directly. Possibly there are cytoplasmic sorting sequences that direct membrane flow (100). The magnitude of membrane flow in primary Ly is not known. Conceivably, primary Ly membrane recycles continually between Golgi apparatus and endocytic vacuoles.

The Golgi apparatus region can participate in endocytosis in addition to its classical roles in exocytosis and posttranslational modification of proteins. Some endocytic tracers, notably cationized ferritin and dextrans, label Golgi elements in many secretory cells (reviewed in references 101, 102; and section 6). With other tracers and/or cell types, delivery of solute to the Golgi apparatus is not observed (e.g., Figs. 1 and 3). In spite of this participation in both biosynthetic and endocytic processes, the Golgi apparatus, like other components of the vacuolar system, maintains its special repertoire of cytologic features,

enzymatic markers (reviewed in reference 103), and proteins recognized by specific Abs (104, 105).

Novikoff et al. (106) have described a reticulum rich in acid phosphatase along the trans aspect of the Golgi complex, termed GERL (Golgi apparatus-endoplasmic reticulum-lysosomes). The GERL may represent a separate organelle specialized for the biogenesis of some Ly hydrolases, or it may represent the most mature element in a biosynthetic pathway that involves other cisterns in the "classical" Golgi complex. There is still no consensus on the relative labeling of GERL and other Golgi saccules with endocytic tracers. In some studies, GERL seems preferentially involved in uptake (107, 108), whereas in others, tracers seem to enter additional Golgi cisterns (101, 102).

2.5 Pathways of Endocytosis, and Membrane Fusion

The pathways followed by endocytic vacuoles vary with the cell type and applied stimulus or tracer. Endocytosed contents may be delivered to Ly, to Golgi elements, or across cells (Fig. 4). As will become evident when each pathway is considered below, the movement of contents is both rapid and directed.

Membrane fusion occurs at many levels in the endocytic pathway. In some cases, as in the formation of endocytic vacuoles there is fusion of the external membrane faces; in
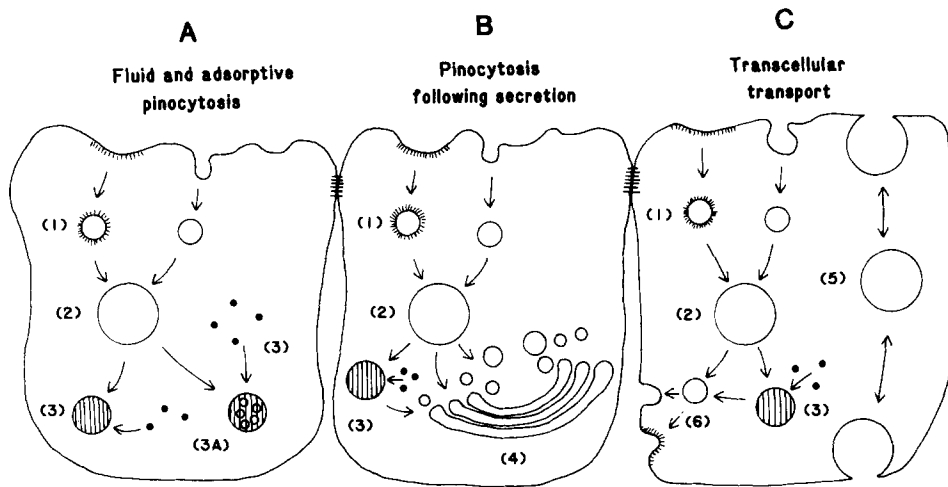
A
Fluid and adsorptive
pinocytosis

B
Pinocytosis
following secretion

C
Transcellular
transport

FIGURE 4 Diagrams of the pathways that can be followed by endocytic vacuoles. In many instances the forming endocytic vacuole (1) is coated, and then the coat is quickly lost. Some nascent endocytic vacuoles may lack coats. The internalized vesicles can fuse with one another (to form a larger intermediate or endosome, (2), with lysosomes (3), with Golgi apparatus (4), or with another domain of the PM. Internalized vesicles may interact with more than one type of organelle, either in series or in parallel. (A) Fluid and receptor mediated pinocytosis (sections 4 and 5). PV fuse with each other to form endosomes, and subsequently with Ly. In some cases, the Ly has the appearance of a multivesicular body (3a). Phagocytosis (not shown) delivers particles to Ly, but does not involve a morphologically distinct, or endosome, intermediate. (B) Pinocytosis following secretion (section 6). In noneuronal secretory cells, endocytosed tracers can be delivered to the Golgi complex (vesicles, saccules, and exocytic granules), to Ly and to endosomes. The exact route seems to vary with tracer and cell type. (C) Transcellular transport (section 7). In endothelia, smooth vesicles (5) shuttle back and forth between basal and apical surfaces, bypassing Ly and Golgi complex. Transepithelial movement of IgA also seems to follow a direct route from one side of a cell to another. Transepithelial movement of IgG, however, may involve Ly and endosomes; the forming and discharging (6) vesicles can both be coated.

other cases, as in PV-PV or PV-Ly fusion, fusion occurs between two protoplasmic faces. It is still very difficult to quantify and manipulate the various fusion events during endocytosis. The fusion of internalized vacuoles with Ly has been studied primarily. One can follow the appearance of internalized contents in acid hydrolase-positive vacuoles determined cytochemically in intact cells, or biochemically in cell fractions. The development of a low pH in an endocytic vacuole can be visualized with acridine orange. Or, Ly can be preloaded with an endocytic tracer, particularly electron-dense colloids, and then one can monitor fusion with a vacuole labeled with a second material. All these methods may be insensitive, i.e., extensive fusion activity may be required before it can be detected.

Kielian and Cohn (109–111) and Kielian et al. (112) used the acridine orange system of Hart and Young (113) to follow the fusion of endocytosed yeast particles in Mϕ. The kinetics of fusion were not influenced by the number of phagosomes and Ly, and were not altered by colchicine or cytochalasin. Substitution of membrane lipids with 19:0 or trans 18:1 fatty acids, which decreases the rate of endocytosis in Mϕ (114, 115) also did not slow fusion. The factors that enhanced fusion rates included addition of phorbol myristate acetate (PMA), prolonged Mϕ culture, or activation of Mϕ by immunologic stimuli. The factors that significantly retarded fusion included: preloading the Ly with polyanions like polyglutamate, sulfatides, and sulfated dextrans; and low temperature (<18°C).

Low temperature also slows fusion of incoming PV and Ly. Dunn et al. (18) perfused glycoprotein ligands through rat liver at <18°C. Receptor-mediated endocytosis occurred, but fusion with Ly was not detectable by several assays. Instead, ligand accumulated in large pre-lysosomal vacuoles or "endosomes." The rate of endocytic vacuole-Ly fusion may also be influenced by the content of endocytic vacuoles. For exampled, PV-Ly fusion was blocked when macrophages interiorized concanavalin A; this lectin accumulated in large, acid phosphatase negative, "endosomes" (116).

We shall now consider the kinetics of membrane flow and

evidence for membrane recycling in each of the pathways summarized in Fig. 4. While these routes are distinguished by operational criteria (type of cell and tracer), there may be many common features that will emerge as the mechanisms which control membrane movement and sorting become better understood.

3 PHAGOCYTOSIS

Phagocytosis is a property of specialized cells, but it provides some of the most direct evidence for membrane recycling and therefore will be considered first. Traditionally, phagocytosis has been viewed as a severe stress on membrane economy. Large portions of the cell surface may be interiorized around particles that are digested slowly and sometimes not at all. Thus, after fusion with Ly, the endocytosed PM was thought to be trapped within a hydrolytic compartment. But the PM and Ly membranes have proved to be much more dynamic as will be evident in three examples of phagocytosis.

3.1 Phagocytosis in Leukocytes

Muller et al. (34) selectively radio-labeled the phagolysosome membrane within viable mouse macrophages. As discussed above (section 2.2a), LPO was covalently coupled to latex and introduced into Ly by phagocytosis. The LPO latex could then iodinate the luminal surface of the phagolysosome membrane at 4°C (Fig. 5a). The label was covalently attached to phagolysosome membrane polypeptides which were identical to those found on iodinated PM. When the cells were warmed from 4°C to 37°C, there was a dramatic redistribution of label from Ly to the PM, as demonstrated by EM autoradiography (117; Fig. 5b). Several observations established that this redistribution resulted from the concerted movement of labeled membrane polypeptides from Ly to PM. (a) The iodinating enzyme, LPO, was covalently attached to the latex beads, and both LPO and beads remained together within intracellular vacuoles throughout the experiment. (b) The protein bound ^{125}I label was nonreusable if digested to ^{125}I-tyrosine or free [125]I. (c) The

FIGURE 5 EM autoradiographic evidence for membrane recycling in cultured Mφ. (A) Mφ were radio-iodinated intracellularly using phagocytosed LPO-latex spheres and exogenous ^{125}I and H_2O_2. The spheres (x) are surrounded by silver grains, and by a rim of dense, peroxidatic (LPO) cytochemical reaction product. Bar, 0.5 μm. × 16,000. (B) Mφ were iodinated intracellularly and then returned to culture at 37°C for 10 min. Radiolabel has redistributed from phagolysosome to the PM. During the 10 min of culture there was little degradation of label, and the LPO-latex (x) remained within the cell. Bar, 0.5 μm. × 16,000.
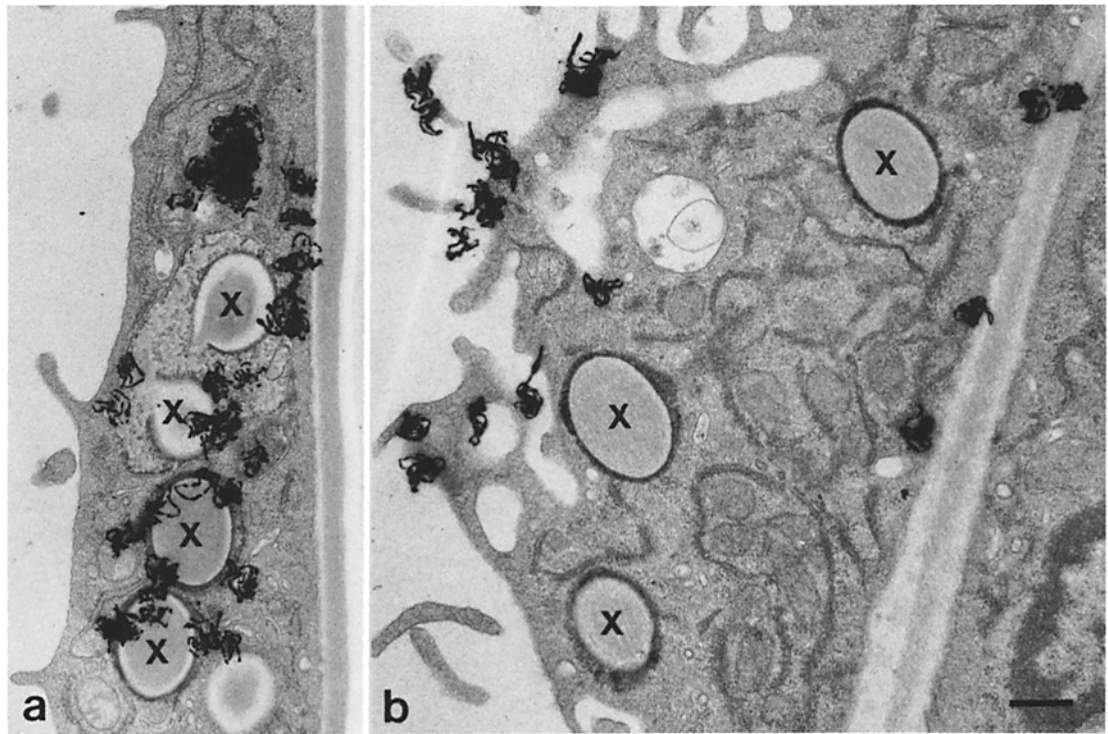
labeled PM was isolated, following recycling, by administering a styrene-butadient bead meal. These styrene butadiene phagosomes could be separated from the LPO latex vacuoles on sucrose density gradients. The same labeled polypeptides that were labeled initially in the latex phagolysosome were thus shown to be present on the PM.

Additional EM-autoradiographic studies have since been performed (41). Movement of label from Ly to PM is extremely rapid, and is complete in 5–10 min at 37°C. Movement may occur at reduced temperatures, even 4°C, although it is markedly slowed. Rapid movement of membrane polypeptides in concert must be mediated by vesicles, although these vesicles have not been visualized.

In addition to a centrifugal flow of membrane (Ly to PM), there is a centripetal flow from PM to latex phagolysosome, mediated by PV. Muller et al. (117) allowed Mφ to phagocytose latex and then found that HRP and colloidal thorium dioxide both were delivered continuously to the phagolysosomes. If the PM of latex laden cells was radio-iodinated, then some labeled PM also was found in the latex compartment (117). Therefore, there is a bidirectional membrane flow (Ly to PM, PM to Ly) which likely is in precise equilibrium, since the latex particles remain tightly surrounded by membrane.

It should not be assumed that all phagocytic vacuole membrane escapes degradation in Ly. Evidence for digestion of PM polypeptides, and enzymatic activities is available (31, 32). The extent of degradation, however, probably is limited by the time endocytosed membrane spends in the Ly.

3.2 Uptake of Yeast Particles in Dictyostelium and Acanthamoeba

Detailed stereologic data of membrane areas during phagocytosis have been obtained in studies of yeast uptake in Dictyostelium (118) and Acanthamoeba (119). Before phagocytosis, roughly equal areas of membrane were present at the cell surface and in the vacuolar system (endocytic vesicles and Ly). Both cells could quickly interiorize a large meal of yeast particles, the uptake step requiring <1 min (120). After phagocytosis, the PM area remained constant despite the fact that phagocytic vacuole surface area was equivalent to 40% of PM area. In addition to phagocytic vacuoles, Bowers et al. (119) measured two compartments of intracellular membranes: large, electron-lucent vacuoles, which were Ly because they stained cytochemically for acid phosphatase, and small vacuoles of uncertain identity. The combined area of the latter large and small vacuoles decreased in parallel to the increase in the area of vacuoles containing yeast. These observations suggest that membrane moves from intracellular vacuoles to the PM to maintain PM area following phagocytosis. Bowers' freeze-fracture studies (120) have documented that newly formed phagosomes are surrounded by small vesicles, many of which are in continuity with the phagosome. These vesicles may contribute membrane to the forming phagosome, and/or may represent recycling vesicles moving to the PM.

3.3 Food Vacuole Formation in Ciliated Protozoa

Feeding in ciliated protozoa yields large food vacuoles that undergo a number of condensation and fusion reactions summarized in Fig. 6, taken from the work of Allen and Staehelin (121). Phagocytosis begins at the cytopharynx by formation of a vacuole (DV I), which then condenses to form the DV II. Ly fuse to form a mature digestive vacuole (DV III), which finally discharges degraded material at the cytoproct. Each stage in the life history of a phagocytic vacuole requires only minutes, and each may be associated with extensive membrane flow and recycling according to the ultrastructural studies of Allen and colleagues (121–124) and McKanna (125).

The area of membrane interiorized during food vacuole formation (DVI) is many times that of the cytopharynx in
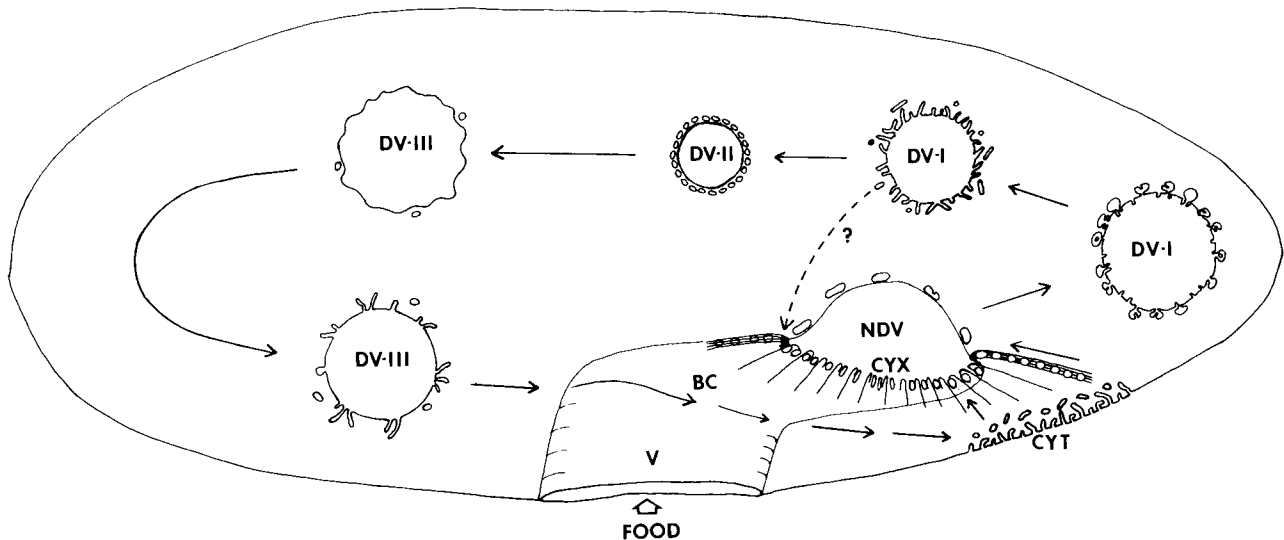
FIGURE 6   A diagram, kindly provided by Dr. Richard Allen (121) illustrating the pathways of membrane flow during endocytosis by *Paramecium caudatam*. Food enters the vestibulum (*V*) of the buccal cavity (*BC*), and a nascent digestive vacuole (*NDV*) arises at the cytopharynx (*CTX*). A digestive vacuole (*DV-1*) forms which is first surrounded by, and then connected to, many vesicles. The DV I condenses to form the highly acidified DV II, which then fuses with lysosomes to form the DV III. The latter exocytoses at the cytoproct (*CYT*).

which phagocytosis occurs. The membrane required for phagosome formation is probably derived from nearby discoidal vesicles (121).

The DV I then condenses to form the DV II, and the pH drops precipitously to <5.0 (126, 127). Recent observations (128) suggest that the development of the DV II is associated with fusion to nearby, non-Ly vesicles. The latter may correspond to the neutral red-positive granules described by Mast (126). Since neutral red is a weak base and will stain acidified vacuoles, it is possible that the DV II acidifies after it interacts with these neutral red-positive granules. Yet, Ly fusion does not occur until later, at the DV III stage (129, 130). During condensation of the DVII, many cylindrical vesicles from (~0.1 × 0.4–0.8 μm); these vesicles might recycle membrane to the cytopharyngeal region to participate in additional food vacuole formation (121).

Formation of DV III occurs by the extensive fusion of Ly with the DV II. In the DV III digestion of phagocytosed bacteria can be visualized and the pH rises gradually to neutrality (126, 127). During digestion, the DV III condenses and seems to give rise to small vesicles (121), which possibly are used in future DV II to DV III transitions. The final event is defecation or the expulsion of contents at the cytoproct. Following exocytosis, the vacuole rapidly (<30 s) collapses into a mass of flattened and tubular PV which can be labeled with exogenous HRP (124). This retrieval of exocytic vacuole membrane by pinocytosis is typical of many secretory systems (see section 6). The fate of the retrieval PV in *Paramecium* has not been established. The PV might contribute to the cytopharyngeal discoidal vesicle and/or Ly pools.

Additional methodology is required to further document the proposed movement of membrane and contents in the digestive cycle of ciliates. Yet this system provides some of the most dramatic images of the extent and rapidity of membrane fusion, vesiculation, and movement during endocytosis. Of particular interest is the data that DV II represent acidified condensing endocytic vacuoles that have *not* fused with Ly. The timing of the acidification and Ly fusion events during endocytosis are

critical unknowns in the study of recycling and will be discussed further below (see section 8.2 and 8.3).

4   FLUID PHASE PINOCYTOSIS

The concept of membrane recycling was proposed sometime ago to account for the enormous volumes of extracellular fluid internalized by cultured cells during pinocytosis. In his original description of pinocytosis in 1931, Lewis (131) somewhat generously estimated that Mϕ interiorized approximately three times their total cell volume per hour. In view of this observation, Palade (132), when he first identified tiny or "submicroscopic" pinocytic vesicles (PV), reasoned: "The quantities (of fluid) involved in pinocytosis in macrophages, as well as the abundance of "submicroscopic" pockets and vesicles associated with the cell membrane in many other cell types render unlikely a unidirectional flow of membrane material from the cell surface towards the interior of the cytoplasm. It is more probable that the membrane is repeatedly circulated between the two locations mentioned." Subsequent experimental evidence has largely confirmed this prediction. In this section, we will review morphologic and biochemical studies of fluid phase pinocytosis which indicate a continuous, rapid, and extensive bidirectional flow of PM.

4.1   Volume of Fluid Internalized by Pinocytosis

The volume of pinocytosed fluid is calculated from the uptake of solutes that are interiorized primarily in the fluid phase, e.g., sucrose (1, 133), insulin (134), dextran (23), horseradish peroxidase (HRP; 5, 6) (In some Mϕ, adsorption of HRP to mannose receptors can contribute to uptake [135]), polyvinylpyrrolidine (PVP; 135, 136), succinylated concanavalin A in the presence of competing sugar (40), and even sugars and amino acids in some amoebae (134). The evidence that these solutes are interiorized without significant adsorption to the plasma membrane (PM) is the following: uptake varies linearly with concentration of solute in the medium, showing no evidence of saturation over a large (mg/ml) range; uptake of

labeled solute is not reduced when unlabeled solute is added to the medium; binding to the cell surface is not detectable in quantitative or morphologic assays at 4°C and 37°C; and when more than one solutes is tested, uptake of each occurs at a similar rate. Fluid phase markers generally accumulate continuously in the cell for the duration of the experiment usually 3–7 h (1, 6, 133, 134, 136, 137) but in some cases 96 h (111). For most solutes, regurgitation back into the extracellular space is difficult to detect with the exception of the brief period after uptake (see section 4.5 below). However, the catabolic products of these solutes (e.g., amino acids) can be found released into the culture medium concomitant with solute degradation.

The volume of fluid pinocytosed by mouse peritoneal Mφ and *Acanthamoeba* is equivalent to 20–25% of their cell volume each hour (1, 134). L-cells and many fibroblasts in culture interiorize 25% of their cell volume every 3–6 h (1). These volumes are even more striking if one considers that the volume of the Ly space into which solute is being delivered is only 1–10% of total cell volume (see below). Yet both cell and Ly volumes change very little, and as mentioned, fluid phase uptake can continue at constant rates for days in culture (1). These volume measurements indicate that internalized fluid must be returned to the extracellular space either by exocytosis or by permeation across vacuolar membranes (see sections 4.5 and 8.4).

4.2 Direct Measurements of PV Dimensions

Measurements of individual PV sizes provides the best information on the magnitude of PM flow during pinocytosis. Bowers and Olsewski (134) tagged *Acanthamoeba* PV using a 20-s exposure to exogenous HRP. The labeled PV were heterogeneous in size and shape but most had an average profile diameter of 0.25 μm. PV of this dimension would be expected to interiorize roughly 10 times the cell surface area (estimated to be 2,200 μm^2) during 1 h of pinocytic activity.

A detailed kinetic study was carried out by Steinman et al. (1) who used stereologic techniques to measure individual PV profiles in mouse Mφ following short pulses (30–300 s) of HRP (Fig. 7). The distribution of sectioned diameters was converted to a distribution of actual PV dimensions using a modification of Wicksell's technique. The mean PV volume thus obtained (0.015–0.025 μm^3) was multiplied by the number of PV which formed (80–120 per cell per min), giving a volume of 1.7 μm^3/cell/min. This value compared favorably with the volume of fluid uptake inferred from HRP solute measurements (1.2 μm^3/cell/min.) Similar data on the volume of PV were obtained using direct point counting (volume-fraction) of the HRP-reactive PV compartment (1; Fig. 7 a)

Accordingly, it could be determined that the area of membrane surrounding an average PV labeled during a 30–300-s exposure to HRP was 0.265 μm^2 (1). Thus, ~27μm^2 of PM, corresponding to 3.1% of the Mφ cell surface, was being interiorized per minute. A similar result was obtained using a simple approach in which the percentage of test lines crossing PV vs. PM was measured (Fig. 7b). The analysis was also carried out using L-cell fibroblasts in which the area of membrane internalized as PV's was 0.9% of the cell surface per min (1). In other words, Mφ and L cells interiorized the equivalent of their entire cell surface area once every 33 min and 2 h, respectively. Clearly, the influx of membrane around PVs is huge, indicating again that recycling must occur, rather than degradation and replacement of internalized PM.
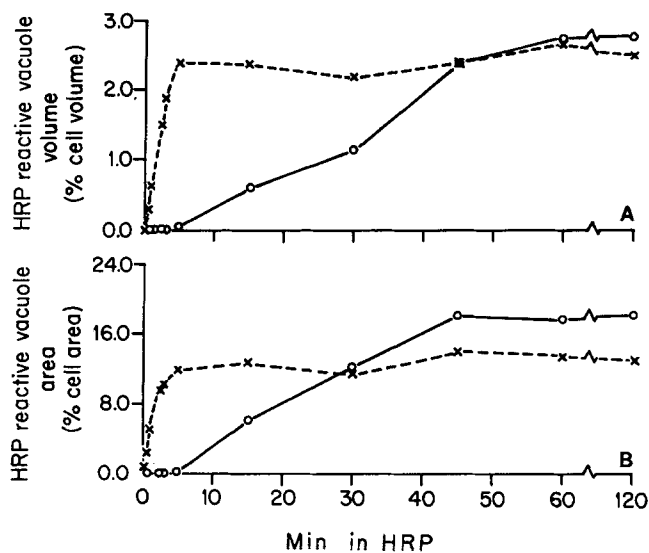

FIGURE 7 The kinetics of formation of HRP reactive pinocytic vesicles (X) and secondary lysosomes (O) taken from Steinman et al. (1). Macrophages were cultured for varying periods of time in 1 mg/ml HRP. The cultures were quickly (15–30 s) washed, fixed, processed for peroxidase cytochemistry, and analyzed by stereologic techniques. To measure vacuole volume relative to cell volume (A), a grid of test points was placed on the micrographs; the percentage of points falling on vacuole vs. cell was measured. To measure vacuole surface area relative to cell surface area, (B) a grid of test lines was used; the percentage of lines crossing vacuole vs. cell surface was measured.

4.3 Delivery to Lysosomes

During the first 3–5 min of exposure to HRP, both the volume and membrane area of the HRP-labeled PV compartment in Mφ expanded at a rapid rate—0.43% of cell volume and 3.1% of PM area per min (Fig. 7). During this time interval, incoming PV underwent little if any fusion with secondary Ly. This was determined by preloading Ly with an electron-dense colloid, thorium dioxide or gold, and then finding that HRP-reactive vacuoles did not acquire colloid during the first 3–5 min after exposure to HRP. At 3–5 min, a new population of cytochemically reactive vacuoles became detectable. These presumptive Ly were smaller (average volume of 0.0075 μm^3 vs. 0.036 μm^3 for the population of PV); and were filled completely with cytochemical reaction product (vs. incoming PV which had only a rim of reactivity). In pulse-chase experiments, HRP moved out of the PV compartment and into the Ly compartment (1, 40). More significantly, the rate of expansion of the total HRP reactive space slowed at 3–5 min, so that recycling of interiorized membrane had begun (Fig. 7). It was postulated that recycling was initiated when the PV fused with Ly and underwent shrinkage, concentration of endocytosed contents, and vesiculation to yield small recycling vesicles. These data are also consistent with the possibility that some internalized fluid and membrane began recycling *before* Ly fusion (see sections 4.5 and 8.4).

After 45–60 min in HRP, the HRP-reactive Ly space also reached its maximum dimensions, corresponding to 2–3% of the total cell volume with an area of 17–18% of the cell surface (Fig. 7). Neither the PV nor the Ly compartments expanded further during an additional 1–2 h in HRP, even though cells continued to interiorize fluid and PM at a rate equivalent to

five to ten times the volume and membrane area of the vacuolar system each hour. These observations quantified the dramatic discrepancy between the volume and area interiorized in PV and the dimensions of the Ly compartment in which HRP accumulated.

Muller et al. (117) performed an analogous experiment. A subpopulation of Mφ Ly was tagged with 1 μm polystyrene latex beads and then the cells were exposed to HRP. The latex-filled phagolysosomes could be purified on sucrose density gradients and studied directly. HRP accumulated continuously for 2 h in this Ly compartment; moreover, accumulation occurred at the same rate as in the total cell homogenate. By EM, the latex particles in intact cells were always tightly surrounded by Ly membrane, so that again solute was being delivered continuously without expanding Ly dimensions.

4.4 Studies of Labeled PV Membrane

The above data show that substantial amounts of PM are interiorized and solutes delivered to Ly, without continually expanding the size of the vacuolar system. The importance of this process is enhanced by evidence that most PM constitutents are susceptible to internalization (see above, section 2.2). Specifically, when Mφ are iodinated intracellularly with LPO and glucose oxidase in PV, the polypeptides that are labeled are similar to those labeled externally (on the PM) with LPO (4). The proteins labeled in PV do not represent a subpopulation of membrane components destined for rapid degradation. It is known that radio-labeled PM proteins turnover slowly ($t_{1/2}$ of 10–80 h; 32, 138, 139). Mellman (140), therefore, compared the turnover of labeled PV membrane with that of labeled PM and found that the label in both compartments turned over at similar slow rates. In contrast, the LPO that was "self-iodinated" during PV labeling was rapidly degraded ($t_{1/2}$ of <3 h vs. $t_{1/2}$ of >20 h for most membrane polypeptides). These experiments showed that LPO, a fluid phase marker, was delivered or sorted to a degradative compartment, while PV membrane escaped degradation presumably by recycling to the PM. Additional experiments are required to document directly the return of PV membrane label to the PM, as was done in the case of cells iodinated intracellularly with LPO-latex (see above, section 3.1).

Storrie et al. (141) have also used the technique of LPO-mediated intracellular iodination in Chinese hamster ovary cells. Cells were fed soluble LPO for 10 min, washed, and iodinated at 4°C by addition of exogenous H_2O_2. Biochemical characterization of the labeled species remains to be detailed. However, the incorporated radio-label was insensitive to protease, whereas 45% of the label introduced by LPO into PM was solubilized by protease. If cells were returned to culture following intracellular iodination, 45% of the label became protease sensitive within 10 min. Thus iodinated proteins rapidly converted from protease insensitive to sensitive, presumably by recycling from within the cell to the PM.

Thilo and Vogel (142) tagged the surface of Dictyostelium with [^{3}H]galactose using galactosyl transferase at 4°C. The labeled species were not characterized but could represent integral PM glycoproteins and/or peripheral or secreted glycocalyx components. Upon warming, 50% of the radio-label became resistant to release by β-galactosidase, indicating that a fraction of [^{3}H]galactose molecules had been internalized. Accordingly an experiment was performed in which [^{3}H]galactose-labeled cells were first warmed to internalize labeled PM, then chilled and treated with β-galactosidase to remove remaining PM label. A small amount of β-galactosidase-sensitive label reappeared on the cell surface, suggesting the recycling of internalized components from within the cell. However, this system was a difficult one since there was a large background of β-galactosidase insensitive label at all time-points.

4.5 Reflux of Internalized Fluid and Contents

If membrane recycling occurs by a vesicular means, a necessary correlate is that some internalized fluid be returned to the extracellular space. In several studies, once macromolecular solutes have been delivered to Ly, little or no exocytosis occurs. Indigestible particles such as latex, colloidal gold, and thorium are retained for days, even weeks, within Ly (137, 143, 144). Soluble macromolecules like HRP (5, 6) and PVP (145–147) also are quantitatively retained with little if any reflux detected using sensitive assays. A solute that may be released from Ly is sucrose. When Mφ interiorize large concentrations of sucrose (10–100 μM), the vacuolar apparatus swells markedly in 2–3 h (148) due to the fact that sucrose is osmotically active. Pinocytosed invertase rapidly digests the sucrose, leading to vacuole shrinkage within 30–60 min (148). However, sucrose vacuoles also disappear slowly during overnight culture in the absence of invertase. This could mean that sucrose is slowly exocytosed, permeates the Ly membrane, or is degraded to permeable monosaccharides. Likewise if cells interiorize radio-labeled sucrose at tracer levels, a slow release ($t_{1/2}$ of 3–12 h) of intact label into the medium is detectable (137, 149). It is not clear whether sucrose, or macromolecular markers like gold and HRP, provides the representative picture of fluid reflux from typical Ly.

On the other hand, a portion of content markers like PVP, sucrose, and colloidal gold may be regurgitated from a PV compartment during the brief (5–15 min) period directly following uptake. Specifically, there are many reports (137, 145–147) in which cells have been exposed to tracers, washed, and then 5–20% of cell associated label was detected in the medium within a few minutes. This work has thus far been difficult to interpret, since none of the studies rigorously controlled for the simple release of dish- or surface-bound solute. Recently, however, Besterman et al. (149) have performed a detailed analysis of [^{3}H]sucrose release from cultured Mφ and fibroblasts. Monolayers were exposed to labeled sucrose for 5–180 min, washed quickly at 4°C and returned to culture at 37°C. Intact sucrose was then released into the medium, apparently by exocytosis. This release was temperature dependent, varied linearly with the concentration of sucrose initially administered, and was not competed for by excess unlabeled sucrose. Invertase degrades sucrose at 4°C but did not release label from chilled monolayers, indicating that there was minimal sucrose adsorption to cells. When the kinetics of sucrose release were analyzed, both slow ($t_{1/2}$ of 3 or more hours, see above) and fast compartments were identified. The rapid compartment had a $t_{1/2}$ of 5 min, became saturated after a 5-min exposure to sucrose, and corresponded to 2.5% of the cell volume. It is known for stereology that internalized membrane must begin to recycle within 3–5 min of pinocytosis (see section 4.3); that the PV compartment fills within 3–5 min in Mφ; and that PV account for 2–3% of the cell volume. Therefore, the rapid release of sucrose could be mediated by vesicles recycling from the PV compartment. Adams et al. (150) demonstrated an analogous release of the macromolecular tracer, HRP, from CHO cells. Again, reflux

occurred at 37°C, but not at 4°C. Exocytosis probably occurred from the PV as opposed to the Ly compartment, since the same amount of HRP was released after 10 min (when most HRP was in PV) and 60 min (when most HRP was in Ly) exposures to enzyme.

A fascinating example of regurgitation following pinocytosis comes from the work of Aley (151) in *Entamoeba histolytica*. When amoeba were exposed continuously to tracers like HRP and fluoresceinated dextran, solute accumulated but reached a plateau in 2 h. Pinocytosis had not ceased at 2 h since exposure to a second fluid phase marker gave the same kinetics of accumulation as the first tracer. Both markers were visualized in large (0.25–1.0 μm) vacuoles that had the same pH as the culture medium. If the cells were pulsed and washed, most of the pinocytosed solute was regurgitated, apparently intact, back into the medium. Only small amounts of solute reached typical Ly which were small, acidified, and perinuclear. Therefore, it is possible that PV can recycle their contents without an obligatory interaction with other organelles like Ly and Golgi apparatus.

5 ADSORPTIVE ENDOCYTOSIS

Adsorption to the PV membrane provides a mechanism for enhancing the efficiency with which substrates are cleared from the extracellular space. This process was appreciated in the early work on amoebae pinocytosis (152–154) and has been evident in many subsequent quantitative studies of mammalian cells. For example, the uptake of HRP in immune complexes (HRP-anti-HRP) can be 100 to 1,000 times the rate of uptake of enzyme in the fluid phase (155). Other adsorbed markers include certain cationized proteins, lectins, antibodies, virions, and a large group of ligands that bind with high affinity to specific PM sites (receptor-mediated endocytosis). In many cases, high rates of adsorptive uptake can continue for hours even with saturating levels of ligand.

Although adsorbed substrate would seem an ideal tool to study membrane flow, there are some difficulties. *A priori*, one cannot assume that simple binding to the PM is necessarily followed by uptake, since the adsorbed material must be attached to a segment of PM that is internalized. For example, certain polypeptide hormones (156) and transcobalamin II (157) bind with high affinity to specific PM receptors but are internalized at relatively slow rates. Furthermore, the adsorption approach is indirect, since the internalization of ligand rather than binding site is usually studied. Nevertheless, a large body of data pertinent to membrane recycling exists and it will be considered in three sections: recycling during receptor-mediated endocytosis; receptors that do not recycle; and recycling during other forms of adsorptive uptake.

5.1 Recycling during Receptor-mediated Endocytosis

Over two dozen examples of high affinity binding sites which mediate the endocytosis of specific ligands are now known (Table I); most of these receptors deliver ligand to Ly. These ligands often have important physiologic functions and offer the potential for studying the dynamics of a specific PM binding site in detail. The amount of ligand on the PM can be distinguished from that internalized into the cell using reagents that quickly elute the PM pool of ligand at 4°C, e.g. EDTA, competitive inhibitors of ligand binding, low pH, and proteases. Furthermore, biologically active conjugates tagged with radio-

label, ferritin, HRP, or colloidal gold can all be prepared, facilitating both quantitative and morphologic analysis.

Kinetic studies of ligand uptake in several instances have suggested that individual binding sites deliver ligand and are reused many times every hour. The most detailed examples are available in the recent work of Schwartz et al. (158) and Zigmond et al. (159). Table II illustrates some of the more extensively studied systems. A comparison of columns *1* and *2* (molecules/cell bound to PM at saturation vs. molecules/cell internalized/hour) indicates that during a continuous exposure to ligand, cells internalize 5–30 times the maximum number of surface-bound molecules. The number of PM binding sites remains constant despite this continued ligand uptake, often for several hours. Furthermore, pulse-chase experiments (ligand binding at 4°C, and cells warmed to 37°C) have indicated that ligands are rapidly internalized, being cleared from the PM with a $t_{1/2}$ of 2–5 min. Accordingly, it has been proposed that the binding site enters the cell, delivers its ligand, and, within minutes, recycles back to the cell surface to mediate another round of uptake. This hypothesis requires that: (*a*) the number of binding sites on the PM approximates the actual number of receptor molecules; (*b*) the binding site enters the cell with ligand; (*c*) there is no large pool of internal receptor capable of replacing interiorized binding sites; (*d*) the binding site can liberate or deliver its ligand to Ly; but (*e*) the receptor is not itself degraded. These assumptions are currently under investigation and will be considered individually.

