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Ira S. Mellman

Ralph M. Steinman

Jay C. Unkeless

Zanvil A. Cohn

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Selective Iodination and Polypeptide Composition of Pinocytic Vesicles

IRA S. MELLMAN, RALPH M. STEINMAN, JAY C. UNKELESS, and ZANVIL A. COHN

The Rockefeller University, New York 10021

ABSTRACT   We describe a method for the specific radioiodination of pinocytic vesicles (PVs) based upon the simultaneous endocytosis of lactoperoxidase (LPO) and glucose oxidase (GO). Initial experiments indicated that LPO was interiorized by the macrophage cell line J774 by fluid phase pinocytosis and without detectable binding to the plasma membrane (PM). Interiorization varied linearly with enzyme concentration and exposure time, was temperature dependent, and was undetectable at 4°C. Employing EM cytochemistry, LPO activity was restricted to PVs after a 3- to 5-min pulse at 37°C. These results formed the basis of the method for iodinating the luminal surface of PVs: 5-min exposure to both LPO and GO at 37°C followed by washes and iodination (addition of ^{125}I and glucose) at 4°C. Enzyme-dependent incorporation of iodide into the polypeptides of both PV membrane and contents occurred.

Several lines of evidence indicated that there was selective labeling of PV as opposed to PM. Iodination did not occur if the pinocytic uptake of LPO and GO was inhibited by low temperature. EM autoradiography showed a cytoplasmic localization of grains, whereas a clear PM association was evident with surface labeling. LPO was iodinated only after PV labeling and was present within organelles demonstrating latency. After PV iodination, >75% of several labeled membrane antigens could be immunoprecipitated by monoclonal antibodies only after cell lysis. In contrast, all labeled antigens were accessible to antibody on intact cells after surface labeling.

The polypeptide compositions of PM and PV membrane were compared by SDS polyacrylamide gel electrophoresis and by quantitative immune precipitation using a panel of anti-J774 monoclonal antibodies. The electrophoretic profiles of iodinated proteins (15-20 bands) were strikingly similar in NP-40 lysates of both PV and PM iodinated cells. In addition, eight membrane antigens examined by immune precipitation, including the trypsin-resistant immunoglobulin (Fc) receptor and the H-2D^d histocompatibility antigen, were found to be iodinated to the same relative extents by both labeling procedures.

We conclude that PV membrane is formed from a representative sample of PM polypeptide components.

Quantitative stereological measurements indicate that mammalian cells interiorize large amounts of plasma membrane (PM)[1] continuously during pinocytosis (see reference 30 for

review). Mouse peritoneal macrophages and L cells internalize the equivalent of their entire cell surface areas every 33 min and 2 h, respectively (32). Because PM components are neither degraded nor synthesized in a correspondingly rapid fashion (2, 5, 11, 13), it seems likely that the majority of internalized

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[1] *Abbreviations used in this paper:* BPA, bovine plasma albumin; DAB, diaminobenzidine; EM-ARG, electron microscope autoradiography; GO, glucose oxidase; HD, half distance; HRP, horseradish peroxidase; LPO, lactoperoxidase; MEM, alpha-modified minimal essential medium; NP-40, Nonidet-P40; PBS, Dulbecco's phosphate-buffered saline (without calcium and magnesium); PBS-G, PBS containing 5 mM

glucose; PM, plasma membrane; PV, pinocytic vesicle; R rat, rabbit anti-rat IgG; SA buffer, 0.3 M NaCl, 0.0125 M potassium phosphate, pH 7.4, 0.02% NaN$_3$; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

membrane is recycled to the cell surface. Many of these considerations make the assumption that a representative sample of membrane is interiorized in pinocytic vesicles (PVs). Unfortunately, little direct information exists regarding the composition of PV membrane, much less its ontogeny. Classical techniques of subcellular fractionation have not been effective in the isolation of a PV preparation suitable for biochemical analysis. Therefore, we have attempted to develop alternative strategies to study internalized membrane. In this paper, we describe an adaptation of the lactoperoxidase (LPO)-glucose oxidase (GO) catalyzed iodination of cell surface proteins (9, 10) which permits the specific labeling of PV membrane proteins in the mouse macrophage cell line J774. Our results, obtained from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and immune precipitation with a panel of monoclonal anti-J774 antibodies, indicate that the polypeptide compositions of PV and PM are virtually identical. These data in combination with previous stereologic work suggest that pinocytosis brings about a large flow of typical PM into and out of cells.

