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High-yield isolation of functionally competent endosomes from mouse lymphocytes

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A discontinuous-sucrose-gradient procedure for isolating endosomes from mouse lymphoma cells has been developed. After centrifugation, most organelles (especially mitochondria and lysosomes) are recovered in the denser fractions of the gradient, whereas a mixture of plasma membrane and endosomes is present at lighter densities. The endosome recovery in this fraction can be increased (by 100%) by (*a*) a mild trypsin treatment of the postnuclear supernatant and (*b*) loading the cell endosomes with a saturating concentration of low-density lipoproteins. Removal of the plasma-membrane contamination was achieved by preincubating the cells with a gold–ricin complex at 4 °C. On centrifugation, the gold-loaded membranes sediment to the bottom of the gradient. The endosome preparation isolated by these procedures is less than 6% contaminated by other organelles and contains 42% of internalized ^{125}I-transferrin. We show that these isolated endosomes are functional, as displayed by their ability to fuse and to acidify in a cell-free system. Endosome fusion was studied by a new assay based on the use of fluorescence resonance energy transfer. This fusion is dependent on ATP and on a cytosolic, thermoresistant but trypsin- and *N*-ethylmaleimide-sensitive, protein factor. Early endosomes fuse more actively among themselves than with late-endocytic vesicles, and they fuse only slowly with plasma-membrane vesicles.

INTRODUCTION

After internalization, either via receptor-mediated endocytosis or via non-specific fluid-phase endocytosis, the sorting of internalized molecules toward their final destination in the cell (the plasma membrane, the cytoplasm, or the lysosome) is made through an intracellular compartment usually called the endosome (Helenius *et al.*, 1983; Hopkins, 1983*a*,*b*). The (prelysosomal) membrane vesicles and tubules which make up this compartment are heterogeneous in shape (vacuolar or cisternal), size and subcellular distribution (peripheral or juxtanuclear) (Helenius *et al.*, 1983; Hopkins, 1983*a*,*b*; Pastan & Willingham, 1983; Hopkins & Trowbridge, 1983; Willingham *et al.*, 1984). Biochemically, endosomal compartments have been only poorly characterized. Subcellular fractionation suggests that it has a different protein composition from the plasma membrane (Dickson *et al.*, 1983; Evans & Hardison, 1985; Marsh *et al.*, 1987; Enrich *et al.*, 1988; Schmid *et al.*, 1988) and that it is highly enriched in cell-surface receptors (Bretscher *et al.*, 1980; Dickson *et al.*, 1983). Endosomes have also an acidic internal pH (5–6) largely maintained by an ATP-driven pump (Merion *et al.*, 1983; Galloway *et al.*, 1983; Mellman *et al.*, 1986). This low pH has important functional implications; in particular, it has been shown to trigger the dissociation of iron from transferrin, a key step in the transferrin cycle (Hopkins & Trowbridge, 1983; Dautry-Varsat *et al.*, 1983). The acidic environment of the endosome also induces the dissociation of low-density lipoproteins (LDL) from their receptor, allowing them to follow different cellular routes: the LDL are degraded in lysosomes, whereas the receptor is recycled back to the plasma membrane (Goldstein *et al.*, 1985).

A wide variety of methods have been developed for endosome isolation (Mullock *et al.*, 1983; Dickson *et al.*, 1983; Debanne *et al.*, 1984; Quintart *et al.*, 1984; Evans & Flint, 1985; Evans & Hardison, 1985; Wall & Hubbard, 1985; Khan *et al.*, 1986; Mueller & Hubbard, 1986; Gruenberg & Howell, 1986; Branch *et al.*, 1987; Belcher *et al.*, 1987; Beardmore *et al.*, 1987; Marsh *et al.*, 1987; Enrich *et al.*, 1988; Schmid *et al.*, 1988; Diment *et al.*, 1988), but to date none of them have been applied to the lymphocyte.

The intracellular processing of endocytosed macromolecules in the lymphocyte is of special interest for drug-targeting applications. For example, during the treatment of leukaemias or graft-versus-host diseases, immunotoxins are being used for the selective removal of lymphocytes (Youle *et al.*, 1986; Pastan *et al.*, 1986; Uckun *et al.*, 1988). The mode of action of these conjugates is incompletely understood, but the most efficient of them are made with a monoclonal antibody against actively internalized antigens (Lesley *et al.*, 1984; Youle *et al.*, 1986; Pastan *et al.*, 1986), and their cytotoxic activity is inhibited by NH_4Cl (Youle *et al.*, 1986). It is therefore probable that entry of immunotoxins in the cytoplasm occurs, like most toxins (Olsnes *et al.*, 1988) from the endosome compartment (Pastan *et al.*, 1986).

As a prerequisite for a detailed study of the immunotoxin penetration into the lymphocyte, we have

Abbreviations used: LDL, low-density lipoproteins; RET, resonance energy transfer; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolonium chloride; DAPI, 4′,6′-diamidino-2-phenylindole; HRP, horseradish peroxidase; PBS, phosphate-buffered saline (137 mM-NaCl, 2.7 mM-KCl, 2.6 mM-KH_2PO_4, 8.6 mM-Na_2HPO_4, pH 7.4); DMEM, Dulbecco's modified minimum essential medium; BSA, bovine serum albumin; Dns-Tf, dansyl transferrin; Rh-Tf, rhodamine transferrin; NEM, *N*-ethylmaleimide; PNS, post-nuclear supernatant.

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therefore developed an endosome isolation procedure from these cells.

This paper describes a fairly rapid (~6 h) preparative method for isolating highly purified endosomes in high yield from mouse leukaemic cells. It is based on an endosome-density shift induced by preincubation of the cells with LDL to lighten the endosomes, in conjunction with a ricin–gold labelling of the cell surface to remove the plasma membrane. The result is that endosomes can be separated by a single discontinuous sucrose gradient. The procedure has been monitored by using enzymic determinations, monoclonal antibodies and electron microscopy.

The isolated endosomes are functional, as shown by their ability to acidify and fuse. The former property was tested with fluorescein–transferrin (Ohkuma & Poole, 1978; Merion *et al.*, 1983; Galloway *et al.*, 1983), whereas endosome fusion was studied by a new and simple method based on the use of fluorescent transferrins and fluorescence resonance energy transfer (RET). Most of the results obtained by this RET assay were similar to those reported by using previously described (Warren *et al.*, 1988; Diaz *et al.*, 1988; Gruenberg *et al.*, 1989) endosome-fusion assays [i.e. dependence on ATP and on a trypsin- and *N*-ethylmaleimide (NEM)-sensitive cytosolic protein factor]. We also found that endosomes can fuse among themselves and (albeit slowly) with plasma-membrane vesicles, as reported by Mayorga *et al.* (1988). Elements derived from the early part of the endocytic pathway fuse more readily than those derived from later, juxtanuclear, stages. The RET fusion test is simpler and more rapid than previously reported fusion assays (Davey *et al.*, 1985; Gruenberg & Howell, 1986; Braell, 1987; Woodman & Warren, 1988; Diaz *et al.*, 1988; Gruenberg *et al.*, 1989).

MATERIALS AND METHODS

Chemicals and reagents

Transferrin (iron-saturated), ricin (RCA_{60}), INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolonium chloride], DAPI (4′,6′-diamidino-2-phenylindole), adenylyl imidodiphosphate, horseradish peroxidase (HRP), dansyl chloride, fluorescein isothiocyanate and rhodamine isothiocyanate (on Celite) were purchased from Sigma (Poole, Dorset, U.K.). Pronase was from BDH (Poole, Dorset, U.K.). Trypsin, soya-bean trypsin inhibitor and all enzyme substrates and cofactors were obtained as previously described (Beardmore *et al.*, 1987). $Na^{125}I$ (carrier free), [2-^{3}H]AMP and UDP-D-[6-^{3}H]galactose were obtained from Amersham International, Amersham, Bucks., U.K.

The I3/2.3 monoclonal antibody (Trowbridge, 1978) against the T 200 trans-membrane glycoprotein used in this study was a gift from Dr. I. S. Trowbridge, and the mouse T lymphoma BW 5147 (Hyman & Stalling, 1974) was kindly provided by Dr. R. Hyman (both from the Department of Cancer Biology, The Salk Institute for Biological Studies, San Diego, CA 92138, U.S.A.). The antibody was purified to homogeneity (according to SDS/polyacrylamide-gel electrophoresis) on Protein A (MAPS; Bio-Rad).

Human LDL were isolated from blood serum (kindly provided by the North London Transfusion Centre) by sequential flotation (Havel *et al.*, 1955) and showed only one band on SDS gels. LDL concentrations are deduced from protein determinations.

Synthesis of transferrin, LDL and T 200 antibody derivatives

Both transferrin and the rat IgG I3/2.3 (anti-T 200) were iodinated by the chloramine-T method (Beardmore *et al.*, 1987) to a specific radioactivity of 7 μCi/μg, whereas LDL were iodinated by the iodochloride method (Contreras *et al.*, 1983) to a specific radioactivity of 1.8–3 μCi/mg. Transferrin was conjugated to fluorescein or rhodamine by using their isothiocyanate derivative coated on Celite (van Renswoude *et al.*, 1982), except that transferrin was allowed to bind only four fluorescent labels per molecule. For the labelling of transferrin with dansyl chloride, we have modified a previously described method (Jacobsen & Jacobsen, 1979). Transferrin (15 mg/ml) was dissolved in 0.1 M-sodium phosphate buffer, pH 7.4, and 0.03 vol. of a 700 mM-dansyl chloride solution made in dimethyl sulphoxide/acetone (1:1, v/v) was added to the transferrin solution while stirring. After 2 h at room temperature the mixture was dialysed for 2 days at 4 °C against phosphate buffered saline (PBS: 137 mM-NaCl, 2.7 mM-KCl, 2.6 mM-KH_2PO_4, 8.6 mM-Na_2HPO_4, pH 7.4) with several changes, and dansyl chloride that had not reacted was removed by centrifugation. The final conjugate was found to contain 2–3 dansyl molecules per transferrin molecule.

The synthesis of the transferrin–HRP complex was performed as described previously (Hopkins, 1983*b*).

Manufacture and handling of ricin–gold complexes

Colloidal gold (10 nm diameter) was produced by the tannic acid method (Slot & Gueuze, 1985), and conjugated to ricin at pH 7 (van Deurs *et al.*, 1985). Usually 100 ml of gold was conjugated to about 1.8 mg of ricin and resuspended after washes in 3–5 ml of PBS.

Cell culture

BW 5147 cells were maintained at (0.5–3) $\times 10^6$ cells/ml in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated horse serum in a 5%-CO_2 atmosphere, and were washed three times with PBS before use.

Binding studies

Cells [(2–3) $\times 10^7$/ml] were labelled with ^{125}I-transferrin (about 10 nM) in DMEM (2–5 ml) supplemented with 100 μg (or 500 μg for LDL-binding experiments) of bovine serum albumin/ml (DMEM/BSA) at 37 °C for the indicated period of time. To determine total cell-associated radioactivity, 200 μl was then loaded on a cushion of dibutyl phthalate (750 μl) and centrifuged for 20 s at top speed in an Eppendorf Minifuge. The bottom of the tube was cut off and counted for radioactivity. To determine the amount of labelling of the plasma membrane, cells were diluted in 1.25 ml of ice-cold PBS, then washed twice and resuspended in 0.5 ml of 0.3% Pronase in PBS (Dautry-Varsat *et al.*, 1983). After 1 h at 4 °C, cells were pelleted as described above. The Pronase-sensitive radioactivity present in the supernatant originated from the plasma membrane (see the Results section), whereas Pronase-insensitive radioactivity was taken to represent intracellular labelling.

Endosome isolation procedure

After labelling with a transferrin derivative and with LDL (150 μg/ml) in DMEM/BSA (20–25 ml) for 30 min at 37 °C, the excess ligand was washed away with cold DMEM/BSA, then with DMEM, and the cells (typically 5×10^8) were incubated for 1 h on ice with gold–ricin (about 10 μl of complex/10^7 cells, a ratio too low to induce significant cell aggregation) in DMEM/BSA (about 5 ml). After this time, binding of the complex reached a plateau (results not shown). The rest of the procedure was performed at 4 °C except when otherwise indicated. After washing twice with DMEM/BSA, then with PBS (15 ml each), and finally with 20 ml of a hypo-osmotic buffer (Goldberg & Kornfeld, 1983) containing 15 mM-KCl, 1.5 mM-magnesium acetate, 1 mM-dithiothreitol and 10 mM-Hepes, pH 7.5, the cells were resuspended in this buffer at a density of about 5×10^7 cells/ml and placed in a nitrogen bomb for 20 min at 750 lb/in^2 (5.17 MPa). We found that this previously described procedure (Goldberg & Kornfeld, 1983) did not achieve a lysis higher than 30% (according to Trypan Blue exclusion), so we added a Dounce-homogenizer treatment (20 strokes) after the cavitation. This produced more than 90% lysis without significant enhancement of nuclear disruption (3–4%, according to DAPI binding). Finally, 0.1 vol. of hyperosmotic buffer (700 mM-KCl, 40 mM-magnesium acetate, 1 mM-dithiothreitol, 10 mM-Hepes, pH 7.5) was added (Goldberg & Kornfeld, 1983), and unbroken cells and nuclei were removed by centrifuging for 5 min at 800 ***g***. After washing the nuclear pellet with 2–5 ml of a buffer containing 85 mM-KCl, 5.5 mM-magnesium acetate, 1 mM-dithiothreitol and 10 mM-Hepes, pH 7.5, the post-nuclear supernatant (PNS) was subjected to a mild trypsin treatment (1μg/ml, for 3 min at 37 °C, stopped with 1.5 μg of soya-bean trypsin inhibitor/ml) and then centrifuged for 20 min at 145000 ***g***. The resulting crude membrane pellet was resuspended in 0.5–1 ml of homogenization buffer (0.25 M-sucrose/1 mM-EDTA/10 mM-Tris, pH 8) and loaded on a discontinuous sucrose gradient (Johnson & Bourne, 1977) made of 0.5 ml of 2.5 M-sucrose in water, then 4 ml of 40%, 4 ml of 30% and 3 ml of 20% sucrose in gradient buffer (1 mM-EDTA/10 mM-acetic acid/10 mM-Tris, pH 7.5). After centrifugation for 2 h at 100000 ***g*** in an SW 40 Beckman rotor, 1 ml fractions were collected (and are numbered in the Figures) from the bottom of the tube.

Measurement of nuclear lysis

This assay relies on the ability of the (non-fluorescent molecule) DAPI to bind to double-stranded DNA, thus emitting a strong fluorescent signal (Russel *et al.*, 1975). The reaction mixture included 15 μl of 0.1% Triton X-100 and 3 ml of buffer (100 mM-NaCl/10 mM-EDTA/10 mM-Tris, pH 7) containing 100 ng of DAPI/ml. Excitation was set at 360 nm and emission at 450 nm. Samples (15 μl) of cellular fractions were added five times, and the mean fluorescence increase was calculated.

Marker enzyme assays

Acid phosphatase, β-*N*-acetylglucosaminidase, 5′-nucleotidase, NADH–cytochrome *c* reductase, galactosyltransferase and succinate–INT reductase assays were performed as described previously (Beardmore *et al.*, 1987)

Endosome-fusion assay

When the fusion of endosomes with plasma-membrane vesicles was studied, the latter were prepared by the centrifugation for 20 min at 145000 ***g*** of the (trypsin-treated) PNS from cells (typically 5×10^8) incubated with rhodamine-transferrin (Rh-Tf) (60 μM) for 30 min at 4 °C, and used without further purification, since transferrin binding at 4 °C is restricted to the plasma membrane (see the Results section). Endosomes for fusion were obtained by labelling two separate batches of cells (5×10^8 in 20 ml of DMEM/BSA) for 30 min at 37 °C, one with dansyl-transferrin (Dns-Tf) at 60 μM and LDL (150 μg/ml) and another one with Rh-Tf at 30 μM and LDL (150 μg/ml). Usually the ricin–gold step was omitted for these experiments (see the Results section). After homogenization, fractions 7–10 of the gradient were pooled, diluted 1:1 with gradient buffer and spun for 30 min at 160000 ***g***. This endosome preparation was resuspended in 0.5–1.5 ml of fusion buffer (110 mM-KCl/15 mM-$MgCl_2$/20 mM-Pipes, pH 7.1), and samples (40 μl) from the two endosome preparations were mixed. Complete fusion medium also contained 100 μl of cytosol (145000 ***g*** supernatant) and 20 μl of an ATP solution (0.1 M-ATP, 0.05 M-$MgCl_2$ in fusion buffer). An ATP-regenerating system was not necessary for the brief incubation periods that we used (results not shown). After various periods of time at 37 °C, 1.25 ml of ice-cold fusion buffer was added, together with 60 μl of a 20% (w/v) formaldehyde solution in PBS. The formaldehyde addition prevents further fusion (see the Results section) and enhances the reproducibility of fluorescence measurements. The latter were performed as described below to determine the efficiency of the transfer, and thus the endosome fusion rate (Fernandez & Berlin, 1976).

Fluorescence measurements (for pH and endosome-fusion assays)

Fluorescein–transferrin fluorescence was used for pH measurements (Ohkuma & Poole, 1978; Merion *et al.*, 1983; Galloway *et al.*, 1983), after labelling the cells as described for Dns-Tf, and using suitable blanks and standards. Excitation and emission were at 450 or 495 nm and 525 nm respectively. For acidification experiments, fluorescein–transferrin-labelled isolated endosomes were incubated for 14 h at 4 °C in fusion buffer (supplemented with 1μg of antipain/ml and 1 μg of aprotinin/ml) to deplete ATP stores and equilibrate the endosome at the buffer pH value. ATP and $MgCl_2$ were added at respectively 5 mM and 20 mM final concns. and the fluorescence was recorded at 525 nm with excitation at 495 nm.

Rh-Tf and Dns-Tf were quantified by using respectively 550 or 335 nm for excitation and 592 or 530 nm for emission. The efficiency of transfer in the RET assay was monitored by using the ratio of 592 nm/530 nm emission (with 335 nm excitation), as previously detailed (Fernandez & Berlin, 1976).

Electron-microscopy processing

After fixation in Karnovsky (1965) fluid, and post-fixation in 1% OsO_4, samples were dehydrated in ethanol, transferred through propylene oxide and embedded in Epon. This procedure does not preserve LDL structure. Samples labelled with HRP–transferrin were treated as described by Hopkins (1983*b*) to demonstrate HRP

activity. Sections were examined after staining with uranyl acetate and lead citrate in a Philips CM12 electron microscope at 80 keV (Beardmore *et al.*, 1987).

Protein determination

Proteins were assayed by the Hartree (1972) method, with BSA as standard.

Statistics

Experiments were performed at least twice (in duplicate), only results of a representative experiment being presented in graphs. Errors are expressed as S.E.M. and statistical differences were studied by Student's *t*-test.

RESULTS

Markers of subcellular compartments

As yet no endosome-specific marker (membrane antigen or enzyme) is available. We have therefore adopted the approach used in several previous studies and monitored endosome purification by using internalized radiolabelled transferrin (Schmid *et al.*, 1988; van Renswoude *et al.*, 1982).

We first assessed the validity of transferrin as an endosome marker in BW 5147 cells, using standard binding experiments in which surface-bound transferrin was digested with Pronase (Dautry-Varsat *et al.*, 1983). The validity of the Pronase treatment to remove all the surface-bound transferrin on BW 5147 cells is shown in Fig. 1, where all the cell-associated ^{125}I-transferrin is Pronase-sensitive for short incubation times (<2 min). We used a 30 min incubation at 37 °C to allow for equilibrium labelling, and at this time more than 70 % of the ^{125}I-transferrin is inside the cell. This result is similar to a previous report on lymphocytes (Vidal *et al.*, 1986). It was confirmed by fluorescence microscopy of cells that had internalized Rh-Tf and by experiments in which gradient analysis of the membrane fraction of cells treated with ricin–gold complexes at 4 °C was performed (see below).

Other organelle distributions were monitored by using conventional enzyme assays for mitochondria (succinate–INT reductase), lysosomes (acid phosphatase and β-*N*-acetylglucosaminidase), Golgi apparatus (galactosyltransferase), endoplasmic reticulum (NADH–cytochrome *c* reductase) and plasma membrane (5′-nucleotidase) (Beardmore *et al.*, 1987). In addition to 5′-nucleotidase activity, we used an additional independent plasma-membrane marker: T 200, a transmembrane glycoprotein (Omary & Trowbridge, 1980). This protein is thought to be bound to the cytoskeleton (Bourguignon *et al.*, 1985) and is thus unlikely to be internalized. In separate studies using a monoclonal antibody against T 200 (I3/2.3), we confirmed this view by showing that this molecule remains restricted to the plasma membrane during prolonged incubations at 37 °C (results not shown).

Optimization of the endosome isolation

The discontinuous gradient used in this study has been extensively used (Allan & Crumpton, 1970; Johnson & Bourne, 1977; Hoessli & Rungger-Brändle, 1983; Bourguignon *et al.*, 1985; Walker *et al.*, 1989) for the isolation of the plasma membrane of lymphocytes. We show here that the fractions described in these studies as plasma membrane (i.e. interfaces 40/30 % and/or 30/20 % sucrose and 20/10 % sucrose) are significantly contaminated by endosomal elements. Thus the ^{125}I-transferrin distribution profile (after a 30 min labelling at 37 °C) in this gradient displays (Fig. 2) a large labelling of fractions 4/5, 8/9 and 12 (respectively interfaces 40/30, 30/20 and 20/10 % sucrose). A similar distribution profile of ^{125}I-transferrin was observed (*a*) after incubation for 1 h at 4 °C to label only the plasma membrane or (*b*) after protease digestion of the label bound to the plasma membrane to leave only the label present in endosomes (results not shown). Since the labelling of the cells with ^{125}I-labelled anti-T 200 at 4 °C also gave a similar ^{125}I distribution on the gradient (see Fig. 2), these experiments show that plasma membrane and endosomes display similar distribution on this gradient.

As reported previously (Johnson & Bourne, 1977) (but not shown there) and displayed in Fig. 3 (electron microscopy not illustrated), the other organelles, es-

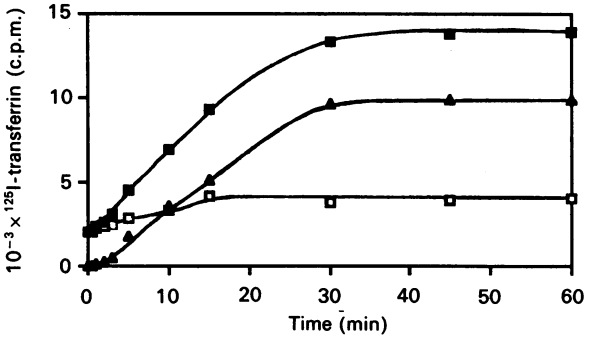

Fig. 1. ^{125}I-transferrin uptake by BW 5147 cells at 37 °C

Cells were allowed to take up ^{125}I-transferrin in DMEM/BSA at 37 °C, then plasma-membrane-bound and internalized ^{125}I-transferrin were determined as described in the Materials and methods section. (□) Plasma-membrane, (▲) intracellular, and (■) total cell-associated ^{125}I-transferrin.

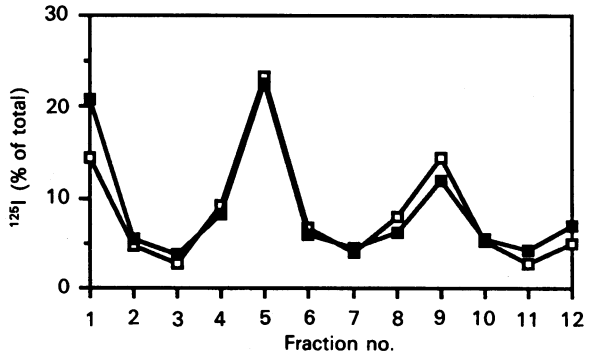

Fig. 2. Distributions of ^{125}I-transferrin (■) and ^{125}I-labelled anti-T 200 (□) on a discontinuous sucrose gradient

BW 5147 cells were labelled for 30 min either with ^{125}I-transferrin at 37 °C or with ^{125}I-labelled anti-T 200 at 4 °C. Excess label was washed away, the cells were lysed and the gradient was prepared as described in the Materials and methods section (but without any trypsin treatment of the PNS). Fraction 1, bottom of the tube.

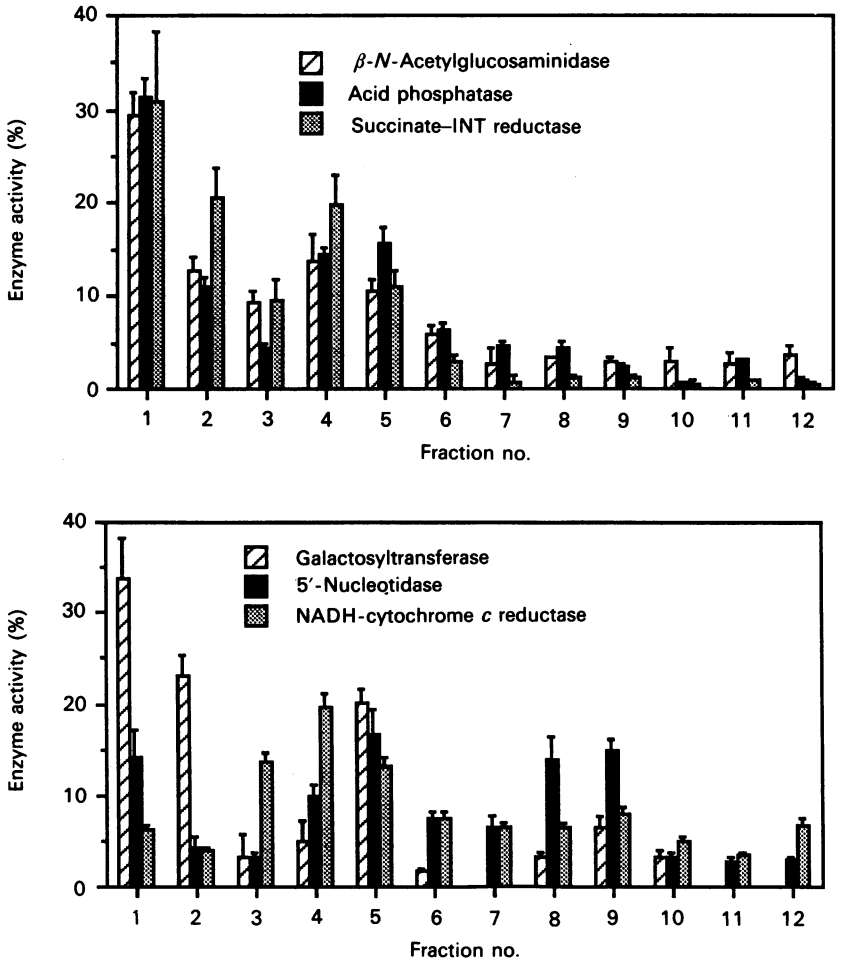

Fig. 3. Distribution of marker enzymes of BW 5147 cells on the sucrose gradient

Untreated cells were homogenized and the gradient was prepared (without trypsin treatment of the PNS) as indicated in the Materials and methods section. Results are expressed as means ± S.E.M., as percentages of the total activity present on the gradient (more than 95% of each enzyme activity loaded on the gradient was recovered in the fractions). Fraction 1, bottom of the tube.

pecially lysosomes and mitochondria, are mainly located in fraction 1 on this gradient (at the bottom of the 40%-sucrose layer), whereas the Golgi and the endoplasmic reticulum are present to some extent (respectively 9 and 14% of the total gradient activity) in fractions 8/9. 5′-Nucleotidase displays a distribution similar to that of T 200 and transferrin.

From these experiments we conclude that fractions 8/9 consist primarily of endosomes with plasma membrane as the major contaminant. We therefore attempted three manipulations to improve the yield and purity of the endosomes in these fractions. Except when otherwise indicated, the distribution of the marker enzymes of the other organelles was unaltered by these manipulations.

The first consisted of a mild trypsin treatment of the PNS, as suggested by previous work (Beardmore *et al.*, 1987). The advantages and potential hazards of this mild proteolysis on endosome integrity had been discussed previously (Beardmore *et al.*, 1987; Marsh *et al.*, 1987; Howell *et al.*, 1989). Fig. 4(*a*) shows that the effect of this trypsin treatment (for 3 min at 37 °C) in enhancing endosome recovery (monitored with ^{125}I-transferrin) in fractions 8/9 is saturated at a trypsin concentration of 1 μg/ml. We used this treatment in all of our subsequent fractionation experiments. Routinely we found that the ^{125}I-transferrin shifted by the trypsin treatment to fractions 8/9 came primarily from more dense fractions of the gradient, mainly fraction 1. This is consistent with the interpretation that microfilaments trap endosomes (in the fraction where the bulk of organelles is present) and that mild trypsin treatment hydrolyses the microfilaments. The distribution of the plasma membrane (monitored with ^{125}I-labelled anti-T 200) was not altered by the trypsin treatment, as shown in Fig. 4(*b*).

The second treatment that increased ^{125}I-transferrin recovery in fractions 8/9 was incubation of the cells with LDL together with the ^{125}I-transferrin (for 30 min at 37 °C). The aim of this treatment was to load endosomes with LDL, thereby decreasing their buoyant density. Fig. 5 shows that this effect is significant and saturated at an LDL concentration of 100 μg/ml. This corresponds to a saturating concentration of more than 5-fold the K_d of the BW 5147 cells for LDL, i.e. 84 nM or 18.5 μg/ml, for 10^7 binding sites per cell (see the Scatchard graph in the insert of Fig. 5). We have used 150 μg of LDL/ml during all our fractionation experiments. Fig. 5 also

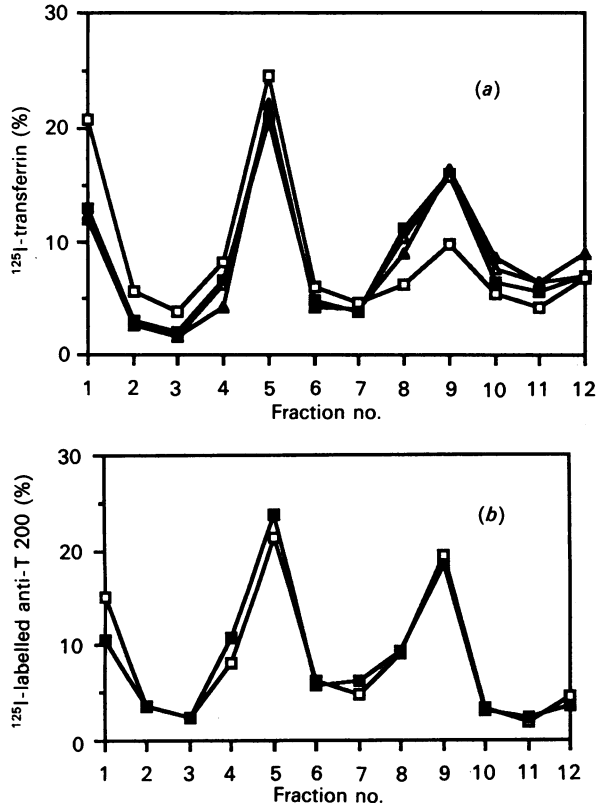

Fig. 4. Effect of trypsin treatment of the PNS on the distributions of ^{125}I-transferrin (*a*) and ^{125}I-labelled anti-T 200 (*b*) on the sucrose gradient

After incubation for 30 min at 37 °C with ^{125}I-transferrin (or with ^{125}I-labelled anti-T 200 for 30 min at 4 °C), BW 5147 cells were lysed and the PNS was treated for 3 min at 37 °C with the indicated trypsin concentration: □, untreated control, ■, 1 μg/ml; ▲, 5 μg/ml; △, 10 μg/ml. After stopping proteolysis with excess soyabean trypsin inhibitor, the gradient was prepared as described in the Materials and methods section. Results are expressed as percentages of the total radioactivity on the gradient.

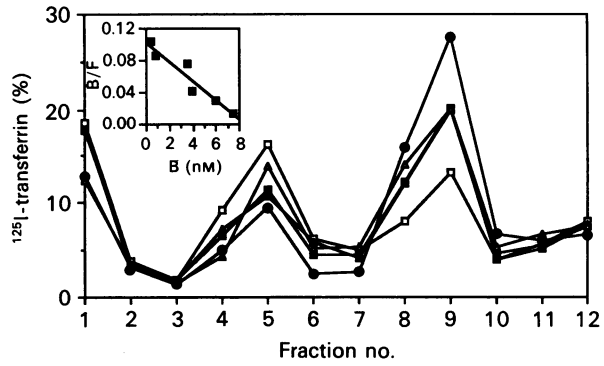

Fig. 5. Effect of incubation of the cells with LDL on the ^{125}I-transferrin distribution on the sucrose gradient: cumulative effects with the trypsin treatment of the PNS

BW 1547 cells were incubated for 30 min with ^{125}I-transferrin, and LDL at the indicated concentration, and lysed with or without trypsin treatment (1 μg of trypsin/ml for 3 min at 37 °C) of the PNS. The gradient was prepared as described in the Materials and methods section. Results are expressed as percentages of the total radioactivity on the gradient. Symbols: □, untreated control; ▲, 100 μg of LDL/ml only; ■, 500 μg of LDL/ml only; △, PNS trypsin treatment only; ●, 500 μg of LDL/ml and PNS trypsin treatment. Inset: Scatchard analysis of ^{125}I-LDL binding (performed for 20 min at 4 °C, the time after which labelling equilibrium was reached).

shows that LDL and trypsin effects are additive and together give a final ^{125}I-transferrin recovery in fractions 8/9 of more than 35% of ^{125}I-transferrin present in the gradient (30% of total cell labelling or 42% of internalized ^{125}I-transferrin). Similar results were obtained with ^{125}I-LDL (results not shown).

The ^{125}I-transferrin shifted by the LDL treatment is seen mainly in relation to fraction 4. Only minor differences are observed in fractions 1 and 12. The distribution of ^{125}I-labelled anti-T 200 was not significantly altered by the LDL treatment (results not shown).

Although the endosome content of fractions 8/9 was increased by 100% by the LDL and trypsin treatments, the distribution of ^{125}I-labelled anti-T 200 indicated that these fractions still contained 15–20% of the plasma membrane present on the gradient (Fig. 2). We therefore introduced a pre-fractionation step to remove plasma membrane from these fractions.

We show in Fig. 6(*a*) that the whole plasma membrane can be density-shifted toward the bottom of the gradient by incubating the cells with gold–ricin for 1 h at 4 °C. Ricin–gold was especially effective in producing this

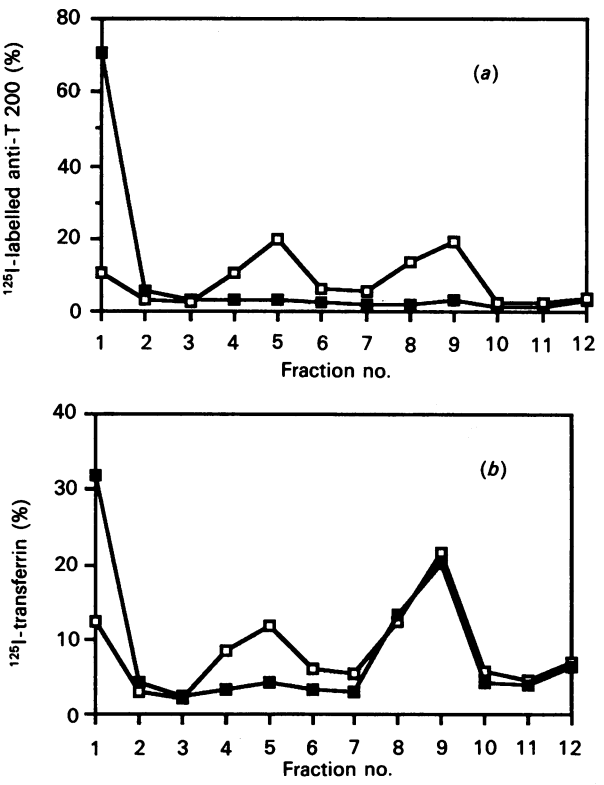

Fig. 6. Effect of incubation of the cells with ricin–gold at 4 °C on the distributions of ^{125}I-transferrins (*b*) and ^{125}I-labelled anti-T 200 (*a*) on the sucrose gradient

BW 5147 cells were incubated with 150 μg of LDL/ml (*b*, and with ^{125}I-transferrin) for 30 min at 37 °C, and after washing, with (■) or without (□) ricin–gold complex for 1 h at 4 °C (*a*, and with ^{125}I-labelled anti-T 200), as described in the Materials and methods section.

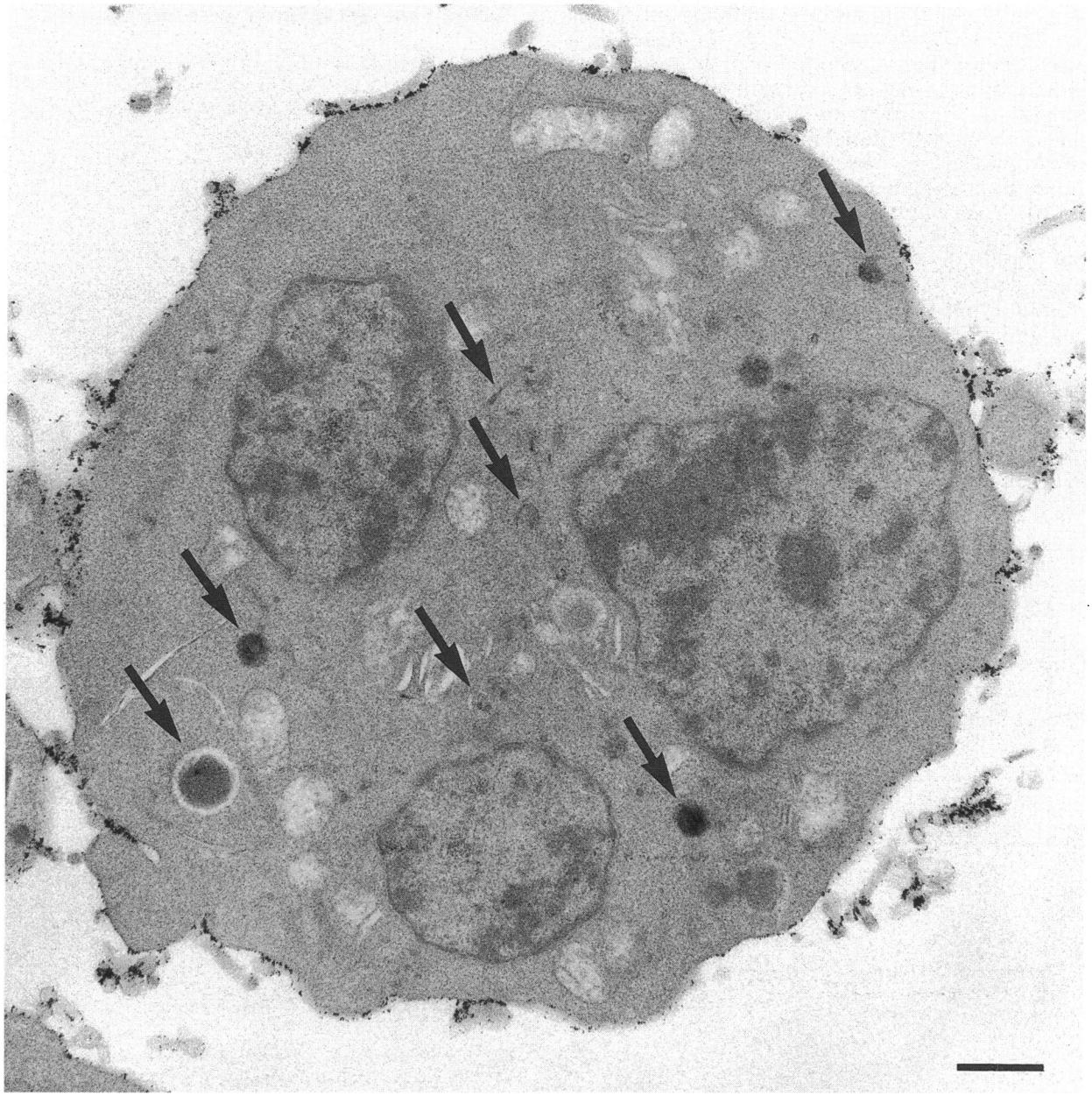

Fig. 7. Electron micrograph of BW 5147 cells after labelling with HRP–transferrin at 37 °C and ricin–gold at 4 °C

BW 5147 cells were labelled with HRP–transferrin for 30 min at 37 °C and, after washing, were incubated with the ricin–gold complex for 1 h at 4 °C. They were then washed, fixed and processed for electron microscopy as described in the Materials and methods section. In these conditions, the gold (–ricin) is bound only to the plasma membrane, whereas HRP (–transferrin) products can be clearly seen in several endocytic elements (arrows). Bar = 1 μm.

shift: neither gold–concanavalin A nor a gold complex prepared from a monoclonal antibody (R17.217; Lesley *et al.*, 1984) directed toward the mouse transferrin receptor achieved a very significant redistribution (results not shown). Ricin–gold pretreatment removes the contamination of endosomes by the plasma membrane in fractions 8/9 without altering their recovery. On the other hand, this pretreatment shifts (Fig. 6*b*) 30 % of the ^{125}I-transferrin (an amount equivalent to the proportion bound to the plasma membrane; see Fig. 1) towards the bottom of the gradient. The viability of the cells was not altered by the incubation at 4 °C with ricin–gold, which, as shown by electron microscopy, remains bound to the plasma membrane at this temperature (Fig. 7).

Initial experiments using ^{125}I-transferrin showed that surface receptors labelled at 4 °C are internalized when the cells are warmed to 37 °C. Morphological studies using transferrin–peroxidase showed that, upon warming, at early time points the receptor is in the peripheral endosomes, but later it is concentrated in elements in the Golgi area. This is displayed in Fig. 7, which shows, in addition to the gold bound on the plasma membrane, that the transferrin–peroxidase label is found only in peripheral and juxtanuclear endosomes.

To show that the ^{125}I-transferrin in fractions 8/9 is present only in endosomes and does not represent a plasma-membrane-bound transferrin fraction, which would be resistant to the ricin–gold shift, we followed the movements of ^{125}I-transferrin initially bound to the plasma membrane at 4 °C (and thus recovered in fraction 1 after the gradient) into fractions 8/9 after warming at 37 °C and thus inducing internalization. A typical result of this experiment is shown in Fig. 8. As seen in Fig. 8(*a*) after binding at 4 °C the ^{125}I-transferrin is recovered in fraction 1, as for the ^{125}I-labelled anti-T 200 (see Fig. 6*a*). We conclude that the radiolabel is bound primarily to the plasma membrane. With warming to 37 °C, the amount of ^{125}I-transferrin in the endosome region (fractions 8/9) increases from 2 to about 20%, as displayed in Fig. 8(*b*). The distribution of the ^{125}I-labelled anti-T 200 remained unchanged (results not shown).

The final yield and purity of the endosome preparation obtained from these fractions are displayed in Table 1. It is noteworthy that with this lysis procedure, although only less than 15% of mitochondria, endosome and plasma-membrane markers are lost in the nuclear pellet, significant amounts of endoplasmic reticulum (60%), lysosomes (70%) and especially Golgi apparatus (75%) are sedimented at this stage. These data were used to transform the distributions on the gradient in final yields.

Electron micrograph of fraction 9 (Fig. 9) shows that transferrin–peroxidase-loaded endosomes are present and greatly enriched in this part of the gradient. The

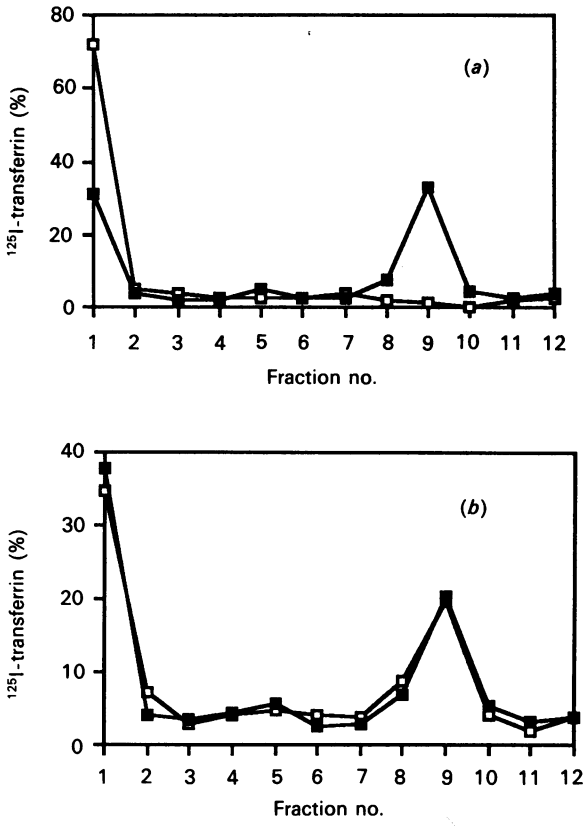

Fig. 8. Effect of the incubation time at 37 °C on the distribution on the sucrose gradient of initially plasma-membrane-bound ^{125}I-transferrin

BW 5147 cells were labelled with ^{125}I-transferrin (and LDL) for 30 min at 4 °C or 37 °C as indicated and, after washing away the excess ligand, cells labelled at 4 °C were incubated at 37 °C (with LDL) for the indicated period of time. All cells were then treated with the ricin–gold complex and lysed as described in the Materials and methods section. (*a*) ^{125}I-transferrin distribution after labelling for 30 min at (□) 4 °C or (■) 37 °C. (*b*) After labelling with ^{125}I-transferrin at 4 °C, cells were incubated at 37 °C for (□) 5 min or (■) 30 min.

Table 1. Final yield of the endosome-isolation procedure

Recovery yields are expressed as percentages (of activities for enzymes and of c.p.m. for radiolabels) of the cellular homogenate present in fractions 8 and 9 of the gradient.

	Final yield (%)
Endosomes	
^{125}I-transferrin	28–32
Plasma membrane	
^{125}I-labelled anti-T 200	2–3
5′-Nucleotidase	2–3
Lysosomes	
Acid phosphatase	1–2
β-*N*-Acetylglucosaminidase	2–3
Mitochondria	
Succinate–INT reductase	2–3
Golgi	
Galactosyltransferase	2–3
Endoplasmic reticulum	
NADH–cytochrome *c* reductase	5–6

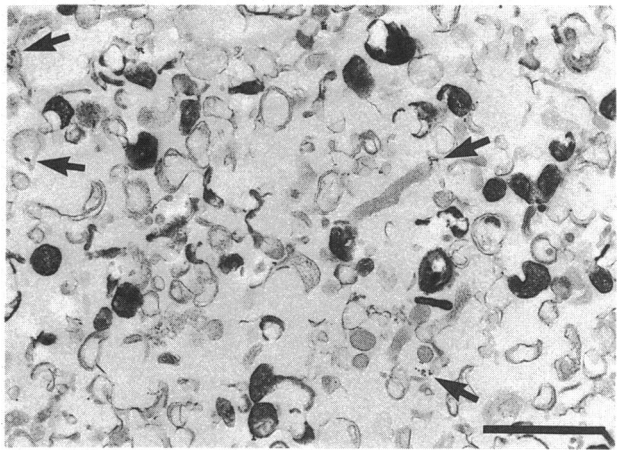

Fig. 9. Electron micrograph of endosomes prepared from HRP–transferrin-labelled BW 5147 cells

BW 5147 cells were labelled with HRP–transferrin and LDL for 30 min at 37 °C, then with ricin–gold for 1 h at 4 °C and submitted to the endosome-isolation procedure. Fraction 9 of the gradient was fixed and processed for electron microscopy as described in the Materials and methods section. This micrograph displays a representative portion of the isolated endosome preparation. The HRP reaction products identify the endosomal elements which became labelled during the incubation procedure. Arrows indicate contaminating fragments of gold-labelled plasma membrane. Bar = 1 μm.

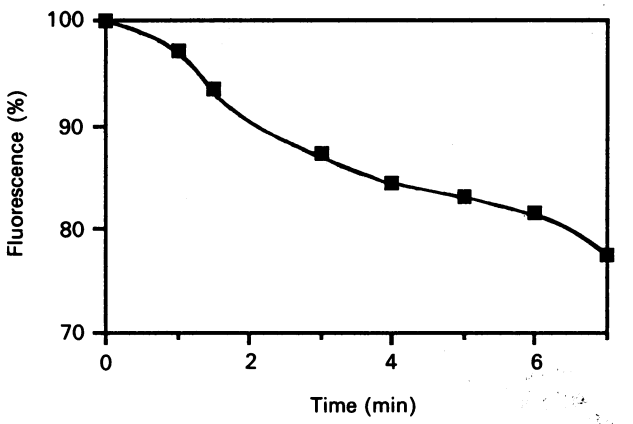

Fig. 10. Endosome ATP-dependent acidification

After labelling BW 5147 cells with fluorescein–transferrin for 30 min at 37 °C, endosomes were purified as described in the Materials and methods section and left for 14 h at 4 °C in iso-osmotic buffer (pH 7.1) to deplete ATP stores and dissipate the pH gradient. Then 5 mM-ATP and 5 mM-$MgCl_2$ were added, and the fluorescence was recorded at 525 nm. Excitation was set at 495 nm.

heterogeneity of endosome morphology is similar to that observed in intact cells (Helenius *et al.*, 1983; Hopkins, 1983*a*,*b*; Pastan & Willingham, 1983; Hopkins & Trowbridge, 1983; Willingham *et al.*, 1984).

Using transferrin–fluorescein as a probe, and in the presence of ATP, we found a pH of 5.2–5.4 for these isolated endosomes from BW 5147 cells. This is in the range of the values reported previously (Merion *et al.*, 1983; van Renswoude *et al.*, 1982). On addition of ATP, endosome acidification occurred at a rate similar to that reported for other cells (Fuchs *et al.*, 1989; Merion *et al.*, 1983; Galloway *et al.*, 1983), as shown in Fig. 10.

Endosome-fusion assay

To examine the suitability of the isolated endosomes for cell-free studies, we developed a method with which to study their ability to fuse selectively. The RET assay for endosome fusion is based on the ability of an excitated fluorophore (the donor) to transfer its excitation to another fluorophore (the acceptor) if (1) the emission spectrum of the donor largely overlaps the excitation spectrum of the acceptor and (2) the two fluorescent molecules are sufficiently close (< 10 nm). Applications of RET have been reviewed by Stryer (1978). In our case the donor is Dns-Tf and the acceptor is Rh-Tf. Hence, by mixing a preparation of endosomes prepared from cells labelled with Dns-Tf and another from Rh-Tf-labelled cells, fusion should bring the fluorophores together. If the dansyl group is excited, the RET induced by the fusion will cause a decrease in Dns-Tf emission (530 nm) and an increase in Rh-Tf emission (592 nm). The emission ratio 592 nm/530 nm thus provides a simple and sensitive way of monitoring endosome fusion (Fernandez & Berlin, 1976).