5.1 a THE NUMBER OF CELL SURFACE RECEPTORS: In most cases (Table II, column *1*), one measures the maximum numbers of ligand molecules bound per cell, rather than the number of receptor molecules. The two parameters need not be identical, since many ligands appear capable of multivalent attachment (e.g., man-6-P-containing glycoproteins, LDL, ASGP), and monovalent binding may be so rapidly reversible that it is not detected by current procedures. While these situations would result in an underestimation of the actual number of receptor molecules, it is also possible that receptor number may in some cases be overestimated by ligand binding studies. Any one class of ligand molecules may in fact bind to more than one site. For example, α_2-macroglobulin binds to 10,500 "high affinity" and 600,000 "low affinity" sites on fibroblasts (160).

A better approach for quantifying the number and distribution of receptors is to prepare a specific Ab to an externally disposed portion of the ligand binding site. A good example is the monoclonal Ab, designated 2.4G2, directed against the trypsin-resistant Fc receptor on mouse leukocytes (161). Fab fragments of this high affinity Ab block the binding of immune complexes containing mouse IgG1 and IgG2b, and have been used to purify the receptor by affinity chromatography (74). The isolated receptor is itself capable of specific binding of ligand (74). Thus the actual number of surface receptor molecules has been measured by quantitating the binding of [125]I-2.4G2 Fab. For the Mϕ cell line J774, the number of receptors/cell at saturation is 500,000–600,000 while the number of soluble IgG immune complexes (ligand) bound is 600,000–800,000 (I. Mellman, unpublished observations). Similarly, Fearon (77) has made an Fab fragment of a heteroantiserum to the human leukocyte C3b (complement) receptor which blocks the binding of complement-coated particles. Using this reagent, human polymorphonuclear leukocytes have been found to have 90,000 receptor molecules/cell (Fab anti-receptor binding sites) as compared to 120,000 ligand binding sites.

TABLE I

Ligands for Receptor Mediated Endocytosis

Category*	Ligand‡	Cell type	Delivery to lysosomes§	Characteristics‖ of receptor
Nutrients				
	Low density lipoprotein	Fibroblasts (2)	Yes (2, 9, 73, 262)	160-kdalton glycoprotein (145, 263, 264)
	Instrinsic factor	Intestinal epithelium (265)	Probably (265)	180-kda glycoprotein (266) (59- and 42-kdalton dimer)
	Transcobalamin II	Many (265)	Yes (14, 157)	50-kdalton glycoprotein (267)
	Transferrin	Many (15)	Probably (15)	180-kdalton glycoprotein (268) (90-kdalton dimer)
	Phosvitin (yolk proteins)	Oocytes (58)	No (58)	—
Virions, toxins				
	Reovirus	Fibroblasts (43, 269)	Yes (43, 269)	—
	Semliki forest virus	Fibroblasts (13, 42, 270)	Yes (13, 42, 270, 245)	—
	Vesicular stomatitis virus	MDCK cells (271)	Yes (271)	—
	Influenza virus	Many (272)	Yes (44)	Sialic acid (272)
	Diptheria toxin	Many nonmurine cells (273, 274)	Probably (254, 255)	—
	Cholera toxin	Many (274, 275)	Yes (276)	G.M. ganglioside (275)
Effector molecules				
	Insulin	Hepatocytes monocytes, adipocytes (181, 182, 277)	Yes (181, 182, 277)	130–135-kdalton and 49–90-kdalton glycoprotein (278)
	Chorionic Gonadotropin	Leydig tumor, ovarian luteal cells (279, 280)	Yes (279, 280)	—
	Chemotactic peptide	Leukocytes (281, 282)	Yes (283)	—
	Complement (C3b)	Leukocytes (27, 77)	Probably (240)	205-kdalton glycoprotein (77)
	Epidermal growth factor	Fibroblasts (7)	Yes (7)	190-kdalton glycoprotein (176)
	Mouse IgG₁, IgG2b	Macrophages, lymphocytes (231)	Yes (8)	47–60-kdalton glycoprotein (74)
	Mouse IgG₂ₐ	Macrophages, lymphocytes (231)	Probably (231)	65-kdalton glycoprotein (284)
	Mouse IgG3	Macrophages, lymphocytes (23, 285)	Probably (231)	—
	Maternal IgG	Intestinal epithelium (170, 251)	Probably (38)	—
	IgA	Leukocytes, intestinal epithelium (221, 223)	No (225)	90-kdalton glycoprotein (226)
	IgE	Basophils, mast cells (286)		45–55-kdalton glycoprotein (286)
Clearance from the extracellular space				
	Galactose-terminal glycoproteins	Hepatocytes (287–289)	Yes (45, 290)	42–59-kdalton glycoprotein (164, 165)
	Mannose-fucose-terminal glycoproteins	Macrophages, endothelial cells (11, 288, 291)	Yes (11)	—
	Man-6-P glycoproteins	Fibroblasts (88, 94–99)	Yes (12, 94, 292)	150-kdalton glycoprotein (76)
	Acetylated low density lipoproteins	Macrophages (244, 293)	Yes (244, 293)	—
	α₂ Macroglobulin-protease complexes	Macrophages (295)	Yes (10)	—

* For simplification, we have classified ligands according to the presumed physiologic consequence of endocytosis.
‡ Some ligands, particularly some hormones and virions, are not listed because they are reviewed elsewhere (43, 294).
§ "Delivery to lysosomes" means that ligand accumulation has been visualized in Ly, or that degradation of ligand occurs intracellularly.
‖ In most cases, receptors have been identified only by ligand-mediated affinity chromatography. Thus, identification should be considered provisional until the isolated molecule(s) have been demonstrated to have functional and/or immunologic identity with cellular receptors.

This general approach will probably be extended to other receptors shortly, since an increasing number of antireceptor Ab are being isolated and characterized.

5.1 b INTERNALIZATION OF RECEPTOR AND LIGAND: Several morphologic studies have indicated that ligand and receptor enter the cell together. Wall et al. (16) used lactosaminated ferritin, which is an electron-dense probe for the hepatocyte ASGP receptor. As expected. the ferritin molecules were closely apposed to the PM. Ferritin was also closely apposed to the membrane of recently internalized PV, suggesting the continued binding of ligand to receptor. These vesicles (coated and uncoated) were judged to be fully internalized because they failed to stain with ruthenium red, a marker for external glycocalyx. Within minutes the PV appeared to fuse with one another, and/or with Ly, and at this time the ferritin was found in the lumen of the resulting vacuole or endosome.

Receptor for:	Cell type	Molecules/cell bound to plasma membrane at saturation	Molecules of ligand internalized/h/cell at saturation	Molecules/cell bound to intracellular membranes	Half-time for internalization of surface-bound Ligand	Half-Life of Receptor	Effect of cyclohex-imide on internalization
					min	h[‡]	
LDL	Human Fibroblast	20,000–70,000 (2, 9)	560,000 *(2, 9)	Few (167)	~5 (2, 9, 45)	25 (2, 9)	None (2, 9)
Phosphomannosyl-gly-coproteins							
β-glucuronidase	Fibroblast	36,800 (12)	406,000 *(12)	>147,000 *(12, 99, 166)	~2.5 (12)	—	None (12)
α-iduronidase	Fibroblast	14,000 (97)	154,000 *(97)	—	~2.5 (97)	—	—
Asialoglycoproteins							
	Isolated rat hepa-tocyte	250,000 (164)	~5,000,000 (164)	300,000 (164)	—	20 (164)	—
	Human hepa-toma	150,000 (158)	900,000 (158)	75,000 (158)	2.2 (158)	—	None (158)
Mannosyl-fucosyl-glyco-proteins	Rat alveolar mac-rophage	~75,000 (11)	2,250,000 *(11)	—	<5 (11)	—	None (11)
α-Macroglobulin-pro-tease complexes	Rabbit alveolar macrophage	~100,000 (10, 295)	500,000–1,000,000 (10)	Few (10)	2–4 (16)	3.3 (10)	None (10)
Transferrin	Mouse teratocar-cinoma	5,700 (15)	73,000 *(15)	—	3 (15)	—	—
Transcobalamin II	Fibroblast	3,000–4,000 (18)	~1,750 *(14)	—	~60 (157)	~8 (157)	None for 3 h (157)

* Estimated from published data.
‡ Measured using protein synthesis inhibitors.

Similar images have been obtained during endocytosis of Semliki Forest virus (13), LDL (45, 49), and α₂-macroglobulin (51). The interpretation of these data is that the ligand-receptor complex dissociates shortly after interiorization.

Additional indirect support for the coordinate internalization of ligand and receptor comes from systems in which ligand enters the cell and simultaneously, binding sites are lost from the PM. This occurs during down regulation of the EGF, insulin, and Fc receptors (section 5.2). Down regulation also occurs during adsorptive uptake of acid hydrolases (12) and LDL (162), as long as chloroquine or monensin are included in the culture medium. It is thought that these drugs raise Ly pH and thereby interfere with ligand discharge and/or receptor recycling. Perhaps the most detailed model is the interaction of chemotactic peptide with neutrophils (159). Kinetic studies indicate that a pulse of ligand enters the cell together with receptor, within minutes, receptors and some of the ligand, reappear on the cell surface by a cycloheximide insensitive mechanism (163).

Biochemical studies on the entry of receptor molecules in PV are hampered by the lack of methods to purify and/or clearly separate PV from other organelles by physical techniques. An alternative approach is to selectively radiolabel PV membrane within living cells (4; see section 2.2). This mode of analysis has thus far been applied only to the Mφ Fc receptor. In the absence of exogenous ligand, identical relative amounts of iodinated receptor were detected in both PM and PV membrane (4). Hopefully this technique can be adapted, e.g., using LPO coupled to specific ligands, to radio-label receptor molecules during ligand uptake.

5.1 c THE INTRACELLULAR RECEPTOR POOL: It is possible that there is a large intracellular receptor pool capable of replenishing surface binding sites following uptake of ligand-receptor complexes. Accordingly, a recently internalized receptor need not itself recycle immediately. Stahl et al. (11) trypsinized alveolar Mφ at 0°C for 30 min and found a 70% reduction in mannosylglycoprotein surface binding sites. Within a minute after warming to 37°C, the surface binding activity had returned. The interpretation was that trypsin destroyed PM binding sites, and that after warming, previously internalized receptors recycled to the PM. An alternative explanation is that

trypsin alters the disposition of receptors capable of multivalent attachment, and that warming restored binding activity by allowing rapid diffusion of receptors within the PM.

Several laboratories have tried to quantitate intracellular receptor pools. In most of these experiments, ligand binding studies have been performed on crude or microsomal membrane fractions to try to compare the number of binding sites on the PM with total cell homogenates. For both man-6-P and ASGP, the total level of binding sites is four- to tenfold greater than can be found on the PM (99, 164, 165, 166). In contrast, values for PM and total binding sites are similar for LDL and α₂-macroglobulin-protease complexes (10, 167). It has not yet been possible to confirm that ligand binding to subcellular fractions and to intact cells can be measured with equivalent efficiencies, so it is difficult to interpret these findings. It would be appropriate to await studies which quantitate or visualize receptors by other assays, e.g., specific antibody binding or radio-immunoassay. In our view, there are still no compelling data on the amount of receptor on the PM relative to the amount in the intracellular pool.

5.2 d DISCHARGE OF LIGAND AFTER ENDOCYTOSIS OF LIGAND-RECEPTOR COMPLEX: For a number of ligands, a decrease in pH to ~5 could provide a rapid mechanism for dissociating internalized ligand from receptor. Thus, after the acidification of incoming PVs, ligand would be discharged into the lumen of the vacuole allowing "free" receptor to return to the cell surface to participate in another round of endocytosis. The best example is the man-6-P glycoprotein receptor, where both intact cells (12) and isolated receptor (76) do not bind at low (intralysosomal) pH. Similar information has been obtained for receptors for ASGP (168) mannosylglycoproteins (169), and transferrin (pH sensitivity of apotransferrin-iron interaction) (15). A variation on this theme is provided by the Fc receptor of the neonatal rat intestine, which may transport primarily maternal IgG from the slightly acidic intestinal lumen across epithelial cells, and not to Ly (see below). This receptor is active at low pH (<6) and inactive at neutral pH (170); thus, even if Ly fusion occurs during transport, the ligand should not be discharged by acidification alone. In all the above examples, the effects of pH on ligand binding are reversible.

It has been difficult to prove that such pH-dependent delivery systems actually function in intact cells. However, there is a decrease in the number of PM man-6-P receptors of fibroblasts exposed to ligand (acid hydrolase) and chloroquine (12). These data suggest that raising Ly or intravacuolar pH interferes with the discharge of ligand and the recycling of receptor. Conceivably other features of the Ly, e.g., acid hydrolases, influence ligand discharge.