MATERIALS AND METHODS

Cells and Cell Culture

The mouse macrophage cell line J774 (25) was maintained in suspension or monolayer culture in alpha-modified minimal essential medium (MEM) containing 10% heat-inactivated (56°C, 0.5 h) fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.), 100 U/ml penicillin and 100 μg/ml streptomycin. The cell density of suspension cultures was maintained between 2 and 8×10^5/ml.

Pinocytosis of Soluble LPO

Bovine LPO (EC 1.11.1.7; purified grade; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was soluble in culture medium or phosphate buffered saline (PBS) and could be administered to cells without toxicity over a concentration range of 0.01–2.0 mg/ml. LPO uptake in monolayer or suspension culture was performed in a manner analogous to that previously described for horseradish peroxidase (HRP) (33, 34). Confluent monolayers containing 10^6 cells in 35-mm dishes were exposed for up to 2 h to LPO dissolved in complete medium, washed five times over a 15-min period with ice-cold PBS containing 5 mM glucose (PBS-G) and then incubated an additional 30 min at 37°C in complete medium to remove dish-bound enzyme. The cells were washed twice more with cold PBS-G, lysed in 0.15% vol/vol Triton X-100, and assayed for LPO enzymatic activity and for protein content (15). Alternatively, suspension cultures, containing up to 10^7 cells/ml of medium, were exposed to LPO and washed by centrifuging the cells at 4°C at 500 g for 7.5 min through 10 ml of 3% Metrizamide (Accurate Chemical & Scientific Corp., Hicksville, N. Y.) in HEPES-saline (10 mM HEPES, pH 7.6, 120 mM NaCl, 7 mM KCl, and 1 mM CaCl₂). The cells were washed three additional times in cold PBS-G at 1,200 g, 2.5 min, and lysed in 0.15% Triton X-100.

A modified triiodide assay was used to quantify cell-associated LPO enzymatic activity (17). Small aliquots (≤10 μl) of Triton lysates were added to 1 ml of reaction mixture containing 33 mM sodium phosphate (pH 7.0), 5 mM KI, and 6 mM H_2O_2 at room temperature. Change in absorbance at 350 nm was followed on a Zeiss recording spectrophotometer (model PM6, Carl Zeiss, W. Germany). The reaction was linear with respect to protein and time (up to 5 min) and could detect as little as 0.5–1 ng of our LPO preparation.

The Graham and Karnovsky diaminobenzidine (DAB)-H_2O_2 technique was used to visualize cell-associated LPO cytochemically (8). Suspension or monolayer cultures were exposed to 1–2 mg/ml LPO, washed in the cold, and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 15 min at room temperature. The fixative was removed with three rinses of PBS, and cytochemistry performed in 0.5 mg/ml DAB tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.)-0.01% H_2O_2 for 15 min at room temperature. The cells were rinsed in PBS, postfixed for 1 h at 4°C in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, rinsed in saline, and stained en bloc with 0.25% uranyl acetate for 15 min at room temperature. Cells in suspension were embedded in 1% agarose before dehydration in graded alcohols. Thin sections were examined in a Siemens 101 electron microscope, with and without staining with lead citrate and uranyl acetate. J774 cells not exposed to exogenous peroxidase were found to lack any cytochemical reaction product under these conditions.

Surface Area of Incoming PVs

To measure the surface area of incoming PV membrane relative to total cell surface area, J774 cells were plated on 35-mm dishes, exposed to HRP (1 mg/ml in complete medium) 5 min at 37°C, quickly washed in cold PBS, and processed for peroxidase cytochemistry. Micrographs of all cell profiles on randomly selected, unstained thin sections were taken. Using photographs at a final magnification of 10,000, the fractional surface area of PV membrane was measured with a grid of parallel lines as described previously (32). Cell volume was determined using a Coulter electronic cell counter (model ZBI) equipped with a Channelizer (Coulter Electronics Inc., