Isolation and Preliminary Characterization of the Major Membrane Boundaries of the Endocytic Pathway in Lymphocytes

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Abstract. Plasma membrane, coated pits, endosomes, and lysosomes were isolated from a mouse T lymphoma cell line using a density shift protocol in which these compartments were selectively loaded with gold conjugates. The plasma membrane was prepared after selective labeling for 1 h at 2°C with gold-ricin and gave a yield of 40% according to enzymatic and antigenic markers. Endosomes were obtained by loading the cells for 2 h at 22°C with gold complexed to an antimouse transferrin receptor mAb. Coated pits were isolated using a similar procedure, but after an incubation at 10°C, which allowed deep invagination of the pits but prevented internalization. The yield (calculated using the recovery of [^{125}I]transferrin) was 32% for endosomes and 10% for coated pits. Finally lysosomes were prepared by loading the cells for 18 h at 37°C with gold low density lipoproteins (LDLs) followed by a 3-h chase at 37°C with LDL alone. The final lysosome yield (based on the recovery of lysosomal enzymes) was 16%. Studies of the protein composition of these cellular compartments on two-dimensional gels showed that while some major proteins are present throughout the pathway, specific proteins can be identified in each of the isolated fractions. The greatest change in the pattern of protein constituents seen along the pathway was between endosomal and lysosomal preparations.

THE pathways involved in the uptake and intracellular processing of hydrophilic macromolecules during receptor-mediated endocytosis have been well documented (55, 58). Several approaches have been developed for isolating the membrane boundaries involved in these pathways, and methods of isolating plasma membrane (3, 14, 19, 34, 41, 49) and lysosomes (1, 16, 17, 28, 39) as well as the intermediate compartments, which include coated vesicles (13, 31, 40) and a variety of endosomal elements (4, 12, 25, 35), have been described. However, because of the pleiomorphic form of the compartments that make up the system no single approach has thus far successfully isolated all of the boundaries from a single pathway.

Knowledge of the endocytic pathway in the lymphocyte is derived mostly from morphological studies (10) because the subcellular fractionation schemes that have been developed (3, 19, 28, 34) have not examined the distribution of endosomal elements. Our preliminary studies (5) found that the most widely used gradient system for isolating the plasma membrane from mouse leukaemic cells (3, 7, 34) yields a preparation significantly contaminated by endosomes.

The most effective fractionation techniques devised to date rely upon density shift procedures (4, 5, 12, 17, 31). We have concentrated (4, 5) on the use of gold conjugates because they have the advantage of not inactivating endosomal proteins (2).

We show in this paper that using the appropriate gold conjugates in conjunction with temperature manipulations designed to deliver the complex to specified intracellular locations, most of the membrane boundaries involved in the endocytic pathway in mouse T lymphoma BW 5147 can be isolated. The plasma membrane and lysosomes can be isolated using a ricin-gold and an LDL-gold complex, respectively, while coated pits and endosomes can be obtained using an antimouse transferrin receptor antibody (AMTR)[1]-gold conjugate.

By tracer, marker enzyme, antigen, and electronmicroscopical criteria these procedures are shown to yield highly purified fractions. It was possible, therefore, to use two-dimensional electrophoresis to characterize them and examine the degree of membrane sorting that occurs along the endocytic pathway.

Materials and Methods

Cell Line and Antibodies

The BW 5147 mouse T lymphoma cell line, the 13/2.3 rat mAb directed

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1. *Abbreviations used in this paper*: AMTR, antimouse transferrin receptor antibody; M6PR, cation-independent mannose 6-phosphate receptor.

against the mouse transmembrane glycoprotein T 200 (CD 45) (44), and the hybridoma producing R17.217, a rat antibody (37) against the mouse transferrin receptor, were gifts from Dr. R. Hyman and Dr. I. S. Trowbridge (Salk Institute for Biological Studies, Division of Cancer Biology, San Diego, CA). The cells were maintained at a density of $<4 \times 10^6$ cell/ml in DME supplemented with 10% horse serum (plus 60 mg/l of penicillin G and 150 mg/l of streptomycin sulfate) at 37°C in a 5% CO_2 atmosphere. The R17.217 antibody was produced in nude mice and purified on an anti-rat IgG-Sepharose 4B column. 13/2.3 was purified on protein A (MAPS; Bio-Rad Laboratories, Richmond, CA). These two antibodies gave only one and two bands when analyzed using nonreducing and reducing SDS/PAGE, respectively (36). The rabbit serum anti-cation-independent mannose 6-phosphate receptor (anti-M6PR) (PMR I, a later bleed of the one used in previous work [47]) was a kind gift of Dr. G. Sahagian (Tufts University, Boston, MA).

Chemicals

Most chemicals including all enzyme substrates and cofactors, RNase, DNase I from pancreas, and ricin (RCA_{60}) were purchased from Sigma (Poole, Dorset, UK). Radioisotopes were from Amersham International (Amersham, UK). Protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden). Human LDLs were purified from normal serum (provided by the north London transfusion center) using the sequential flotation technique (30). They showed only one band on (reducing or nonreducing) SDS-PAGE corresponding to the 200 kD molecular mass of apoB. Their concentration is expressed as protein.

Manufacture and Handling of Gold-Protein Complexes

10-nm diameter colloidal gold was made as described by Slot and Geuze (51), except for gold-LDL complexes which were made as detailed elsewhere (27), and used within 24 h. Ricin (≈1.9 mg), Con A (≈1.3 mg), and R17.217 (≈1 mg) were complexed to colloidal gold (100 ml) as previously described (54, 6, 4) after determination of the amount of protein required for stabilization of the gold (21). The 10-nm gold complexes were finally resuspended in 3–5 ml of PBS supplemented with 0.05% of polyethylene glycol 20.000 (final OD_{520} = 30–18), whereas the gold-LDL complexes were directly resuspended in cell culture medium.

Radiolabeling

Human transferrin and the protein-gold complexes were labeled as described previously (4), using ^{125}INa (carrier-free; Amersham International). It is useful to note that ^{125}I gold complexes are labeled on the gold moiety simply by mixing the iodine with the gold-protein complex and then thoroughly washing by centrifugation (4).

Incubation Protocols

For the isolation of the plasma membrane $\sim 10^8$ cells were labeled by incubation for 1 h on ice with gold-ricin (≈200 μl) in 5 ml of DME supplemented with 100 μg BSA/ml (DME/BSA).

Endosomes were isolated after labeling the cells (10^8) for 2 h at 22°C in 5 ml of DME/BSA with AMTR-gold (≈130 μl). Coated pits were labeled using a similar protocol but performed at 10°C to avoid internalization.

To prepare lysosomes, cultured cells ($2–5 \times 10^8$ in DME supplemented with 1% horse serum and 5 mg BSA/ml) were treated directly in culture dishes for 18 h with gold-LDL (≈0.8 mg of LDL) followed by a chase for 3 h in DME supplemented with 150 μg LDL/ml and 5 mg BSA/ml. This LDL concentration saturates the BW 5147 receptors for LDL (i.e., corresponds to seven- to eightfold the K_d [5]).

After incubation with the gold complexes, cells were washed twice with DME/BSA, then once with PBS, and taken through the lysis procedure.

Temperature Dependence of Transferrin Internalization

To measure internalization, cells were incubated with radiolabeled transferrin for up to 120 min at temperatures from 2°C (ice bucket) to 37°C, and the amount of label remaining on the surface was then determined by its accessibility to digestion with 0.3% pronase. This procedure using BW 5147 cells has been described in detail in a previous publication (5) and was carried out for the present study without modification.

Lysis Procedure

The BW 5147 cell lysis was carried out as detailed elsewhere (5) using hypotonic shock and nitrogen bomb treatments. In contrast with the procedures we have previously employed (4, 5), mild trypsinization did not improve yield and was thus omitted. The postnuclear supernatant was subjected to an RNase (0.1 mg/ml) and DNase (0.2 mg/ml) treatment, 3 min at 37°C, to allow both an easier homogenization of the crude membrane pellet and avoid artefacts during the two-dimensional protein analysis (42), and was then cleared of aggregates by centrifugation for 5 min at 1,500 *g*. Recovery of all organelles in the postnuclear supernatant was similar with or without gold labeling. The crude membranes were prepared by centrifugation of the supernatant at 120,000 *g* for 30 min and were resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris, pH 8, before layering on a continuous sucrose gradient (0.5 ml of 2.5 M sucrose in water, then 12.5 ml of 60–30% sucrose in gradient buffer: 1 mM EDTA, 10 mM acetic acid, 10 mM Tris, pH 7.5). After centrifugation for 16 h at 200,000 *g* in a swinging rotor, 1-ml fractions were collected (and are numbered in the figures) from the bottom of the tube. The density change of the gradient is approximately linear from 1.25 (bottom) to 1.08 g/ml (top). The pink pellet was resuspended in the buffer suitable for its subsequent analysis. After sedimentation of gold-loaded coated pits a slight pink color was seen near the top of the gradient. In all other gradients a pink color was seen only in the pellet.

Fraction Analysis

Cells and fractions were assayed for marker enzyme activities and processed for EM and SDS-PAGE as described (4, 5).

The validity for these cells of using [^{125}I]transferrin and [^{125}I]anti-T 200 (the T 200 is a transmembrane glycoprotein which is bound to the cytoskeleton [7]) as an endosome and a plasma membrane marker, respectively, has been documented elsewhere (5). Throughout this study the marker enzyme 5'-nucleotidase gave results similar to the T 200 antigen localization, even though in other systems this enzyme has been shown to be endocytosed (53).

Single and Two-dimensional Gel Electrophoresis

Single and two-dimensional protein gel electrophoresis was performed as described (36, 20), except that IEF gels were polymerized using Temed (N,N,N',N'-tetramethylethylenediamine) and ammonium persulfate in 2.5-mm diameter tubes and the SDS-PAGE gels were 8–15% acrylamide.

Protein detection was made by autoradiography after ^{125}I labeling of the purified fractions solubilized in SDS (11). After dilution with gradient buffer, SDS was added to the fractions (500 μl) up to 5%, and, after boiling for 3 min to expose all the susceptible amino acids and cooling to room temperature, the samples were freed of most of the gold by centrifugation for 5 min in a minifuge (Eppendorf, Hamburg, FRG). Iodination was then performed using the chloramine T method (23) and 200 μCi of ^{125}INa per fraction. Labeled proteins were then freed of unreacted iodine by dialysis for 8 h at room temperature against 50 ml of PBS containing 0.5% SDS, with seven to eight changes, and stored as aliquots at −75°C until electrophoresis.

The position of spots corresponding to the protein used for organelle isolation (namely ricin for the plasma membrane, R17.217 for coated pits and endosomes, and apoB for lysosomes) was determined after labeling of the protein in the conditions described above, adding some of the iodinated protein to the corresponding fraction and determining which spots become more intense when compared to the gels of untreated fractions. The only proteins giving rise to a spot present on the gel of the purified organelles were the chains of ricin (A and B). The number of spots (three because of the existence of two glycosylation structures for the A chain) was the same on single and two-dimensional gels. This also indicates that under our conditions the chloramine T catalyzed iodination did not significantly alter the pI of the analyzed proteins, as further shown by the fact that the shape of the actin spot was the same on autoradiographs as on silver-stained gels of crude membrane proteins (data not shown).

In preliminary experiments similar results were obtained using either this ^{125}I method or overnight labeling of the cells with [^{35}S]methionine.

Under the conditions we used (20, 42) the IEF range does not cover the basic pI range. We thus performed a complementary analysis using non-equilibrium pH gradient electrophoresis allowing the separation of basic proteins (43). However, these gels showed only a limited number of basic proteins which displayed little, if any, pattern differences between the different organelles.

Localization of the Mannose 6-Phosphate Receptor

The cation-independent mannose 6-phosphate receptor was assayed quantitatively in both organelle preparations and in whole cells by immunoprecipitation. The assay was performed exactly as described previously (47) except that protein A-Sepharose was used as immunosorbant. All extracts were precleared by rabbit serum/protein A-Sepharose. The final immunoprecipitates were treated with boiling SDS (5% in PBS) and the resulting antibodies and receptors were labeled with ^{125}I as described above for the isolated membrane preparations. Iodinated proteins of either isolated membranes or whole cell preparations were analyzed in reducing conditions using 6% acrylamide gels. Migration was monitored using prestained markers (Bio-Rad Laboratories) and terminated when proteins <100 kD (i.e., antibody chains and contaminating BSA) had run off the gel, which was then prepared for autoradiography. To obtain quantitative data the lanes of the gel were cut into 0.3-cm segments and counted in a gamma counter. Two bands were observed on the autoradiographs and on the radioactivity profiles: a 215- and a 180-kD band. The relative amounts of these two bands, which have been noted previously (47), were always the same. The 180-kD band is presumed to be an immature form of the M6PR and was not included in our analysis.

Other Methods

Protein concentrations in the absence of gold were determined using the Hartree method (29). When gold was present SDS was added to the samples (to 1%). They were then boiled and the gold was removed by spinning for 5 min at 160,000 *g* in an Airfuge centrifuge (Beckman Instruments, Inc., Palo Alto, CA); protein assay was then performed on the supernatant using bicinchonimic acid (BCA; Pierce Chemical Co., Rockford, IL). This method avoids interference from the gold but overestimates the protein content of the fractions because they include the protein of the gold conjugate. This, in turn, will mean that our calculations for membrane purifications are underestimates.

All data were obtained at least twice and results of a typical experiment are shown in the accompanying figures.

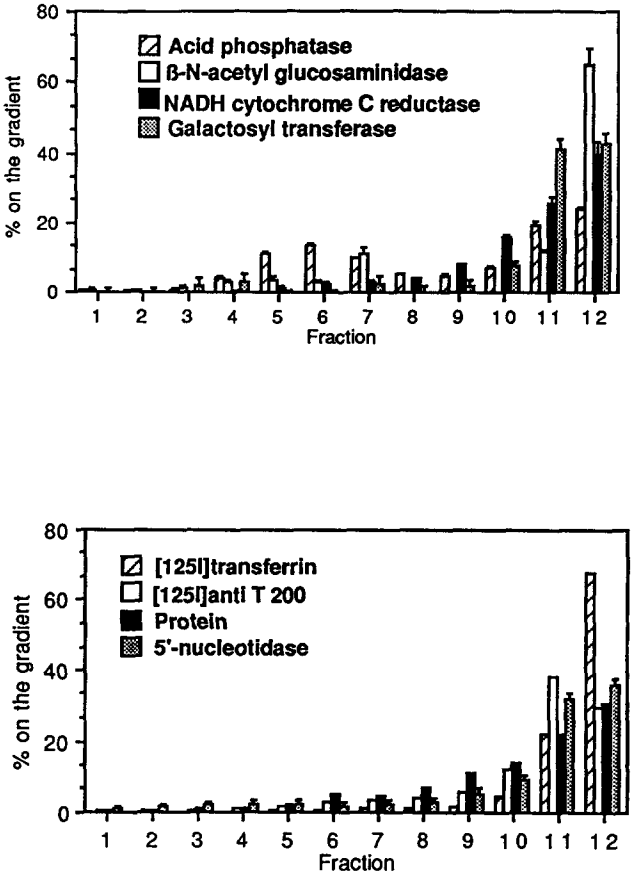

Figure 1. Distribution of marker enzymes or tracers of organelles of BW 5147 on a sucrose gradient. Cells were homogenized as indicated in Materials and Methods and the membranes were layered on the sucrose gradient. When ^{125}I labels were used they were allowed to bind and/or endocytose for 30 min at 37°C before washing and fractionation. Results are expressed as mean ± SE of the total activity (or counts) present on the gradient. Fraction 1 is the bottom of the tube.

Results

Fractionation of the Cells without Gold Loading

Fig. 1 shows the results of the analysis of the gradient prepared from crude membranes of BW 5147 cells. No gold conjugate has been introduced and so no selective separation of the cellular organelles is observed on this gradient. All marker enzyme activities: 5'-nucleotidase for the plasma membrane; NADH cytochrome C reductase for the ER, galactosyltransferase for the Golgi apparatus; acid phosphatase and β-*N*-acetyl glucosaminidase for lysosomes (the latter being a better lysosome marker than the former [28]); and succinate INT (2-(4-iodo-phenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolonium chloride) reductase for mitochondria, are found in fractions 3–12 as are [^{125}I]transferrin (for endosomes) and [^{125}I]anti-T 200 (for the plasma membrane).

Plasma Membrane Isolation

After labeling the BW 5147 cells for 1 h at 2°C with ricin–gold the plasma membrane is seen to be heavily labeled (Fig. 2 *a*), and the cellular viability is unimpaired (over 95%).

When cells labeled using this procedure were disrupted and their crude membranes separated on the sucrose gradient, the distribution (displayed in Fig. 3) of T 200, 5'-nucleotidase activity, and [^{125}I]gold–ricin were almost identical. These distributions show that 50% of the plasma membrane present on the gradient has been density shifted to the bottom (fraction 1). The [^{125}I]transferrin profile was only slightly altered by this ricin–gold treatment (this probably arises from plasma membrane bound label [5]) and assays of the marker enzymes of the other subcellular compartments (Table I) confirmed that only the plasma membrane was sedimented by gold–ricin. The morphological purity of the plasma membrane preparation as shown by EM is displayed in Fig. 2 *b*. It is clear from this micrograph that most of the membrane is in the form of large sheets and thus typical of plasma membrane. Not all of the membrane surfaces are coated with gold but in most instances gold-labeled membrane is continuous with unlabeled membrane. Serial sectioning shows that membrane profiles which are apparently free and unlabeled are usually continuous with gold-labeled domains not included in the section plane being examined (data not shown). The amount of free gold in these fractions is negligible and we do not believe, therefore, that the gold has dissociated from the membrane domains which are unlabeled. It should be noted, however, that the profiles of intact cells shown in Fig. 2 *a* display the densest amounts of gold label seen. In most intact cells, as in fractionated membranes, stretches of unlabeled membranes frequently occur.

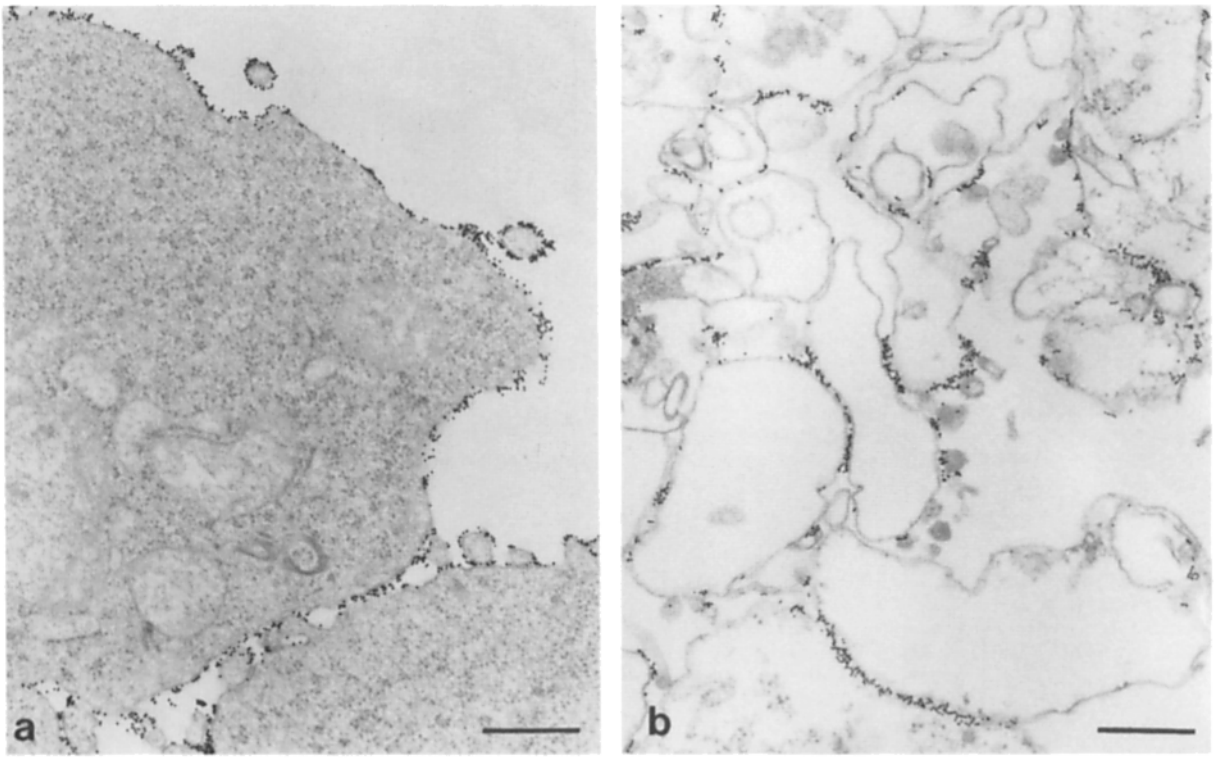

Figure 2. Electron micrographs of BW 5147 cells labeled with gold–ricin at 2°C and of the plasma membrane preparation from these cells. BW 5147 cells were labeled for 1 h at 2°C before washing off excess ligand. They were then either fixed or lysed. In the later case their crude membranes were separated on the gradient. Whole cells (*a*) and fraction 1 of the gradient (membrane preparation, *b*) were prepared for EM. Bars, 0.5 μm.

After correction for losses (20%) in the nuclear pellet the final yield of this procedure is ~40% of the cell plasma membrane. This preparation contains <0.7% of total cell proteins and achieves a purification of at least 57-fold. As noted in the methods section this is likely to be a significant underestimate because it is based on a total protein content which includes the ricin present in the gold complex (see Fig. 10).

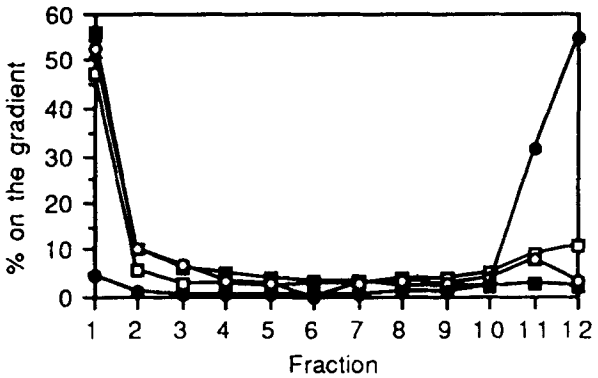

Figure 3. Plasma membrane and endosome tracers and [^{125}I]gold–ricin distribution on the gradient following ricin–gold binding to the BW 5147 cells. After treatment for 1 h at 2°C with gold–ricin, cells were lysed as described in Materials and Methods, and the crude membranes were layered on the gradient. In experiments involving ^{125}I labels, [^{125}I]anti-T 200 was added with the ricin–gold, whereas [^{125}I]transferrin was incubated with the cells for 30 min at 37°C before washing, cooling down, and ricin–gold addition. (●) [^{125}I]transferrin; (□) [^{125}I]anti-T 200; (○) 5′-nucleotidase; (■) [^{125}I]-gold–ricin. Data are percentages of total gradient activity (or counts).

Endosome Isolation

The R17.217 monoclonal antibody against the mouse transferrin receptor is an IgG (37). Neither the antibody nor its gold conjugate compete with transferrin binding (data not shown). It can be seen in Fig. 4 *a* that upon incubation with the cells at 22°C for 2 h this AMTR–gold complex labels plasma membrane and endosomes. As expected, there is more gold in the endosomes since it has been reported that the transferrin receptor is 30-fold more concentrated in this

Table I. Purity of the BW 5147 Plasma Membrane Prepared Using the Ricin–Gold Procedure

Enzyme	Percent in fraction 1
β-*N*-acetyl glucosaminidase	1 ± 2
Succinate INT reductase	0.5 ± 1
Galactosyltransferase	0 ± 1
NADH cytochrome C reductase	0.5 ± 0.2
5′-nucleotidase	48 ± 4

The gradient derived from cells labeled with gold–ricin for 1 h at 2°C was prepared and fractionated, and then enzyme assays were performed as described in Materials and Methods. Data (mean ± SEM) are percentages of the total activity present on the gradient which is recovered in fraction 1 (to be compared to Fig. 1 data). n = 4.

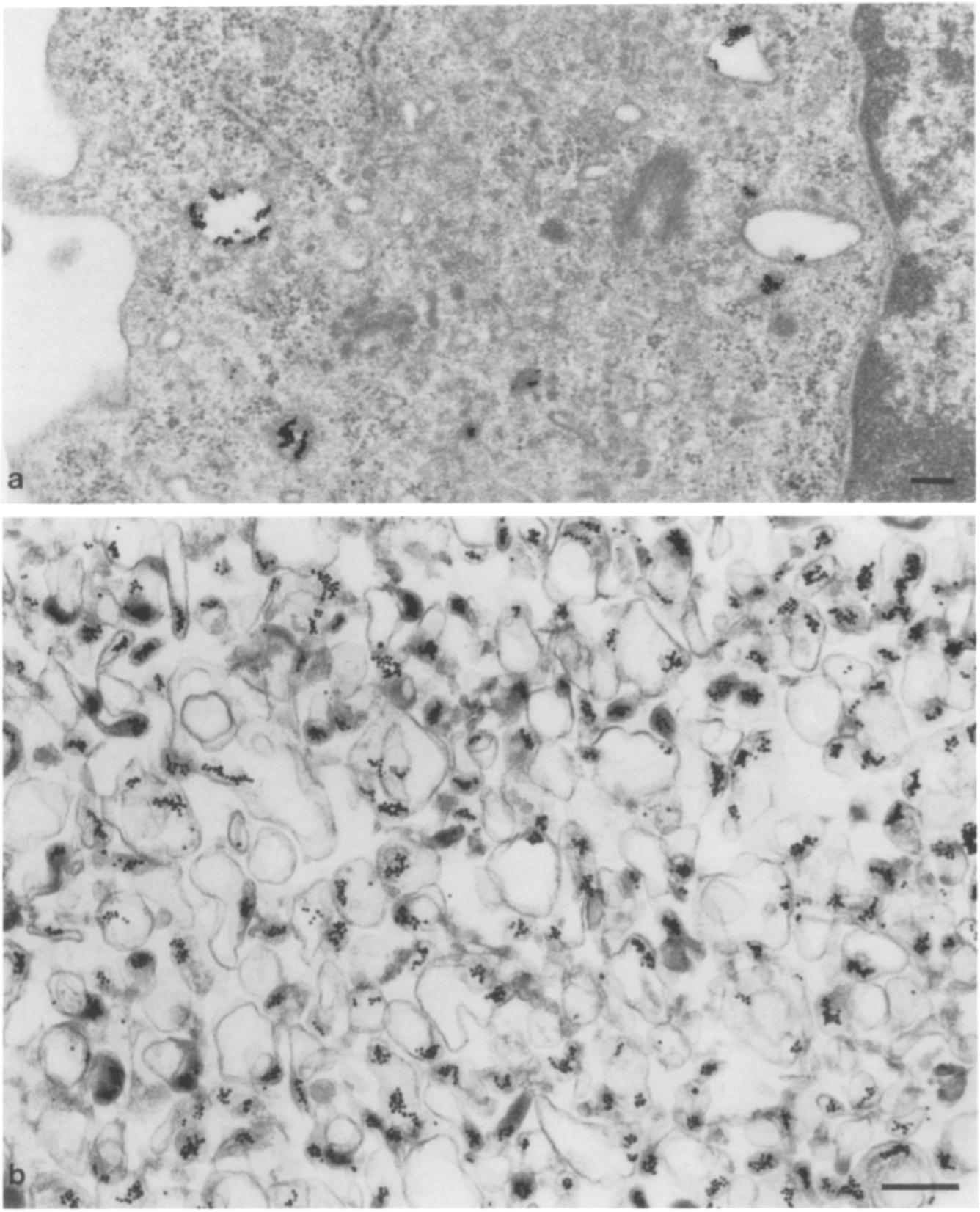

Figure 4. Electron micrographs of BW 5147 cells labeled with gold–AMTR at 22°C and of the endosome preparation. Cells were labeled for 2 h at 22°C before washing off excess ligand, and then either fixed or lysed. In the later case their crude membranes were separated on the gradient. Whole cells (*a*) and fraction 1 of the gradient (endosome preparation, *b*) were prepared for EM. Bars, 0.2 μm.

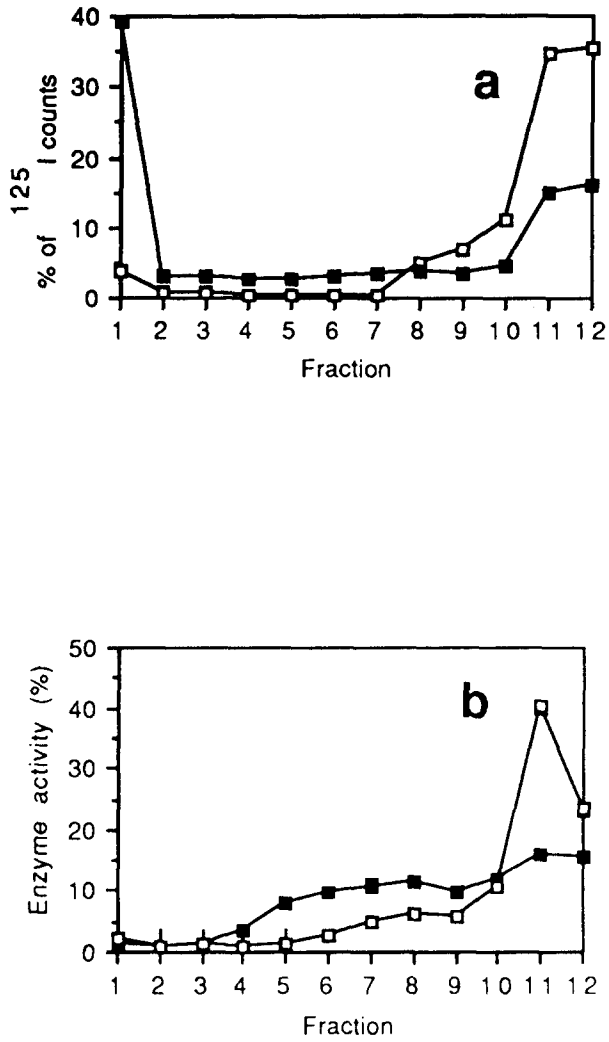

Figure 5. Endosome, plasma membrane, and lysosome marker distribution on the gradient following AMTR-gold labeling of the BW 5147 cells at 22°C. After incubation for 2 h at 22°C with AMTR-gold (and with a ^{125}I label if indicated), cells were lysed as described in Materials and Methods, and the postnuclear pellet was layered on the gradient. *a*: (■) [^{125}I]transferrin; (□) [^{125}I]anti-T 200. *b*: (□) 5′-nucleotidase; (■) acid phosphatase. Data are percentages of total gradient activity (or counts).

cellular compartment (15). 22°C was chosen because it slows recycling and the transfer from endosomes to lysosomes (58, 4, and data not shown) and thus promotes the accumulation of the AMTR-gold complex within the endosomes. Upon gradient separation of crude membranes from these cells it can be seen (Fig. 5) that only endosomes (an amount corresponding to 40% of the [^{125}I]transferrin) are sedimented. The distribution of T 200 (Fig. 5 *a*), 5′-nucleotidase, and acid phosphatase (Fig. 5 *b*) are not significantly different from their profile on control gradients (see Fig. 1). Other marker enzymes confirmed the purity of the endosome preparation (data not shown). As displayed in Fig. 4 *b*, the morphology of the endosomes is as expected from their appearance in intact cells (Fig. 4 *a*). The yield of the procedure, expressed as a function of [^{125}I]transferrin initially bound to the cells, is 32%. Less than 0.5% of total cell proteins are obtained and a purification factor of at least 64-fold is thus achieved.

Endocytic Pit Isolation

Earlier work (33) and preliminary morphological studies on lymphocytes showed that when cells were incubated at 10°C with AMTR-gold complexes coated domains on the plasma membrane became heavily labeled, but they did not appear to pinch off to form free vesicles. To confirm these observations we used [^{125}I]transferrin to assay for internalization over the range of 2, 10, and 15°C. As shown in Table II the internalization of transferring is not significant at 10°C because even after 2 h at this temperature pronase can still remove >90% of the radiolabel.

The cell displayed in Fig. 6 *a* shows that in BW 5147 incubated for 2 h at 10°C the gold is present only in coated pits at the periphery of the cell (Fig. 6, *a* and *b*). Even where the gold appears to be some distance from the cell boundary (Fig. 6 *a*) it is associated with a coated membrane domain and is thus almost certainly still connected to the plasma membrane (i.e., it is a deeply invaginated coated pit). We anticipated that the plasma membrane would break preferentially at the border of the gold-loaded pits during homogenization, allowing them to sediment. As shown in Fig. 7, a significant amount of [^{125}I]transferrin (12%) is recovered in fraction 1 of the gradient prepared from these cells, whereas the amount of plasma membrane, as indicated by T 200, is low (similar to that obtained with cells incubated without gold [see Fig. 1]). EM of the isolated fraction shows the gold to be contained within small (80–100 nm) tube-like vesicles which are often open at one end. At their closed ends these tubular elements are dilated into flask-like shapes (Fig. 6, *c* and *d*). A clathrin lattice is not normally present on these structures but since they contain gold particles and have the dimensions of the gold-loaded coated pits seen in intact cells we believe them to be derived from the clathrin lattice-coated domains of the plasma membrane shown in Fig. 6, *a* and *b*. Since the sucrose gradient used for isolation is above neutral pH it seemed likely that the clathrin lattice may have been lost during centrifugation. To explore this possibility we fixed an aliquot of crude membranes in 0.05% glutaraldehyde for 2 min at 2°C and then ran it on the gradient. In the pellet there was extensive aggregation because of the prefixation, but gold-loaded vesicles with clathrin-like coats were identifiable (Fig. 6 *e*).

From the literature it seems that up to 70% of the transferrin receptors on the plasma membrane could be in coated pits at 5–10°C (33). However, although >50% of them are shifted from the fraction at the top of the gradient only 12%

Table II. Percentage of [^{125}I]Transferrin Remaining on the Cell Surface after Incubating up to 2 h at 2, 10, and 15°C

	0 min	30 min	60 min	120 min
2°C	97.6	96.4	97.3	94.9
10°C	97.6	96.6	96.7	93.7
15°C	97.6	94.0	93.7	81.1

Cells were incubated with radiolabel for the times and temperatures shown, surface label was stripped using pronase and cells separated by centrifugation. At 37°C for 120 min, 35% of the label remained on the surface.

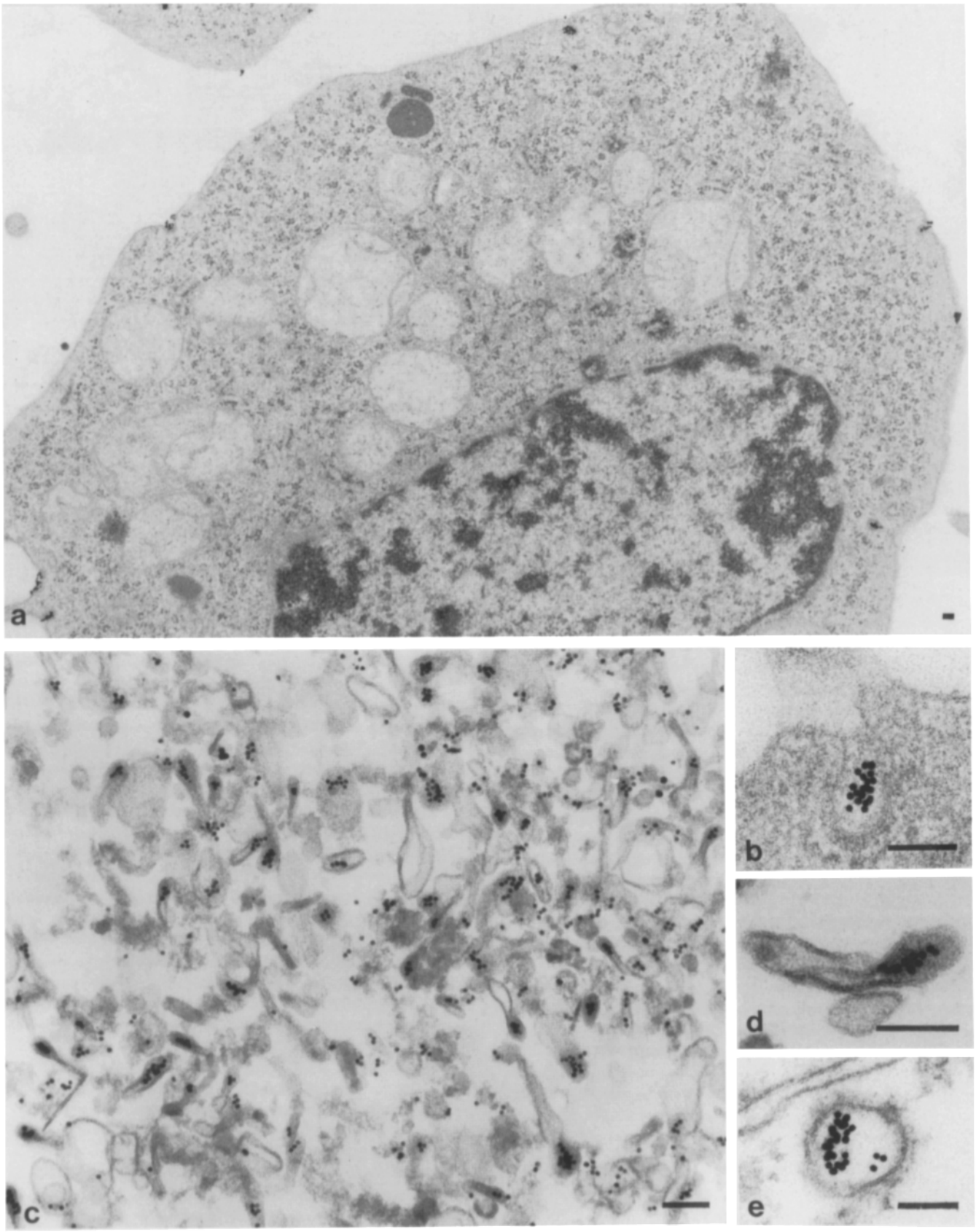

Figure 6. Electron micrographs of BW 5147 cells labeled with AMTR–gold at 10°C and of coated pit preparations. Protocol as in Fig. 4 except that labeling was performed at 10°C. (*a*) Whole cell; (*b*) higher magnification of coated pit in intact cell; (*c* and *d*) endocytic pits obtained in fraction 1 of the gradient without glutaraldehyde fixation of the crude membranes; (*e*) isolated coated pit achieved using a 2-min treatment at 2°C of the crude membranes by 0.05% glutaraldehyde before the gradient. Bars, 0.1 μm.

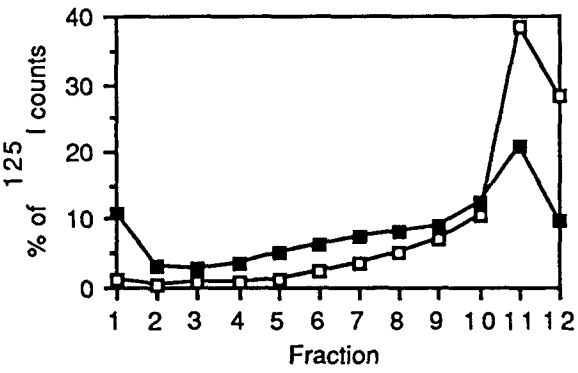

Figure 7. Distribution of the endosome and plasma membrane markers on the gradient following AMTR-gold labeling of the BW 5147 cells at 10°C. After incubation for 2 h at 10°C with AMTR-gold, cells were lysed as described in Materials and Methods, and the crude membranes were layered on the gradient. (■) [^{125}I]transferrin; (□) [^{125}I]anti-T 200. Data are percentages of total gradient counts.

sedimented in fraction 1. Based on these estimates the final yield of isolated pits (~10% of the initially bound [^{125}I] transferrin) is therefore quite low. The purification factor (<0.45% of total cell proteins are recovered) is ~22-fold.

Lysosome Isolation

After 18 h of cell incubation with LDL-gold and a further 3-h chase with excess free LDL, the gold is present only in lysosomal elements (Fig. 8 *a*). This is presumably because gold particles accumulate as the LDLs are degraded (27). When crude membranes from cells labeled in this way are separated on the sucrose gradient, fraction 1 is highly enriched in lysosomal enzymes (Fig. 9). Both acid phosphatase and the β-*N*-acetyl glucosaminidase show 36–52% of total enzymatic activity present in fraction 1, together with >80% of the LDL-gold complexes. However, this yield needs to be corrected for the large losses of lysosome enzyme activities in the nuclear pellet (70%). The estimate of a 16% yield (calculated using β-*N*-acetyl glucosaminidase as a lysosome marker [28]) is not as high as for the plasma membrane or the endosome fraction, but the amount of material obtained (<0.45% of total cell proteins, purification factor >36-fold) is actually much greater. This is probably because lysosomes comprise a relatively large fraction of total cell membrane boundaries. Only lysosomes were sedimented by the LDL-gold loading protocol as monitored using the markers of the other cellular compartments (data not shown).

Electron micrographs of this preparation (Fig. 8 *b*) show typical lysosomes with negligible contamination by other elements. However, the external membranes of the isolated lysosomes are frequently interrupted and there are considerable amounts of free gold. These heavily gold-loaded elements are thus probably very fragile.

The Plasma Membrane, Coated Pits, Endosomes, and Lysosomes Display Different Protein Compositions

A two-dimensional protein analysis of the isolated plasma membrane, endocytic pits, endosomes, and lysosomes is shown in Fig. 10. These are autoradiographs of gels obtained from iodinated purified fractions prepared as described in Materials and Methods. In preliminary experiments, biosynthetic [^{35}S]methionine labeling was found to be too low to give well-labeled two-dimensional gels. Both labeling techniques nevertheless gave similar one-dimensional gel patterns (data not shown).

These two-dimensional autoradiographs show that in each fraction there are protein species that are unique or at least highly enriched. However, most of the major proteins are represented in all fractions from the plasma membrane to the endosome. The greatest difference in the spectrum of proteins observed in the different membrane boundaries of the sequential pathway is between the endosome and the lysosome, which contains the largest number of exclusive proteins. The main results of the gel analysis are summarized in Fig. 11.

The major protein of the whole cell and the crude membranes is a 46-kD pI ~5.5 protein which is presumably actin. This protein dominates profiles obtained from purified plasma membranes but is greatly reduced in those obtained from endocytic pit, endosome, and lysosome fractions. On our gels the large amounts of actin in the plasma membrane probably obscure many of its less well-represented protein constituents.

Within the plasma membrane fraction there is a protein at 200 kD (spots a,b) which is not present in the endocytic pit profile. This is probably T 200, because although it is known to be a major constituent of the plasma membrane from these cells (44) it is not thought to be endocytosed (5). This is in agreement with the distributions of [^{125}I]anti-T 200 shown in Figs. 3 and 7. Ricin is also present in the plasma membrane fraction.

In the endocytic pit fraction there is no suggestion of a 180-kD species (as expected from the absence of a clathrin lattice indicated by EM), but there is a significant increase in 100-kD, pI 6 range proteins (spot g), which could include both adaptins (46) and the transferrin receptor (37).

The endosome fraction is not very different from the endocytic pit preparation, but there is at least one spot (70 kD pI 6.1, i) which is absent in endosomes, while another (j, in the 30-kD pI 6.15 range) is absent from endocytic pits. A previous study by Schmid et al. (48) also found the endosome fraction to be enriched with 30-kD protein species. Other changes are only quantitative (like the increased representation of two spots corresponding to the same molecular weight [56 kD] in the pI 5.9 region) and may reflect only the higher purity of the endosome fraction. In the present study no attempts were made to separate "early" and "late" endosomes.

Most of the slightly acidic 15–50-kD range endosomal proteins are absent in lysosomes while two intense spots (k) appear in the 120-kD, pI 5.85 range. The intensity and molecular weights of these spots indicates that they probably correspond to the major lysosomal glycoproteins identified by others (9). The presence of the M6PR was tested for using a well-documented antiserum (47) for immunoprecipitation. The gel profiles obtained were essentially the same as those described previously (47). The mature form of the receptor (215 kD) was present in all the membrane boundaries we isolated. Table III shows the distribution of the 215-kD receptor