Using the protocol described in the Materials and methods section, we verified that the fusion of endosomes purified from BW 5147 cells was linear with acceptor concentration and time, as shown in Figs. 11(*a*) and 11(*b*) respectively.

To evaluate the proportion of vesicles fusing during

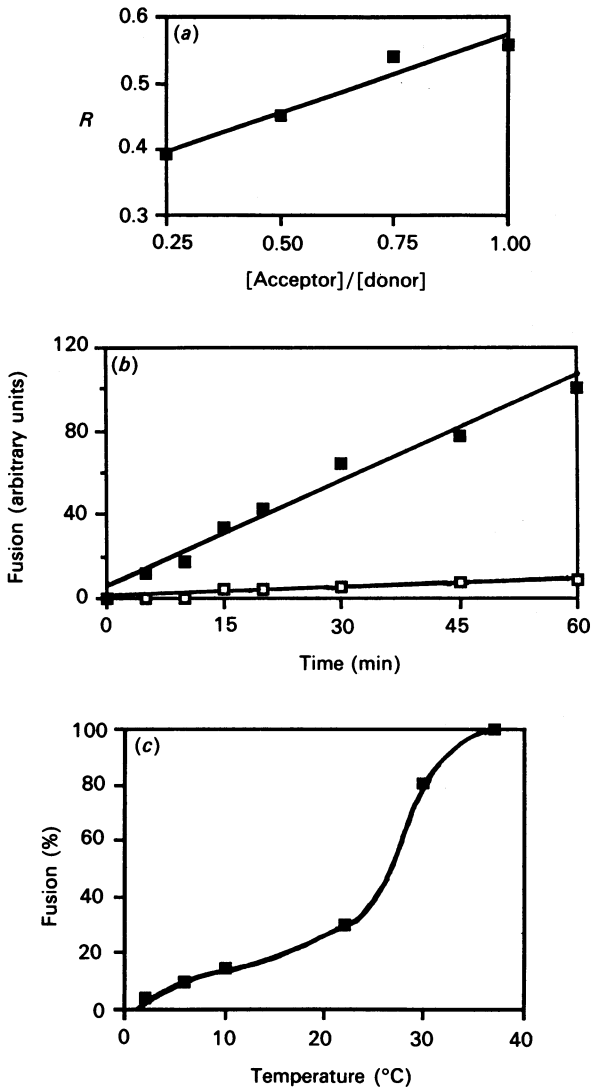

Fig. 11. Linearity of the endosome-fusion rate as a function of acceptor concentration and time: temperature-dependence of the fusion

Endosomes were purified and fusion was assayed as described in the Materials and methods section (except that no ricin–gold treatment was used for endosome isolation). When indicated, membranes prepared from cells labelled with Rh-Tf at 4 °C were used as acceptor. In this case results were normalized to the same number of starting cells and Rh-Tf concentration. *R*, emission ratio 592 nm/530 nm, with excitation at 335 nm. (*a*) Linearity of the endosome-fusion assay as a function of the concentration of Rh-Tf (acceptor). (*b*) Time dependence of fusion of Dns-Tf-labelled endosomes with Rh-Tf-labelled purified endosomes (■) or membranes (□). (*c*) Temperature-dependence of endosome fusion.

the time of the assay, we performed experiments in which cells were preloaded with a mixture of Dns-Tf and Rh-Tf. This procedure simulates a 100% fusion, and a more than 130% increase in *R* value was observed compared with controls. These results show that about 30% of the vesicles have fused after the 45 min incubation used in the fusion assay. This value is in agreement with previous reports (Diaz *et al.*, 1988).

To simplify the procedure, we did not routinely use the

ricin–gold treatment for preparation of endosomes for the fusion assay. In preliminary studies we evaluated the participation of the plasma-membrane-bound transferrin (about 15% with the trypsin and LDL treatment, but without the ricin–gold treatment) in the purified endosome preparations (fractions 7–10 of the gradient) in generating the RET fusion signal. This was performed by binding the acceptor (Rh-Tf) to the plasma membrane (at 4 °C) and testing its ability to fuse with isolated Dns-Tf-containing endosomes. As shown in Fig. 11(*b*), fusion does occur (linearly with time) between endosomes and plasma-membrane vesicles. However, this fusion rate was less than 7% of that observed in endosome–endosome fusion (where an identical number of cells and acceptor concentration were used). Hence, less than 1% of the RET signal observed during endosome-fusion experiments is likely to be due to plasma-membrane contamination of the endosome preparation. The ricin–gold treatment was thus considered unnecessary for the purification of endosomes in fusion experiments.

Fusion between plasma-membrane vesicles and endosomes is low, but it is both ATP- and cytosol-dependent (results not shown). It is to be expected that cell homogenization yields both right-side-out and inside-out vesicles (Mayorga *et al.*, 1988; Howell *et al.*, 1989), and only the fusion of the latter with endosomes is expected in this assay. The actual rate of fusion between endosomes and plasma-membrane vesicles may therefore be higher than that detected in our assay if plasma-membrane right-side-out vesicles are fusion-competent.

Fig. 11(*c*) shows that the endosome fusion is negligible below 10 °C, as reported previously (Diaz *et al.*, 1988). Previous endosome-fusion studies (Braell, 1987; Diaz *et al.*, 1988) reported that fusion is greatest between early endosomes, and that fusion ability decreases as a function of time as the endocytosed probe moves deeper into the endocytic pathway. We have carried out a similar experiment, the results of which are presented in Table 2. When only early endosomes are labelled (for 6 min at 37 °C) they readily fuse. If early endosomes are allowed to fuse with (*a*) late endosomes (labelled with a 30 min incubation followed by a 10 min chase), fusion is decreased by 60%, or (*b*) plasma-membrane vesicles, fusion is decreased to 8%. The degree of fusion that we observed between early and late endosomes is similar to that reported by using a similar chase period for labelling late-endocytic vesicles (Braell, 1987; Diaz *et al.*, 1988). It should be noted that, in order to obtain high levels of labelling, all of our experiments use a labelling protocol (described in the Materials and methods section) which introduces the probes into both early and late endosomes (see Fig. 1).

To determine the requirements of endosome fusion in this cell-free system, we have tested the effect of ATP and cytosol. As shown in Table 3, some fusion occurs after 45 min between purified endosomes in the control. However, this baseline value is greatly enhanced with the addition of ATP, and a further additional increment is seen with cytosol addition, indicating that the cytosolic fusion-enhancing factor is not ATP alone. This is supported by the effects of NEM and of trypsin-treated cytosol (shown in Table 4). These treatments completely (trypsin) or largely (NEM) inhibit fusion. The cytosol cannot be replaced by an exogenous protein (BSA)

Table 2. Specificity of endosome fusion

Early endosomes were loaded with Dns-Tf for 6 min at 37 °C, endosomes were prepared as described in Fig. 11 and allowed to fuse with (1) Rh-Tf-loaded early endosomes or (2) endosomes labelled with Rh-Tf for 30 min at 37 °C (total endosomes) or (3) late endosomes in which Rh-Tf was loaded by an incubation for 30 min at 37 °C, followed by a chase of 10 min with 1 mg of transferrin/ml, or (4) crude membranes prepared from cells to which Rh-Tf was bound at 4 °C for 30 min to label the plasma membrane. Data are means ± S.E.M. ($n = 4$).

Acceptor	Fusion (arbitrary units)
(1) Early endosomes	100 ± 12
(2) Total endosomes	50 ± 6
(3) Late endosomes	39 ± 5
(4) Plasma membrane	7 ± 5

Table 3. Effect of ATP and cytosol addition on the endosome-fusion rate

Fusion assay was performed as described in the Materials and methods section for 45 min, but ATP and cytosol were omitted and the indicated additions were made. Control value (tubes kept on ice) of *R* (ratio emission at 592 nm/emission at 530 nm) was 0.34 ± 0.01. Data are means ± S.E.M. ($n = 3$).

Addition	*R*
None	0.38 ± 0.02
ATP	0.44 ± 0.02
Cytosol	0.47 ± 0.03
ATP + cytosol	0.57 ± 0.03

Table 4. Effect of cytosol or endosome pretreatments on the rate of fusion

Trypsin treatment of the cytosol was performed for 1 h at 37 °C with 1 mg of trypsin/ml. Endosome trypsin treatment was done similarly, but at 0 °C. Proteolysis was stopped by addition of excess soyabean trypsin inhibitor. NEM treatment was performed for 30 min at 0 °C with a final NEM concentration of 1 mM, then NEM was quenched by adding 2 mM-dithiothreitol. BSA was prepared at 10 mg/ml in fusion buffer. Values were corrected for non-cytosol-dependent fusion.