5.1 e THE TURNOVER OF SURFACE RECEPTORS: The concept that receptors recycle during rapid ligand uptake also presupposes that receptors are not rapidly degraded and synthesized. In the absence of ligand, receptor turnover appears to be slow, similar to turnover of most PM proteins. The number of binding sites for LDL (9), TC-II (157), and macroglobulin-protease (10), has been measured following treatment with cycloheximide to block protein synthesis. The measured $t_{1/2}$ were 25, 8, and 3.3 h, respectively. Similarly, slow turnovers were obtained by monitoring the degradation of surface radioiodinated ASGP receptor ($t_{1/2}$, 20 h) (164) and Fc receptor ($t_{1/2}$, 10 h) (36) in the absence of cycloheximide. Turnover of the ASGP receptor has also been studied in the presence of ligand. The turnover rate ($t_{1/2}$, 20 h) was unaltered (164), as would be expected for receptors that recycle.

Another approach was employed by Doyle and co-workers (171, 172). Membrane vesicles containing either crude hepatocyte membrane proteins or purified ASGP receptor were fused with polyethylene glycol to mouse L-cells, which do not normally express receptor activity. The transferred ^{125}I-receptor was stable ($t_{1/2}$ of 50 h) and at least 30–50% of the molecules could bind and mediate ligand internalization. In the case of crude membrane vesicles, it was estimated that each individual receptor could mediate several rounds of ligand internalization. These receptors were presumably reused, since the L-cell recipient lacked preexisting receptor pools and could not synthesize receptors de novo. However, the physical integration of the "transferred" receptor into the L-cell PM was not demonstrated.

5.1 f RECYCLING OF LIGAND: There is recent evidence that ligands interiorized by adsorptive pinocytosis are in part recycled to the cell surface (15, 163, 173–175). Usually this recycling pool empties in minutes. Conceivably intact ligand receptor complexes recycle and/or recycling occurs in the fluid phase, analogous to the sucrose studies of Besterman et al. (149).

5.1 g SUMMARY: The current evidence indicates that receptors mediating adsorptive endocytosis can enter the cell, deliver ligand, and themselves escape degradation. The magnitude of ligand influx (Table II), when compared with the apparent number of binding sites on the PM and on intracellular membranes, further suggests that individual receptors may be reused several times via membrane recycling. However, critical pieces of information remain unknown regarding each of the five assumptions delineated above. Many of these unknowns will be solved through the use of specific antireceptor antibodies so that the internalization and fate of individual receptor molecules can be studied. It will also be important to identify and quantitate receptors on intracellular membranes.

5.2 Receptors That Do Not Recycle—The Role of Ligand

5.2 a THE MACROPHAGE FC RECEPTOR: Using monoclonal antibodies against the mouse MØ trypsin-resistant Fc receptor (FcR) and other unrelated MØ PM proteins, Mellman et al. (36) have recently followed the dynamics of specific proteins during the internalization of IgG-coated erythrocyte ghosts. Receptors were found to be selectively interiorized, i.e., 70% were removed from the cell surface as compared to 10–20% decreases in the levels of other surface antigens. Moreover, the amount of surface FcR remained low for more than 24 h, suggesting that internalized receptors were degraded rather than recycled. This was confirmed by turnover studies in which the MØ PM was first radio-iodinated and the fate of the FcR then analyzed by immune precipitation. In the absence of ligand, the receptor was degraded with a $t_{1/2}$ of 10 h; however, in the presence of ligand, some 50% of the total ^{125}I-labeled FcR was degraded much more rapidly, $t_{1/2}$ of <2 h. The anti-red cell IgG and the red cell PM proteins were also digested rapidly. In contrast, other PM polypeptides turned over at similar rates ($t_{1/2}$, 18–22 h) irrespective of whether IgG-ghosts were ingested. Thus, uptake of an antibody-coated particle results in the selective internalization and degradation of FcR. In contrast, in the absence of ligand, FcR enters the cell but probably escapes degradation by recycling (4).

5.2 b EPIDERMAL GROWTH FACTOR: EGF is a polypeptide hormone which binds with high affinity to a 150-kdalton PM glycoprotein (176). Bound EGF is cleared from the cell surface, often in large PV, with a $t_{1/2}$ of <5 min and is then rapidly degraded (177, 178). After internalization, however, EGF uptake is greatly reduced and there is a loss of surface binding sites. It appears that this failure of EGF receptors to be reused results from the receptor's rapid degradation following ligand binding. However, these data were obtained by photoaffinity labeling a small fraction of the presumptive EGF binding sites (179).

5.2 c INSULIN RECEPTOR: The phenomenon of down regulation of surface receptors has been most extensively studied in the insulin system. Recently it has been shown that ligand (insulin) binding induces enhanced degradation of the insulin receptor (180). These data, however, were obtained with large (μM) concentrations of insulin. Conceivably degradation follows ligand-receptor uptake into Ly (181, 182).

5.2 d ACETYLCHOLINE RECEPTOR: Fambrough and colleagues have measured a $t_{1/2}$ of 17 h for the ACh receptor of cultured muscle cells. If anything, the $t_{1/2}$ was slowed (23 h) following addition of saturating levels of α-bungarotoxin, a cholinergic ligand which essentially binds irreversibly (K_A 10^{15} M^{-1}). Regeneration of receptor activity after ligand binding required synthesis and insertion of new receptors. It was concluded that ACh receptors do not recycle but must be replaced by new synthesis following ligand binding (183). However, the addition of bungarotoxin was performed over prolonged time periods, so that it is quite likely that ligand gained access to intracellular vacuoles capable of replacing surface binding sites. Thus receptors may have recycled while complexed to ligand. It is also possible that rapid receptor discharge and recycling may occur with physiologic ligands, since ACh binds to its receptor with a much lower affinity than α-bungarotoxin.

5.3 Recycling during Other Forms of Adsorptive Endocytosis

5.3 a NONSPECIFICALLY ADSORBED ANTIBODIES: Schneider et al. (184) allowed fibroblasts to pinocytose goat anti-rabbit Ig and then exposed cells to rabbit Ig. Over a 4 h period, some 10–20% of the goat anti-rabbit Ig appeared in the medium as an immune complex with rabbit Ig. It was con-

cluded that rabbit Ig entered the cell attached to PV membrane, picked up the goat anti-rabbit Ig after Ly fusion, and recycled the complex to the cell surface for discharge. It is not clear why this recycling was relatively slow (requiring 4–24 h).

5.3 *b* SPECIFIC ANTIBODIES: Widnell et al. (185) have incubated rat fibroblasts with a polyclonal antibody against a well-known PM ectoenzyme, 5' nucleotidase. This antibody inactivated "patent" enzyme activity defined as enzyme activity present on the PM. Evidence was obtained that surface nucleotidase was replaced by enzyme from an intracellular compartment, and also that antinucleotidase could recirculate from intracellular vacuoles to PM. Analogous results have been reported by Stanley et al. (186) using rat hepatocytes.

Louvard (19) has used specific antibody and indirect immune fluorescence to study the endocytosis and recycling of another PM marker, aminopeptidase, an ectoenzyme located on the apical surface of MDCK epithelial cells. Anti-aminopeptidase was first bound to the cells and visualized using a rhodamine-conjugated second antibody. The cells were warmed to 37°C, and the internalization of the antibody-antigen complex was documented using a third, fluorescein-labeled antibody directed against the rhodamine labeled reagent. While most of the rhodamine-labeled material remained in an intracellular compartment (presumably Ly), a fraction returned to the apical PM, as indicated by the reappearance of binding sites for the fluorescein-tagged antibody.

5.3 *c* CATIONIZED FERRITIN: Thyberg et al. (187) studied membrane recycling in Mφ's using cationized ferritin (CF), a probe first employed for this purpose by Farquhar and Herzog (see references 101, 102), (see section 6). Mφ were exposed to HRP and then maintained in enzyme-free medium. HRP cytochemical reaction product was visualized only in presumptive Ly, and was not detected in Golgi apparatus or on the PM. Cultures were then exposed to CF which was internalized by adsorptive pinocytosis and concentrated in Ly. Within 30 min, CF in association with HRP reaction product was detectable both on the PM and in Golgi elements. These results suggested that CF had complexed some molecules of HRP in Ly and had recycled, possibly via the Golgi apparatus, to the PM.

6 PINOCYTOSIS AFTER SECRETION

In several systems, exocytosis is followed by a burst of pinocytic activity. Holtzman et al. (188) showed that HRP accumulated in synaptic vesicles at stimulated neuromuscular junctions. This was confirmed by Ceccarelli et al. (189) and Heuser and Reese (190). Amsterdam et al. (191) noted that the apical cytoplasm of stimulated parotid cells became filled with smooth vesicles. They suggested that the vesicles were retrieving zymogen granule membrane that had discharged at the apical PM. Membrane retrieval must be considerable: e.g., cells in the exocrine pancreas can discharge granules with a surface area of 300 μm^2 during a single round of secretion. Nevertheless, the apical PM regains its surface area of ~30 μm^2 (192, 193). The pathways and kinetics of membrane retrieval and recycling in secretory cells have been studied primarily by morphologic techniques. These data will be reviewed for three categories of secretory cells.

6.1 Neurons

The most detailed initial studies of membrane recycling at nerve terminals are those of Heuser and Reese (190) who measured the areas of PM, synaptic vesicles, and membrane cisternae in frog neuromuscular junctions. Within a minute of stimulation, synaptic vesicles were exocytosed and the adjacent PM increased in area. By 15 min, the area of the terminal PM had decreased, while the area of cisternae increased. Finally, the areas of synaptic vesicles, PM and cisternae returned to prestimulation levels. HRP was then added to monitor endocytic events. Within a minute of stimulation, HRP was found in coated vesicles, which budded from regions of the nerve terminal covered by Schwann cell processes. These were not adjacent to the presynaptic densities where exocytosis had predominantly occurred, suggesting that the synaptic vesicles themselves were not immediately retrieved by pinocytosis. HRP was next found in large cisternae, and within 15 min, 30–50% of the synaptic vesicles were labeled. To show that the HRP-tagged synaptosomes could again be used in exocytosis, the HRP-labeled junctions were bathed 1 h in HRP-free medium, and the number of HRP-labeled synaptic vesicles decreased, suggesting that the HRP-containing synaptic vesicles had exocytosed. Schaeffer and Raviola (194) obtained similar results in an elegant study of pinocytosis in turtle retina stimulated with light. The first structures labeled with HRP were coated vesicles, cisterns, and 0.10–0.15 μm large smooth vesicles ("endosomes"). Later, most of the synaptic vesicles became HRP-reactive. Ceccarelli and Hurlbut (195) obtained the best evidence for recycling by using black widow spider venom to stimulate massive vesicle discharge from neuromuscular junctions. When Ca^{++} and HRP were added to the bathing medium, synaptic vesicles reappeared that were all labeled with tracer. Since ACh release continued at substantial levels, it is likely that HRP-labeled vesicles were being reused or recycled for neurotransmitter release. However, coupling of exocytosis and endocytosis at nerve terminals may not require that the vesicles contain ACh. Ceccarelli and Hurlbut (196) observed typical recycling in hemicholinium-treated junctions, in the absence of detectable ACh release.

In addition to rapid recycling at the nerve terminal, there is a slow retrograde movement to the perikaryon (197–198). This flow takes hours and may lead to degradation of synaptic vesicle membrane.

6.2 Endocrine and Exocrine Cells with Secretion Granules

The initial studies of pinocytosis following exocytosis utilized tracers like ferritin and HRP. In all cases, the tracer accumulated in typical Ly implying that exocytosed granule membrane was retrieved and then digested (reviewed in references 199, 200). Subsequent studies primarily by Farquhar and others (reviewed in references 101, 102), extended the concept from one of membrane retrieval and digestion to one of membrane recycling or reuse. They found that certain tracers, such as dextrans and CF, labeled not only PV and Ly but also the Golgi apparatus including saccules, condensing vacuoles and secretion granules (201–204). The findings varied considerably with the tracer and the cell type studied. For example, in the pancreas, dextran was observed primarily in Golgi apparatus while HRP was in Ly (204). In the thyroid, CF was primarily in Ly, with small amounts noted in Golgi elements (201). These observations imply that substances which adsorb to the PV membrane in some instances trace the movement of endocytosed membrane through the Golgi region. Whether internalized membrane and content flow directly to the Golgi elements, through a Ly intermediate, or to Ly after delivery to the Golgi elements is not yet clear.

The delivery of endocytosed materials to Golgi elements was first described by Gonatas and co-workers (107, 108) who studied the uptake of ricin-HRP conjugates in cultured neuroblastoma cells. They noted delivery to Ly and GERL. Conceivably membrane flow was secondary to exocytosis in neuroblastoma cells. A more intriguing possibility is that ricin bound to some PM component that is specifically required for flow through GERL or Golgi elements.