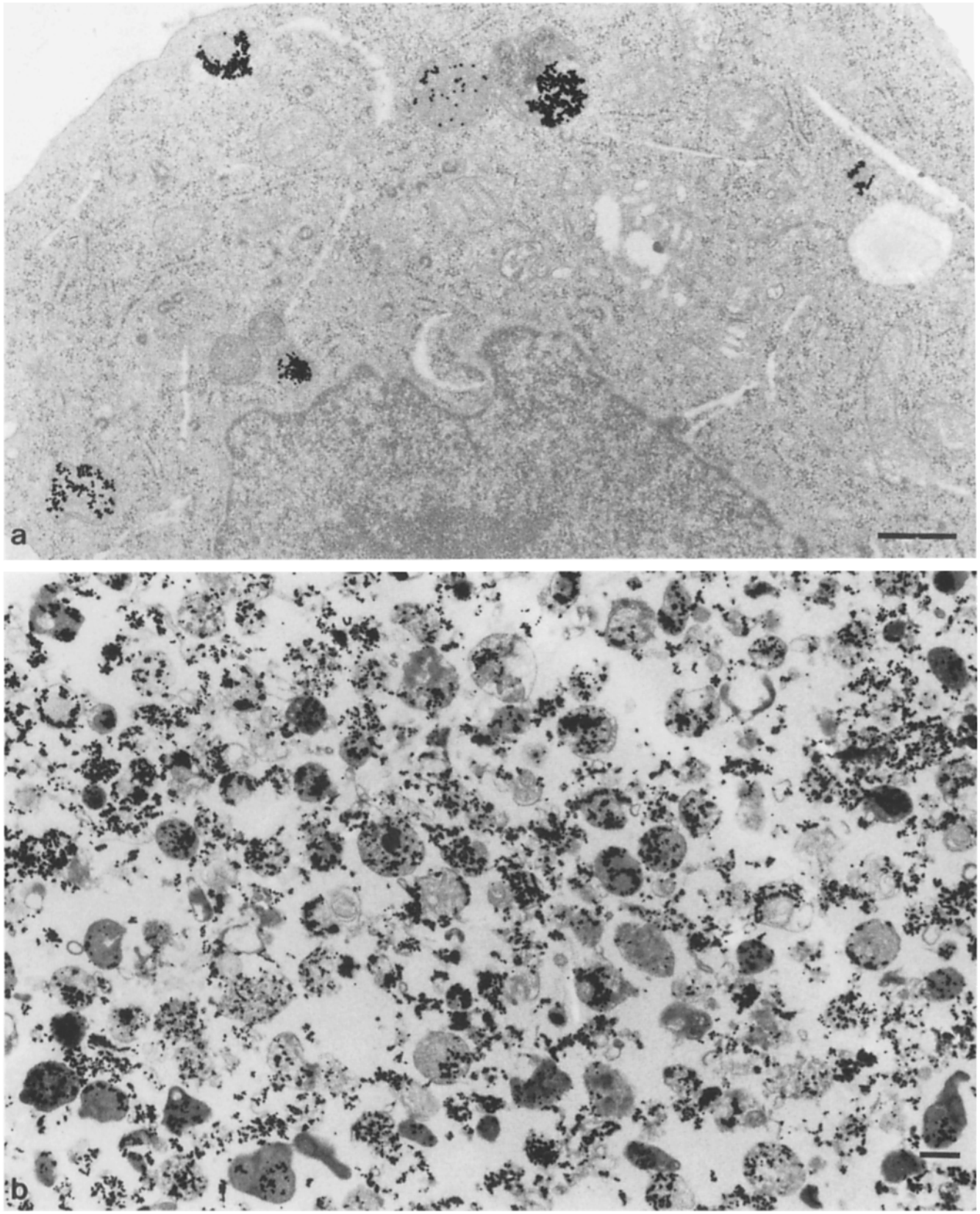

Figure 8. Electron micrographs of BW 5147 cells labeled with gold–LDL and of the lysosome preparation. Cells were labeled for 18 h at 37°C with gold–LDL, the excess ligand was washed away and cells were fixed or lysed after a 3-h chase at 37°C performed with 150 μg LDL/ml. In the latter case their crude membranes were separated on the gradient. Whole cells (*a*) and fraction 1 of the gradient (lysosome preparation, *b*) were then prepared for EM. Bars, 0.5 μm.

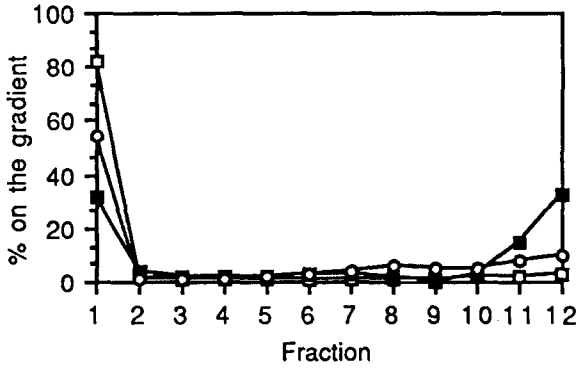

Figure 9. Distribution of lysosome enzyme markers and of [^{125}I] gold-LDL on the gradient following LDL-gold labeling and chase. After incubation for 18 h at 37°C with LDL-gold and chase for 3 h at 37°C with 150 μg LDL/ml, cells were lysed and the gradient prepared as described in Materials and Methods. (■) Acid phosphatase; (○) β-*N*-acetyl glucosaminidase; (□) [^{125}I]gold-LDL. Data are percentages of total gradient activity (or counts).

band as a percentage of total cell receptor. Significant amounts (10–20%) of M6PR are found in the plasma membrane, coated pits, and lysosomes, but endosomes (with up to 30%) contained the highest concentration.

Discussion

Methods for delivering drugs to selected cell types are of considerable therapeutic interest. Immunotoxins are already in clinical use for purging bone marrow cultures (52) and since lymphocyte plasma membranes are among the best characterized immunochemically, further opportunities will undoubtedly become available once the processes of uptake and intracellular processing of cell surface components are better understood. Furthermore, since it is now clear that the endocytic pathway plays a central role in antigen presentation (57), means of preparing lymphocyte membrane boundaries for detailed molecular analysis are required.

Because of its large nucleus to cytoplasm volume ratio the lymphocyte has not been a widely used system for fractionation studies. Good purifications of both plasma membrane (3, 19, 34), Golgi apparatus (22, 26), and lysosomes (28) have nevertheless been achieved. Unfortunately while these preparations have been well characterized in terms of protein content and enzyme activity, gel electrophoretic analyses of the kind used in the present study have not been made and membrane protein profiles for comparative purposes are not available. In an earlier fractionation study designed to isolate lymphocyte endosomal elements (5) we reported that in the absence of density shift procedures plasma membrane and endosomes showed indistinguishable distributions on the gradient used in published methods to isolate the plasma membrane (3, 7, 34). Moreover, except in the hepatocyte (31), coated vesicles originating only from endocytosis (and not mixed with coated vesicles from the exocytic pathway) have not been isolated (8). The density shift method described here for endocytic pit isolation is, hence, more selective than the use of anticlathrin antibodies (40). This preparation should be especially useful for studying the earlier events of endocytosis and in particular the membrane protein sorting occurring during coated pit formation (46).

Ricin-gold was used to selectively alter the plasma membrane density enough to shift it to the bottom of the gradient. The fact that this effect of ricin cannot be obtained using another lectin like con A, or the AMTR, is probably due to the high number of binding sites of ricin on the cell plasma membrane, ~10^7/cell (50). Hence, the critical gold density level needed on this membrane to significantly shift its density is reached only by ricin and not by the AMTR-gold complex (in accordance with our [^{125}I]transferrin binding Scatchard analysis showing 10^5 sites per BW 5147 at 2°C). The ability of the AMTR-gold complex to isolate selectively endocytic pits and endosomes presumably arises because of the concentration step which occurs in coated pits (~30-fold for the transferrin receptor [15]).

Our present studies have achieved reasonable yields of very highly purified fractions of plasma membrane, endocytic pits, endosomes, and lysosomes. The main loss occurs in the nuclear pellet. Recently described manipulations for inducing organelles and especially lysosomes to move out of the juxtanuclear area (32) should allow this problem to be reduced in the future. The purity of all the fractions obtained is at least equal to those achieved in the same (3, 5, 19, 26, 28) or in other systems (1, 4, 12–14, 16–18, 25, 31, 35, 38–41, 48) and was sufficient for a comparative analysis of their protein constituents to be made. This is, necessarily, a preliminary description but it includes most of the boundaries of the endocytic pathway and it compares well with the best achieved from other cell systems (18, 38, 48).

Insofar as they are representative of their membrane boundaries, the two-dimensional gel profiles obtained suggest that there is a major subset of proteins distributed throughout the endocytic pathway. The finding that one or more proteins are exclusive to each fraction confirms the indications obtained from radiolabeled tracers, marker enzymes, and electronmicroscopic analysis, namely, that the membrane boundaries in the different fractions are effectively separated. It also indicates the scale of the molecular changes which take place at each step during the progression along the endocytic pathway. These changes seem to take place by addition as well as by deletion of proteins and result in a distinct protein pattern of each compartment studied. This can be seen in Fig. 11. The constituents of the plasma membrane are the least well presented in 2D gel profiles because the autoradiograph is underexposed to allow clearer comparison with the endocytic pit fraction. The main value of this profile is therefore in showing that while T 200 is present in the isolated plasma membrane it does not enter the pit fraction. In the endosome fraction there are too few compartment-specific proteins for us to determine the extent to which the endosome compartment is a stable system. The two-dimensional protein profile of the lysosomal compartment is clearly very different from that of the endosome, confirming the results of one-dimension analysis (38). However, the long incubations employed for the gold loading are likely to isolate the more distal regions of the lysosomal compartment preferentially and we cannot conclude that the transition from endosome to "early" lysosome will be equally abrupt.