	Fusion efficiency (% of control)
Cytosol pretreatment	
56 °C, 1 h	85–90
Freezing/thawing	90–100
Trypsin treatment	0–5
NEM	10–16
Replaced by BSA solution	0
Endosome pretreatment	
Freezing/thawing	90–100
Trypsin treatment	90–100
NEM	90–95

Table 5. Effect of pH-gradient brakers, bivalent-cation chelators, formaldehyde, NEM and nucleotide addition on the rate of endosome fusion

Each chemical was added at the indicated concentration to the complete assay system, except when nucleotides were tested, in which case ATP addition was omitted. Values were corrected for non-ATP-dependent fusion.

Addition	Fusion efficiency (% of control)
Formaldehyde* (1 %, w/v)	0
Formaldehyde† (1 %, w/v)	100
NEM (10 mM)	0
Bivalent-cation chelator (10 mM)	
EDTA	90–100
EGTA	90–100
pH-gradient braker	
Nigericin (5 μM)	100
Chloroquine (20 μM)	80–85
NH_4Cl + methylamine (20 mM each)	100
Nucleotide (10 mM)	
AMP	10–15
ADP	55–62
ATP	100
GTP	12–19
Adenylyl imidodiphosphate	0–8

* Added at the beginning of the assay.
† Added at the end of the assay but before fluorescence measurements.

solution. On the other hand, endosome fusion ability was not sensitive to either trypsin or NEM pre-treatments, at least at 4 °C.

The mild trypsin treatment of the PNS that we used during the endosome-isolation procedure did not inhibit endosome or cytosol properties in fusion assays (results not shown). Endosome fusion was not affected by treatments which equilibrate pH (as determined by using fluorescein–transferrin; results not shown) across the endosome membrane (displayed in Table 5). This shows, as previously reported (Warren *et al.*, 1988; Diaz *et al.*, 1988), that the acidic internal endosomal pH is not required for endosome fusion. Formaldehyde does not affect the RET value if added after the assay, whereas it efficiently inhibits fusion. Formaldehyde addition was thus considered as a convenient way of terminating the fusion until fluorescence measurements were made.

Nucleotide requirement showed strict dependence of the fusion assay for adenine nucleotide, of which ATP was the most efficient. GTP was as poorly efficient as AMP. The hydrolysis of the γ-phosphate of ATP seems to be required for endosome fusion, since adenylyl imidodiphosphate did not support fusion. Finally, bivalent cations (except Mg^{2+}, which was always in excess) did not seem to be implicated in the fusion process.

DISCUSSION

In the present study our purpose was to devise a method which would allow us to isolate the intracellular pathway(s) involved in the processing of internalized immunotoxins in lymphocytes.

Studies from other cell types suggest that the endosome compartment involved in the intracellular processing includes a very diverse collection of vesicular and tubular elements (Helenius *et al.*, 1983; Hopkins, 1983*a*,*b*; Pastan & Willingham, 1983; Hopkins & Trowbridge, 1983; Willingham *et al.*, 1984) lacking a characteristic buoyant density (Marsh *et al.*, 1987; Beardmore *et al.*, 1987). Hence, conventional cell-fractionation methods cannot easily be applied for the isolation of these elements (Dickson *et al.*, 1983; Mullock *et al.*, 1983; Evans & Hardison, 1985; Wall & Hubbard, 1985; Khan *et al.*, 1986; Belcher *et al.*, 1987; Branch *et al.*, 1987; Diment *et al.*, 1988; Enrich *et al.*, 1988), and it has therefore been necessary for other approaches, such as free-flow electrophoresis (Evans & Flint, 1985; Marsh *et al.*, 1987; Schmid *et al.*, 1988) and affinity methods (Debanne *et al.*, 1984; Mueller & Hubbard, 1986; Gruenberg & Howell, 1986) to be developed (for a review see Howell *et al.*, 1989). The former of these methods has relatively low yields, whereas the latter usually requires the cells to be virally infected (Gruenberg & Howell, 1986). Density-shift methods which exploit the access provided by endocytic uptake mechanisms to load the system with exogenous labels (so that the density of the endosomes is moved significantly away from that of all other organelles) offer another approach. However, for our purposes, these too have their disadvantages. Those employing peroxidase reaction products to achieve the density shift (Quintart *et al.*, 1984) severely inactivate endosome lumen proteins (Ajioka & Kaplan, 1986), whereas those using the gold conjugates which have been developed thus far have introduced the conjugate via the transferrin receptor (Beardmore *et al.*, 1987). Unfortunately, for drug delivery this receptor is potentially one of the most exploitable of all internalizing cell-surface proteins (Lesley *et al.*, 1984). We therefore considered it important to devise a method which did not compromise the ability of this particular receptor to bind ligand.

The method that we have described uses LDL loading of the endosome and gold-conjugate binding to the plasma membrane. In uptake studies the isolated endosomes are shown to contain 30 % of the ^{125}I-transferrin initially bound to the cell, a proportion considerably higher than that of the internalized ligand reported in previous studies [6–12.4 % (Mullock *et al.*, 1983; Dickson *et al.*, 1983; Evans & Flint, 1985; Beardmore *et al.*, 1987; Marsh *et al.*, 1987)]. Our objective was to combine the speed of a step-sucrose gradient with the efficiency of density-shift methods. The endosome-isolation procedure described in this paper is the first reported for lymphocytes. Subcellular fractionation of lymphocytes has been previously described for plasma membrane (Allan & Crumpton, 1970; Ferber *et al.*, 1972; Johnson & Bourne, 1977; Hoessli & Rungger-Brändle, 1983), Golgi (Goldberg & Kornfeld, 1983) and lysosome (Harms *et al.*, 1981). However, yields are usually limited because of the high nucleus/cytoplasm volume ratio (see Fig. 7), and the danger of nuclei disruption leading to the formation of DNA-induced aggregates (Allan & Crumpton, 1970; Ferber *et al.*, 1972; Harms *et al.*, 1981). In the present study cell lysis has been optimized to give better than 90 % cell breakage while leaving nuclei mostly intact (i.e. 3 % of nuclei lysis).

The density-shift manipulations that we have used have allowed us to transform a rapid but relatively

inefficient gradient purification method into a procedure giving both high yield and good purity. The use of LDL loading has been used previously in the isolation of endosomes from rat hepatocytes (Belcher *et al.*, 1987), but it has not previously been used with cultured cells. The use of ricin–gold is a very effective means of removing plasma membrane from subcellular fractions of low density, and may be of general utility in the cell fractionation. In a very recent study (Gupta & Tartakoff, 1989) a similar approach, which used wheat-germ haemagglutinin–gold to remove plasma membrane from Golgi fractions, was also shown to be relatively effective (35–40%, versus 70% in this study).

Because endosomes are poorly characterized biochemically, it is not possible to compare directly the purity of our isolated fraction with the different preparations that have been described from other systems. With regard to the endosome preparations that have been prepared from rat liver, it is possible to say: (1) compared with conventional subfractionation, it contains significantly less lysosomal-marker acid phosphatase [1–2 versus 20% (Khan *et al.*, 1986)] and less Golgi glycosyltransferases [2–3 versus 9.5% (Evans & Flint, 1985)], and (2) compared with the peroxidase density-shift procedure, it has significantly less Golgi-marker galactosyltransferase [2–3 versus 6% (Quintart *et al.*, 1984)].

The RET endosome-fusion assay that we have developed is far quicker and easier to perform than those previously reported (Warren *et al.*, 1988; Diaz *et al.*, 1988; Gruenberg *et al.*, 1989). Unlike most other assays (Davey *et al.*, 1985; Braell, 1987; Diaz *et al.*, 1988; Woodman & Warren, 1988; Gruenberg *et al.*, 1989), it does not require a final solubilization of the endosomes, a procedure which causes mixing of content and may contribute to increased background measurements; and since it does not require enzymic activity or antibody binding, it is functionally very versatile. For example, by using different fluorescent ligands (e.g. transferrin and epidermal growth factor or LDL), it should now be possible to follow the emergence of fusion-incompetent subcompartments in the endocytic pathway (Gruenberg *et al.*, 1989) as ligand–receptor complexes are sorted into their separate trafficking routes.

The RET assay enabled us to confirm on this lymphocyte model the results obtained with other cell types (Warren *et al.*, 1988; Diaz *et al.*, 1988). Thus endosome fusion is dependent on ATP and on a trypsin- and NEM-sensitive cytosolic factor, but it is not (or only poorly) affected by a NEM treatment of the endosome, or by agents equilibrating its acidic pH with the fusion buffer or complexing bivalent cations. Fusion could occur between plasma-membrane vesicles and endosomes (as reported by Mayorga *et al.*, 1988), but this reaction was negligible compared with endosome–endosome fusion.

Together, these data suggest that we have developed an isolated endosome preparation which is functionally competent and which will allow us to study toxin and immunotoxin translocation across the limiting membrane of the endocytic pathway in detail.

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