6.3 Cells That Secrete Constitutively

Several cells in culture secrete proteins at constant rates and lack obvious storage granules with secretory product. Examples include collagen and glycoproteins in connective tissue cells, lysozyme in macrophages, and immunoglobulins in myeloma cells. Membrane flow in myeloma cells was studied by Ottosen et al. (205). Again, HRP and CF appeared in Ly; cationized tracers were also found in small amounts in Golgi saccules and presumptive secretory vacuoles. If the myeloma cells were given HRP and then CF, only the latter appeared in the Golgi apparatus, suggesting sorting of membrane (CF) from content (HRP). In contrast, Thyberg (187) reported delivery of HRP to Golgi in cultured Mϕ that were sequentially exposed to HRP and then CF. He concluded that CF-HRP complexes moved from Ly to the Golgi (see Section 5.3c).

The current state of the Golgi apparatus pathway in endocytosis depends almost entirely on morphologic studies with highly charged multivalent molecules that could induce rather than trace membrane movement. However, most observers think that recycling during secretion is a major pathway whereby cells recapture and reuse secretory granule membrane. However, it has been difficult to study the composition and turnover of secretion granule membrane. Some evidence is available that granule membrane turns over much more slowly than granule contents (206), suggesting that recycling of membrane must occur. Possibly the movement of endocytic vacuole membrane through the Golgi apparatus only occurs when that membrane is used to repackage newly synthesized secretory products from the RER. For instance, the Golgi apparatus pathway may not be required in synaptosomes, where vesicles can acquire neurotransmitter without immediate RER involvement; or in adsorptive endocytosis, where RER products need not be included in recycling vesicles.

7 VESICULAR TRANSPORT ACROSS CELLS

Three examples of vesicular transport across cells will be discussed: movement of plasma across capillary endothelium; IgG transport across neonatal rat intestinal epithelium; and IgA transport across liver and mammary epithelium. In each of these cases, vesicles arise at one surface of a polarized cell, traverse the cytoplasm, and fuse with the PM at the opposite pole thereby discharging contents. On the one hand, transcellular movement of PV represents the clearest example of PM recycling, i.e. the interiorization and reinsertion of PM derived vesicles. However, to establish that recycling occurs, it must be shown that membrane components in the transporting vesicles are ultimately returned to their pole of origin to participate in additional rounds of endocytosis. It should be noted that since the two surfaces involved in transcellular transport represent distinct PM domains, such recycling must involve an efficient sorting of membrane components to preserve cell polarity. Thus far, neither the biochemical nor the morphological features of this return flow have been studied in detail.

7.1 Endothelium

Capillary endothelium mediates the bidirectional flux of enormous volumes of water and dissolved solutes between plasma and interstitial fluid. Movement of most fluid and small solutes likely occurs by nonendocytic means via gaps at intercellular junctions, large fenestral openings present in certain visceral capillary beds, and channels formed by the fusion of two or more endocytic vesicles thus bridging plasma and tissue fronts of the cell (reviewed in reference 207). Vesicular transport probably is responsible for the movement of larger molecules, particularly in nonfenestrated endothelium.

The PM of endothelium is studded with 60–70 nm smooth surfaced plasmalemmal vesicles (PLV). In the endothelium of many visceral capillary beds, two-thirds of the PLV are always open at blood and tissue fronts (207). PLV can occupy as much as 30–40% of endothelial cell volume (207), in contrast to the 2–3% occupied by PV in Mϕ (1). PVL-mediated transport from plasma to interstitial fluid has been demonstrated using several tracers: colloidal gold, ferritin, myoglobin, HRP, and heme-peptides or microperoxidases. The transport of ferritin from interstitial space to plasma has also been shown (208). Data obtained with sensitive cytochemical tracers have shown that transendothelial transport requires less than a minute (209, 210).

Several major differences between endothelial transport and pinocytosis in cultured cells are evident. In endothelium, solutes internalized in PLV move across cells with little accumulation in Ly (211). Interestingly, coated vesicles usually constitute a subpopulation of all PLV and seem to deliver their contents to the Ly compartment (212).

The magnitude and rapidity of the membrane movements involved in PLV-mediated transcellular transport necessitates some mechanism of recycling. PLV may represent a highly specialized compartment, biochemically and functionally distinct from the PM (see section 8.5). Simionescu et al. (212) have recently found that cationic tracers, such as CF or alcian blue, do not bind to nascent PLV but do bind to adjacent PM. In addition, tracers that were bound at the apical PM were not transferred to the tissue front, even after prolonged incubations (212); however, binding sites for these tracers could be demonstrated on the tissue front (213). It was hypothesized that PLV do not form from random invaginations of the PM, which should have included at least some of the bound cationic ligands. Instead, PLV probably constitute differentiated microdomains of the PM, that continually shuttle between apical and basal surfaces of the endothelium.

7.2 IgG Transport across Intestinal Epithelium

Maternal IgG is transported to the developing young across several epithelia including yolk sac, placenta, and intestine. Neonatal rat intestine has been studied in most detail by Rodewald and colleagues who have used IgG coupled to ferritin or HRP (38, 214), or HRP-anti HRP immune complexes (215). Uptake of IgG occurs at the base of microvilli on the apical surface, and discharge occurs at the basolateral surface of the columnar epithelial cell. The most recent data suggest that both uptake and discharge steps are accomplished by coated vesicles.

Transport is highly selective for the Fc portion of IgG. Saturable binding of IgG occurs and is competed for by Fc but not by F(ab)$_2$' fragments. This Fc receptor is distributed over the entire surface of dissociated epithelial cells and its activity

is pH dependent (170). IgG binding occurs at pH 6 and is minimal at pH 7.4. Thus, the receptor would be charged with IgG on the mucosal surface (where the pH is slightly acid) and discharged on the basolateral surface (where the pH likely is neutral). To complete a recycling process, the Fc receptor would have to return to the apical surface in vesicles, since the alternative of diffusion in the PM would require that the receptor traverse the apical tight junction.

Abrahamson and Rodewald (38) have reported experiments in which content (HRP) and ligand (ferritin: IgG) were administered simultaneously. Both entered the cell in the same apical PV. Ferritin IgG reached the basolateral surface (and probably Ly in addition, see Discussion in reference 38), whereas HRP was delivered only to Ly, and not across the cell. The amount of ferritin IgG reaching Ly has not been determined. CF which adsorbed nonspecifically to the apical surface, was delivered both to Ly and across the cell (216). The authors predict that there is an efficient sorting of fluid content and membrane container such that HRP is delivered to Ly while IgG and its Fc receptor traverse the cell. This situation appears different from adsorptive pinocytosis (see section 5.1) where both content and ligand accumulate in Ly. However, the differences may relate primarily to the properties of the intestinal Fc receptor rather than different pathways of membrane flow. Thus ligand may remain attached to the PV membrane as it recycles through the cell; net flux occurs because the receptor only discharges ligand when exposed to elevated pH at the basolateral surface.

7.3 IgA Transport

Serum IgA, is transported to bile across hepatic parenchymal cells (217, 218); IgA synthesized locally by tissue plasma cells is also transported directly across epithelia lining most body surfaces (reviewed in reference 219). In bile and other secretions, the IgA is complexed with a 70–80-kdalton glycoprotein termed secretory piece (SP). The interaction between SP and IgA is highly specific for IgA in most species, but in man, IgM also binds SP. The interaction may be reversible but of high affinity (as in rabbits) or stabilized by disulfide bonds (as in man) (e.g., reference 220). Recent studies have shown that SP not only is bound to IgA in the secretions, but probably acts as the receptor for IgA in the epithelial membrane.

Crago et al. (221) showed that IgA bound specifically to sections of intestinal epithelium and to cultures of a colon carcinoma. IgA that was complexed with SP did not bind. Fisher et al. (222) showed that IgA was the principal type of Ig transported from serum to bile. Again, IgA complexed with SP was not transported, and transport of IgA was blocked by free SP. The only IgG that was transferred was anti-SP Ab. Additional evidence for the binding of IgA to cell associated SP was obtained in isolated hepatocytes (223, 224).

After binding of IgA to cell-associated SP, the IgA-SP complex must move across the cell. Renston et al. (225) found that IgA crossed the liver cell in typical endocytic vacuoles and was discharged at the bile canaliculus; IgA was not found in Ly. HRP, a presumptive content marker, moved in tandem with IgA. Both traversed the hepatocyte in minutes.

There is no published information characterizing PM-associated SP biochemically. However, Mostov et al. studied the synthesis of SP in a cell free system and immunoprecipitated a 90-kdalton molecule rather than the 70-kdalton product found in secretions (226). In the presence of microsomes, SP was synthesized as a transmembrane protein with a 16-kdalton

cytoplasmic extension. They proposed that after transit through the cell, the 90-kdalton membrane SP is cleaved by proteolysis delivering a 70-kdalton SP-IgA complex into the lumen. Recycling of membrane SP would not occur, since it would have to be replaced by new synthesis. Information on the turnover of PM SP is needed, but possibly SP is an IgA receptor that is degraded following ligand attachment.

8 DISCUSSION

We have seen that the intracellular flow of membrane during endocytosis is rapid, is associated with the interiorization of large amounts of PM, and involves organelles such as Ly and Golgi complex. Few of the underlying molecular details are apparent. In this section we discuss some of the current issues regarding the mechanisms and regulation of membrane recycling.

8.1 Initiation of Endocytosis

The main unknowns regarding the initiation of endocytosis are: do endocytic vacuoles form constitutively or are they always induced by some extracellular agent, e.g., the particle or ligand being interiorized? How does clathrin and/or its associated proteins contribute to the interiorization process? What features distinguish nascent endocytic vacuole membrane from the remaining PM?

8.1a INDUCED VS. CONSTITUTIVE ENDOCYTOSIS: The studies of Griffin, Silverstein and colleagues established that phagocytosis of Ab coated particles by Mφ is a local event initiated by the binding of Ig to Fc receptors (227, 228). Two types of particles were attached to the PM—one with a ligand that did not mediate phagocytosis (e.g., complement) and another with an endocytically active ligand (IgG). Only the latter particles were interiorized. This led to the hypothesis that ligand binding generates a local transmembrane signal which activates contractile elements beneath the particle; this would trigger the observed movement of PM and its receptors around the ligand-coated particle in a zippering fashion, until engulfment was complete (229). The role of contractile elements is emphasized by the sensitivity of phagocytosis to cytochalasins (reviewed in reference 230). Clathrin may also be involved in latex phagocytosis (72), but coats have yet to be seen in Fc receptor-mediated particle uptake.

A striking feature about the phagocytic system is that the same receptor molecule mediates particle uptake in some cells but not in others. B lymphocytes, for example, are known to have the same Fc receptor as Mφ (161, 231). While B-cells bind immune complexes, can internalize anti-Ig antibodies, and contain most contractile proteins as well as clathrin, the phagocytosis of bound IgG-coated particles does not occur. An analogous situation can be found in Mφ complement receptors. Activated Mφ phagocytose particles opsonized with C3b, whereas resident Mφ bind but do not interiorize. Thus, the same receptor (possibly the same molecule) behaves very differently in the two types of Mφ, even though both cells actively phagocytose other test particles. The events which link binding and phagocytosis remain important unknowns.

The initiation of pinocytosis following secretion also seems closely regulated. There seems to be little or no endocytic activity in exocrine cells that store secretory product until exocytosis is triggered. In some cases, the discharging exocytic vacuole membrane is itself immediately retrieved by pinocytosis, as in trichocyst and food vacuole defecation in ciliates; in

other cases, the PV develops at a "distal" segment of PM, as in neuromuscular junctions. Factors which stimulate pinocytosis following secretion are unknown.

In contrast, fluid phase pinocytosis is a constitutive activity in most cells. Fluid uptake continues at constant rates for long periods (2 h–4 d) even in the absence of serum in some cases. However, pinocytic activity does appear to cease during mitosis (23, 232). Some agents are known to trigger enhanced accumulation of fluid phase markers as well, e.g., concanavalin A and phorbol myristate acetate in Mϕ (233, 234), antidiuretic hormone in bladder epithelium (235), and EGF and platelet-derived growth factor in fibroblasts and smooth muscle cells (177, 236). These agents may increase the rate of pinocytosis per se, and/or they may reduce the extent of reflux of internalized fluid (see section 4.5) resulting in an enhanced rate of solute accumulation.

The endocytosis step in receptor-mediated pinocytosis need not be initiated by ligand binding. Several investigators have used fluid phase markers to monitor the rate of PV formation after addition of ligand. There is no detectable increase in fluid phase solute uptake following administration of SFV to BHK cells (17), IgG to neonatal rat intestine (38), IgA to rat hepatocytes (225), and macroglobulin-protease complexes to Mϕ (237). Maneuvers which reduce the rate of adsorptive uptake may also produce a parallel fall in the rate of fluid pinocytosis (237). Thus ligand binding need not induce PV formation, as measured by fluid phase markers, although ligand may aggregate receptors to the forming PV (e.g., coated pits).