The presence of the M6PR at the plasma membrane and in coated pits has been observed in many previous studies

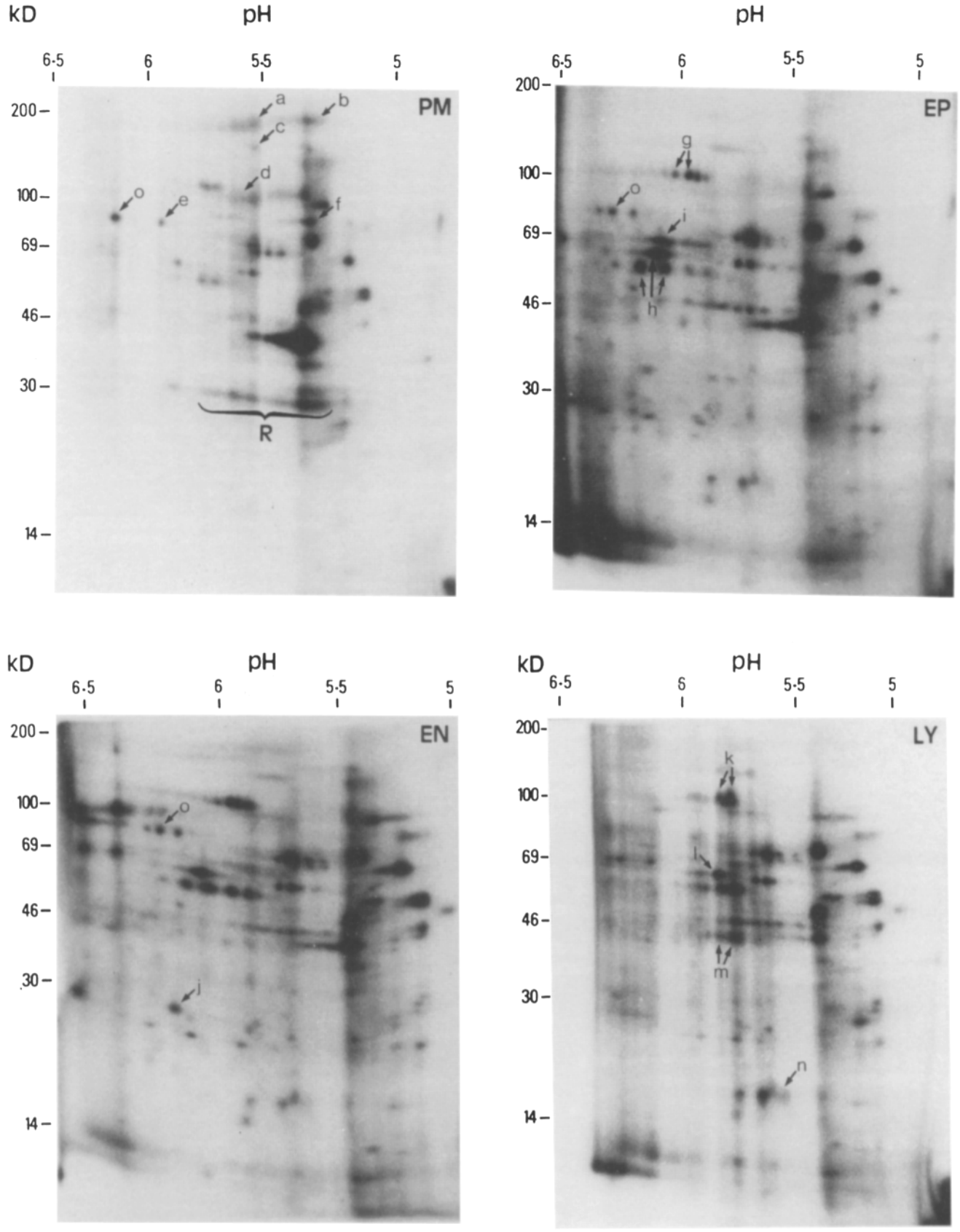

Figure 10. Two-dimensional protein gels of purified plasma membrane, endocytic pits, endosome, and lysosome preparations from BW 5147 cells. IEF and SDS-PAGE were performed as described in Materials and Methods. *PM*, plasma membrane; *EP*, endocytic pits; *EN*, endosomes; *LY*, lysosomes. The pI are indicated in the linear range of the pH gradient measured on the IEF gel. Lettered proteins (kD, pI): *a*, (200, 5.6–5.8), *b* (200, 5.4), *c* (175, 5.65), *d* (90, 5.65), *e* (83, 6.1), *f* (90, 5.4), *g* (105, 5.95), *h* (55–65, 6.1–6.2), *i* (69, 6.1), *j* (33, 6.15), *k* (120, 5.85), *l* (69, 5.8), *m* (40, 5.7–5.9), *n* (20, 5.5), *o* (90, 6.1–6.3). *R*, ricin. The light chain of R17.217 was also found to give a spot in the 30-kD area of our two-dimensional gels, but this spot was not present on endocytic pits or endosome gels. The heavy chain of R17.217 and the apoB were not observed using this two-dimensional system, probably because of a basic pI (see Materials and Methods).

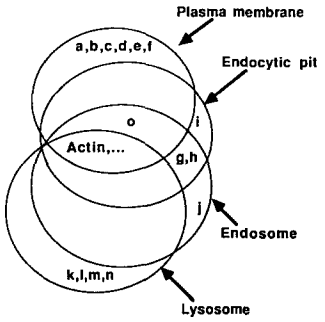

Figure 11. Schematic representation of the distribution of selected proteins between different organelles of the endocytic pathway. Organelles are represented as circles containing or excluding proteins which are lettered as in Fig. 10.

and the estimate of 10% of total receptors in this location is similar to that of an earlier cell fractionation analysis (47). Our finding that the endosomal compartment contains the highest concentration of M6PR is also in agreement with earlier work (47). However, it should be noted that the endosomes we isolate are reached by AMTR conjugates at 22°C and they are not, therefore, likely to include the highly M6PR-enriched prelysosomal elements described by Griffiths et al. (24). Our finding of significant amounts (10%) of M6PR in elements loaded with LDL-gold after chase times of 3 h is unexpected because a previous study by Sahagian and Neufeld (47) detected essentially no activity (as determined by β-galactosidase binding) in a lysosome fraction from CHO cells. The reason for this discrepancy is not clear at the present time.

The electron microscope observations show that the isolated endosomal elements are well preserved; in all respects similar to their counterparts in the intact cell. With this fraction it should be possible to carry out cell-free studies of the intracellular processing of immunoconjugates and to deduce the mechanisms of translocation available for their escape to the cytoplasm in the intact cell.

The endocytic pathway fractionation system developed in this study will also be very valuable to follow the intracellular destination and processing of immunotoxins since the lymphocyte is a major target of these drugs (45).

The comments of Dr. H. Hugson on the manuscript and the help of Clare Futter for binding experiments were greatly appreciated. We also owe special thanks to Pr. A. Bienvenüe for allowing us to perform some complementary work in Montpellier.

This work was supported by a grant from the Leukemia Research Fund to C. R. Hopkins and by supplementary funds from the Commission des Communautés Européennes and the Centre National de la Recherche Scientifique to B. Beaumelle.

Received for publication 4 January 1990 and in revised form 10 July 1990.

Table III. Distribution of the Mannose-6-phosphate Receptor in the Isolated Membrane Fractions

	% of total cell receptor
Plasma membrane	13 ± 2
Endocytic pits	12 ± 2
Endosomes	31 ± 3
Lysosomes	16 ± 5
Cells	100 ± 5

Receptor was immunoprecipitated from membrane fractions or whole lysates. The immunoprecipitates were then iodinated and electrophoresed on 6% acrylamide gels. Radioactivity in the 215-kD band was quantitated by slicing the gels and counting in a gamma counter. In calculating the data presented (± SEM, n = 4) the yield of each isolation procedure is taken into account.

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