On the other hand, the clustering of ligand and receptor on the PM is believed to initiate endocytosis in some systems. When monovalent Fab antibody fragments bind to certain cell surface receptors, e.g., Ig on B lymphocytes (238, 239) or the C3b receptor on neutrophils (240), the Fab is neither digested nor cleared from the PM. In contrast, intact antibody or bivalent F(ab)'$_2$ fragments are removed from the surface and digested. The interpretation of these findings is that the bivalent (or multivalent) reagent induces endocytosis, via receptor clustering. Alternatively, the bivalent ligand may induce: only the more efficient interiorization of receptor due to a redistribution of clustered receptors to coated pits; or (b) the removal of the antibody-receptor complex from a constitutive recycling pathway. In other words, monovalent Fab-receptor complexes may be continuously internalized and recycled intact whereas F(ab)'$_2$-receptor complexes do not recycle resulting in intracellular accumulation. This possibility should be considered in view of the data that the macrophage Fc receptor is a component of fluid phase PV (no ligand added; reference 4) but is selectively cleared from the PM and degraded following the binding of Ab-coated erythrocytes (36).

In summary, it is often thought that endocytosis is induced by the contents to be interiorized. Indeed, this seems to be the case in phagocytosis. Pinocytosis, however, often functions like a continuously operating conveyor belt or escalator, i.e., segments of PM are constantly being internalized independent of ligand signals. Solutes, then, influence their rate of entry and accumulation by the extent to which they bind to the PM, move to regions of forming PV, and become discharged from their binding sites once inside the cell.

8.1 b CLATHRIN AND COATED VESICLES: Many kinds of endocytic vacuoles form at segments of PM that are lined by clathrin-containing coats. This is readily evident in receptor mediated pinocytosis, since one can examine the nascent PV during and shortly after pinocytosis, i.e., during the first sec-

onds of uptake. Coated membrane and clathrin may also play a role in some types of particle phagocytosis, and possibly pinocytosis of fluid phase markers, exocytic granule membrane, and nonspecifically adsorbed tracers (see section 2.2). However, there is very little quantitative data distinguishing the contribution of coated and smooth vesicles.

Cell-free systems have shown that clathrin can assemble from soluble precursors into typical baskets (64–67). In view of these findings, it is possible that the formation of clathrin baskets at coated pits provides the driving force for the formation of endocytic vesicles. There is still little evidence on several key questions: Is clathrin attachment to the PM random, or does it only occur at areas with specialized lipid or protein composition? Does the 100-kdalton clathrin-associated protein help define regions of clathrin attachment or does clathrin recognize domains of receptor molecules? Do coated pits assemble from monomeric precursors, or is there attachment and detachment of large preformed coats?

There are also unknowns with respect to the ligand in coated pit function. Does the receptor, and/or ligand binding, simply guide the ligand to the coated pit, or can ligand/receptor trigger pit formation and internalization? Some ligand binding sites, particularly LDL receptors, are enriched in pits, but the mechanism is unclear. Useful models might be (a) the internalization-defective mutant fibroblasts which can bind LDL at noncoated regions, but do not internalize LDL at high rates (241); (b) cultured A-431 cells which have large numbers of LDL binding sites, but again, the sites are in noncoated regions and do not mediate a high level of uptake (242); (c) ligands, like SFV, which seem to move to the coated pit and are then internalized (13). The SFV model may be applicable to many plasma membrane proteins, i.e., a protein is internalized only when it enters a coated region of the membrane. Localization to coated pits may occur as a consequence of a membrane protein's lateral diffusion in the bilayer; or ligand binding may slow the rate of diffusion through coated pits thus resulting in net accumulation at these regions without triggering coat formation or function.

8.1 c PROPERTIES OF THE NASCENT ENDOCYTIC VACUOLE: A good deal of information is available that the overall polypeptide composition of endocytic vacuole membrane (labeled with lactoperoxidase, references 4, 32, 34) is similar to the PM. Thus PM literally flows into the cell during endocytosis. Obviously, the internalized domains of PM must be specialized in some ways. Most studies have focused on the enrichment of ligands, receptors, and clathrin in nascent endocytic vacuoles. There may be other lipids or proteins that are important in allowing PM to form vesicles, or for receptor and clathrin enrichment. Conceivably, there are stable microdomains of PM that continually enter and recycle through cells. A significant unknown, in this regard, is the contribution of lipid composition to endocytic activity. When Mϕ membranes were "stiffened" by enrichment with exogenous 18:0 and 19:1 fatty acids, the rates of both pinocytosis and phagocytosis were reversibly diminished by 60% (114).

8.2 Formation of Endosomes and Secondary Lysosomes

When PV enter cells as coated vesicles, the clathrin coat is rapidly lost by an as yet unknown mechanism. Soon after their formation, at least some primary PV first undergo fusion among themselves to give rise to larger, electron-lucent,

smooth-surfaced vacuoles that we and others have chosen to call endosomes (52). Endosomes are readily found in cultured cells, especially Mφ's and fibroblasts, in the absence of known ligand-receptor interactions. Also, it is not yet known if the size of the endosome compartment is increased during receptor-mediated endocytosis. It has been suggested that the endosome is the principal site of receptor recycling (16, 50, 51). However, there is still no direct evidence that fusion of PV into larger vacuoles is required for membrane and receptor recycling, or even if most PV fuse with one another before fusion with other organelles (Ly, Golgi apparatus). Without quantitative data, it is even possible that larger endosomes are atypical PV that are relatively slow to recycle and/or to fuse with Ly.

As discussed above, endosomes contain the contents of two or more primary PV (see section 2.2c). For many receptors, internalized ligands already appear to have been discharged from the endosome membrane (see above), suggesting that the endosome has already acquired an acid pH (see below). Endosomes may be considered "pre-lysosomal" in the sense that fusion with Ly, labeled with previously endocytosed colloids (1) or with acid-hydrolase cytochemistry (243), is not detectable. The experiments of Dunn et al. (18), who found that low temperature (<18°C) could inhibit the fusion of Ly with PV (or endosomes), further demonstrates the pre-Ly nature of endosomes. An additional possibility, raised by the work in *Paramecium* (section 3.3), is that endosomes have fused with a distinct intracellular vacuole that is important in intravacuolar acidification and perhaps membrane recycling and sorting.

The kinetics of fusion of PV with primary or secondary Ly need more definition. However, it should be emphasized that fusion can begin very rapidly, certainly within 5 min after endocytosis. Steinman et al. (1) showed that dense perinuclear granules, presumptive Ly, acquired HRP within 5 min of exposure to enzyme. Hubbard et al. (46) observed that in hepatocytes, lac-fer (internalized via the ASGP receptor) was detectable in arylsulfatase-positive vacuoles beginning 5 min after entry; the $t_{1/2}$ of movement out of PV was estimated to be 6-7 min (18). Similar rates were measured by Besterman et al for the movement of HRP from PV to Ly (149). Digestion of ^{125}I-acetyl LDL to ^{125}I-moniodotyrosine by Mφ is detectable with 10 min after the tracer is administered (244). Similarly degradation of immune complexes (IgG-^{125}I-BSA), internalized via the Fc receptor in J774 Mφ, begins within 5 min of uptake (I. Mellman, unpublished observations). Thus, even with current relatively insensitive assays, Ly fusion seems to begin very early; however, in most cells 30-60 min are required to convert fully a pulse of endocytic vacuoles to dense, acid hydrolase-rich, granules (1, 40).

8.3 Acidification of the Endocytic Vacuole

The rapidity of PV-Ly fusion notwithstanding, the kinetics of vacuole acidification are under active study. While traditionally viewed as a unique characteristic of Ly, food vacuoles in ciliates and amoeba probably acidify prior to Ly fusion (175; and section 3.3). Recent observations in mammalian cells also suggest that endosomes may have a reduced pH. The most detailed are studies with Semliki Forest virus (SFV) in BHK fibroblasts. This work by Helenius and colleagues (17, 41, 245) has demonstrated that virus infection requires an acidic environment which catalyzes the fusion of the viral envelope with cellular membranes thus releasing the nucleocapsid into the cytosol. Fusion, and hence infection, is blocked by treatment of cells with "lysosomotropic" amines (NH₄Cl, chloroquine)

which would be expected to elevate almost immediately the intravacuolar pH of Ly (80), or any other acidic compartment. Interestingly, NH₄Cl cannot inhibit infection if added 3-4 min after virus pinocytosis, suggesting that incoming virions have already begun to reach an acidic compartment (pH <6) by that time. Moreover, virus nucleocapsids can be detected in the cytosol soon after virus entry (15 min), while the degradation of viral spike glycoproteins commences only after a considerable lag (30-60 min) (245). Thus, the low pH-dependent discharge of nucleocapsids may occur in a compartment relatively devoid of proteolytic activity, i.e., a pre-lysosomal vacuole such as the endosome.

Murphy et al. (246) have documented the rapid entry of an adsorbed protein following pinocytosis into an acid compartment of Chinese hamster ovary cells. pH dependent shifts in the spectrum of fluorescent histone were detected within 5-10 min of exposure to solute at 37°. Tycko and Maxfield (243) reported that "receptosomes" were acidified 20 min after exposure to fluorescein-tagged α₂-macroglobulin. Again, it is still not clear whether the acidified compartment containing histone or α₂-macroglobulin is truly pre-lysosomal.

Discovering the mechanism of vacuolar acidification will be an important objective of future work. One possibility is that acidification is mediated by a PM-localized proton pump or Na⁺-H⁺ exchanger (reviewed in reference 247). If internalized with bulk membrane during endocytosis, the pump might begin to acidify the tiny confines of the endocytic vesicle as soon as it enters the cytoplasm. On the other hand, there is evidence that newly formed phagocytic vacuoles are not acidified and required up to 30 min to acquire a low pH (248).

8.4 Kinetics, Sites, and Mechanism of Membrane Recycling

Several lines of evidence indicate that endocytic vacuole membrane can begin to recycle minutes after entry into the cell (see sections 4.3, 4.5, 5.1). Although it is possible that recycling membrane becomes part of a large intracellular pool, it is evident that endocytic membrane can move directly, and within minutes, back to the PM. Specifically, when LPO-latex vacuole membrane is radio-iodinated, 80% of the label redistributes to the PM in 5-10 min (41, 117). Regurgitation of pinocytosed solutes and ligand, presumably from intracellular vacuoles, occurs with a $t_{1/2}$ of minutes (149, 150, 163, 173, 174). The kinetic data of Schwartz et al. (158) also indicate that the recycling of the ASGP binding site requires but minutes.

Data suggesting that recycling occurs from a pre-Ly compartment (Fig. 8A) are the studies of Wall and Hubbard (16, 18, 79). They perfused isolated liver with ASGP and showed that fusion of PV with Ly was not detectable when the experiments were performed at 16°C. Nevertheless, ligand accumulated continuously in typical PV ("endosomes"), and the number of PM ASGP binding sites remained constant. This maintenance of PM binding sites was attributed to receptor recycling from PV formed during the exposure at 16°C. Alternatively the PM may have been replenished from another intracellular pool, since 80-90% of ASGP binding sites in hepatocytes are estimated to be inside the cell (165). Stereologic data on the rate of expansion of the PV compartment during continuous exposure to ligand at 16°C might provide more direct information on whether recycling is occuring. Another relevant model is the food vacuole of ciliates. It appears that a condensing vacuole (DVII in Fig. 7) shrinks and recycles membrane without Ly fusion, although acidification does occur (see
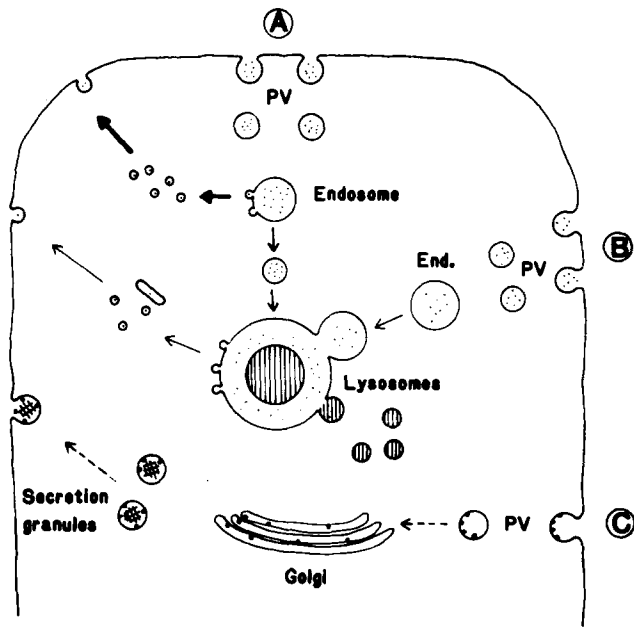
FIGURE 8   Diagram illustrating possible sites for the generation of recycling vesicles. In prelysosomal recycling (A, thick arrow), incoming PV fuse to form endosomes; recycling vesicles, with a high surface to volume ratio, return membrane and some content to the cell surface, leaving behind a residual vacuole that fuses with lysosomes. Recycling vesicles may form from lysosomes (B, thin arrow), here shown with a latex particle as in the study of Muller et al. (117). One pathway through the Golgi apparatus is illustrated (C, dashed arrow) in which endocytosed membrane is used to package secretory products. The endosome-lysosome pathways (A and B) may also require a Golgi apparatus product, such as primary lysosomes or specialized "sorting" vacuoles.

section 3.3). There is also the recent work of Aley et al. on *Entamoeba histolytica* (151, see also section 4.5) in which pinocytosed solutes are likely regurgitated from a nonacidified, nondegradative compartment.

Despite indirect evidence that membrane receptors and pinocytosed solutes can recycle from a prelysosomal compartment, it is clear that solutes and ligands typically accumulate in Ly. Therefore a distinct membrane vesicle (distinct from PM) delivers PV contents to the Ly (Fig. 8A), or pre-Ly recycling is quantitatively small and most recycling vesicles form after rather than before Ly fusion (Fig. 8B). The first moments of fusion are difficult to detect with current techniques, i.e., acid hydrolase cytochemistry, digestion of solute to small molecular weight products, buoyant density shifts. In fact, direct evidence for recycling from Ly is available in the studies of Muller et al. (117). LPO-derivatized latex beads were sequestered in acid-phosphatase positive Ly and used to iodinate the surrounding membrane. The labeled membrane then flowed rapidly to the PM upon reculture at 37°C.

Elements of the Golgi apparatus can also be involved in the endocytosis/recycling pathway (Fig. 8C), although this may apply primarily to the retrieval of exocytic vacuole membrane. The exact route from the PM to the secretory compartment is not established. A Ly intermediate may be important in many systems since the predominant labeling by endocytic tracers, particularly fluid tracers such as HRP and ferritin, is in Ly as opposed to Golgi apparatus.

A priori, recycling must involve vesicular movement of membrane from an endocytic compartment back to the PM. In view

of this fact, this discussion still avoids a principal unknown which may bear upon the mechanism of recycling; i.e., how can solutes like HRP, sucrose, dextrans, and many ligands accumulate continuously in Ly without causing the swelling of the vacuolar system or a considerable reflux of internalized solutes and Ly hydrolases?

One possible explanation for solute accumulation concomitant with recycling is that there is a filtration mechanism whereby membrane-impermeable solutes like sucrose, HRP, or specific ligands, are sequestered in Ly and then the recycling vesicle returns to the cell surface exocytosing membrane, fluid and salts. To us, a more likely alternative is that there is a concentration mechanism within the vacuolar system, the net effect of which is to allow impermeable solutes to accumulate continuously and membrane to recycle. Our hypothesis is that internalized fluid and salts penetrate the vacuole and plasma membranes (1). In this way, vacuolar contents—including ligands discharged from their receptors—are concentrated, allowing for net intracellular accumulation in spite of the reflux of some content when recycling vesicles return to the PM. During this concentration step, the vacuole must shrink and it may vesiculate to form small recycling vesicles with a high surface to volume ratio. The necks/diaphragms of the forming recycling vesicles may restrict entry of macromolecules, as may occur during pinocytosis in capillary endothelium (207). Possibly the formation of exocytic granules in the Golgi apparatus involves a similar concentration, shrinkage, and formation of recycling vesicles that return to the RER.

If shrinking and vesiculation preceeds Ly fusion (Fig. 8A), then one still has to identify the trigger for recycling. Possibly it is the accumulation of protons that could begin once the PV is formed; or incoming PV may fuse with a specialized sorting or recycling vacuole. If shrinkage and recycling are triggered by the fusion of endocytic vacuoles with Ly (Fig. 8B), one still has to explain why there is so little, if any, reflux of acid hydrolases or previously pinocytosed solutes from the secondary Ly compartment.

One approach to identify the mechanism of membrane recycling is to define inhibitors. Weak bases, particularly chloroquine and NH_4Cl (12, 169) and the Na^+-H^+ ionophore monensin (53, 162), trap ligands and their receptors intracellularly, diminishing levels of adsorptive uptake. How these inhibitors work is not clear. Wilcox et al. (249) found that HRP or fluid uptake was severely but reversibly inhibited by monensin, so that bulk membrane flow was blocked. Chloroquine, NH_4Cl, and monensin all produce extensive cytoplasmic vacuolization so that a large proportion of the cell's pool of recycling membrane can be trapped within the cell. The majority of the vacuoles are swollen Ly (249, 250) but Golgi apparatus swelling also occurs after monensin treatment (251). Vacuolization may be the result of osmotic forces. The concentration of protonated chloroquine in the vacuole is about 0.1 M (81), and monensin induced Na^+-H^+ exchange may interfere with the shrinkage of endocytic vacuoles (see above). In addition, weak bases and monensin increase vacuole pH which, among other things, might block ligand-receptor dissociation. Conceivably both vacuolization and increased pH contribute to the sequestration of membrane within treated cells, and prevent the formation of recycling vesicles.

8.5   Sorting of Membrane from Membrane

There are two kinds of sorting that occur in endocytosis. One involves sorting of content (i.e., solutes which accumulate in

the cell) from membrane. The possible roles of concentration vs. filtration mechanisms were discussed above. The other is sorting of membrane from membrane. Endocytosis and recycling proceed without compromising organelle integrity and diversity. PV, Ly, Golgi elements, and specialized PM domains retain their distinctive morphologic, physical, and biochemical features in spite of the continuous flow of membrane which interconnects these compartments. This would appear to invite a randomization of their components as a consequence of membrane fluidity. Thus, the repeated membrane fusion events which occur must be exquisitely specific.

One often thinks that membrane flow is directed by transmembrane extensions of specific proteins, or of peripheral proteins which interact with the cytoplasmic aspect of vacuole membrane. The man 6-P receptor, which may sort Ly hydrolases from other secretory proteins, (section 2.4), could also direct the movement of Ly themselves. Another example is the large transmembrane extension of secretory piece, which may direct bound IgA to the bile canalicular front of hepatocytes (section 7.3) while in the same cell, the ASGP receptor may direct its ligand primarily to Ly.

Another view of the sorting question is that portions of PM that are internalized and recycled represent microdomains that are stabilized by special proteins or lipids, other than the receptors for specific ligands. The plasmalemmal vesicles of endothelial cells are good candidates for microdomains. The data of Simionescu et al. (213) indicate that these vesicles retain their distinctive surface charge (relative to the remaining PM) in spite of repeated movement between blood and tissue fronts. If this line of reasoning is applied to other cells, one would propose that some sort of membrane microdomain persists as endocytic vesicles form, fuse, and move through the cell. Different microdomains might then direct the different pathways of endocytosis diagramed in Fig. 4. For example, secretion granule membrane may be very different from bulk PM (252); following exocytosis, this specialized membrane may guide internalization and movement through the Golgi apparatus for re-use.

8.6 Consequences of Membrane Recycling

8.6 a DELIVERY AND CLEARANCE: Membrane recycling, by allowing repeated use of the PM and individual PM binding sites, enhances the ability of the cell surface to deliver solutes and adsorbed ligands to the vacuolar system. This delivery system may have two consequences. In some cases, the primary effect is to clear the extracellular space of ligand, e.g., proteases in α_2-macroglobulin-protease complexes, and possibly hormones and altered glycoproteins. For other ligands, the vacuolar system is an intermediate between extracellular space and cytoplasm. Examples would include: (a) nutrients such as cholesterol bound to LDL and other carriers, vitamin B_{12} bound to plasma transcobalamins, and iron in heme or iron binding proteins; (b) the toxic fragment of diphtheria and other polypeptide toxins; (c) infectious agents like virions, chlamydia, and trypanosomes; and possibly (d) growth factors, which could require entry in the cytoplasm to initiate their effects. Delivery to the cytoplasm may require localization in intracellular vacuoles for several reasons. (a) Acidification may trigger the penetration event. Nucleocapsids of enveloped viruses and toxic polypeptides will not enter the cytoplasm in chloroquine and monensin treated cells, and entry across the PM can be induced if extracellular pH is lowered to 5–6 (13, 42, 44, 253–257). (b) A substance like cholesterol may be concentrated in Ly following endocytosis, so that the net flux of cholesterol is

able to take place. (c) Finally, although no specific example is available, the vacuolar membrane may contain novel constituents required for penetration.

Obviously, molecules like cholesterol and vitamin B_{12} themselves have direct metabolic consequences following endocytosis. It is less clear, however, if endocytosis generates "second messengers," such as cyclic nucleotides, Ca^{++}, phosphorylated proteins, which then exert metabolic effects. For example, when polypeptide hormones bind to PM receptors it is thought that signaling is accomplished entirely at the cell surface. Is internalization also needed to transduce information; or does endocytosis only regulate receptor metabolism?

8.6 b PLASMA MEMBRANE BIOGENESIS AND MOVEMENT: Since membrane surrounding Ly can move to the cell surface (117), it is conceivable that PM biogenesis could proceed though a vacuolar intermediate, rather than by direct transport from Golgi apparatus to the PM. For example, if newly synthesized PM components were segregated in primary Ly, new membrane could reach the cell surface after entering the endocytic pathway. On the other hand, transit through a Ly might be harmful since exposure to a low pH might alter the membrane, e.g., the fusogenic activity of certain viral glycoproteins such as the influenza hemagglutinin may be irreversibly inactivated by acid pH. (A. Helenius, personal communication).

There is currently no information on the directionality of membrane flow during recycling. In cultured MØ, the movement of radiolabel from iodinated phagolysosome to the PM appears to be random (117). However, directed insertion of recycling vesicles may play a role in mediating cell motility and in the formation of pseudopods as in phagocytosis.

8.6 c INTRACELLULAR PROCESSING: By definition, recycling vesicles return a portion of vacuole membrane and content from within the cell to the PM. In all cases, the membrane and solute that recycle appear to be intact and unmodified. However alterations or processing may occur during recycling. The most plausible target are substrates that are sensitive to the decreased pH that may develop shortly after endocytosis. Another possibility has recently been proposed by Regoeczi et al. (258). They found that a fraction of parenterally administered ^{125}I-asialotransferrin, which is rapidly cleared by hepatocytes, reappears in the circulation in a resialylated form. An area where processing is frequently invoked is in cellular immunology. Here it has been proposed that antigenic determinants are "selected" and/or "complexed" with immune response gene associated (Ia) antigens intracellularly and then reexposed on the PM (reviewed in references 259, 260). However, there has been no direct identification of processed antigens.

8.6 d PLASMA MEMBRANE SURVEILLANCE AND TURNOVER: We have stressed evidence that membrane continuously recycles through the vacuolar system without significant degradation. Yet the movement of PM through the cell may also provide opportunities for the regulation of membrane turnover. The best example would be the selective degradation or down regulation of the MØ Fc receptor during phagocytosis of antibody coated red cell ghosts (36). Uptake of the particle occurs within minutes of binding, so that the subsequent burst of Fc receptor degradation occurs within the cell. Other examples of accelerated receptor turnover following ligand or antireceptor Ab binding are the insulin and EGF receptors (179, 180). Enhanced degradation is likely to be selective for these receptors as well and may occur intracellularly.

Selective degradation of PM constituents after endocytosis may occur because altered, damaged, or ligand-associated mol-
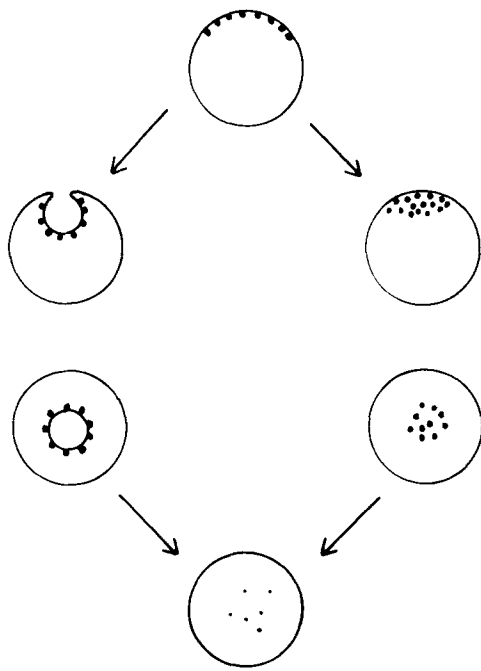
FIGURE 9 Two mechanisms for the selective degradation of a PM constituent, e.g., in a receptor-ligand complex, within the lumen of a secondary lysosome. Both mechanisms begin with the clustering of the component to be degraded. On the left, a vesicle enriched in receptor-ligand complex, is pinched off to form a multivesicular body. On the right, the receptor-ligand complex is sloughed and then degraded. In both, the receptor is selectively degraded while the bulk of the internalized membrane can recycle. This diagram is modified from reference 261.

ecules are recognized as substrates for Ly hydrolases. Alternatively, degradation may begin with the clustering or capping of the PM component(s) to be degraded. The capped components might be extruded or sloughed into the Ly space; alternatively and more likely we feel, the receptor caps before or after entry into the cell, and then a receptor rich segment of membrane pinches off to form a multivesicular body (Fig. 9). Multivesicular bodies, by degrading whole segments of PM, may also be responsible for the basal level of PM turnover in which PM constituents are degraded slowly and in tandem. However, most internalized membrane proteins can avoid degradation, by cycling through the Ly compartment very rapidly and/or by recycling from pre-lysosomal vacuoles.

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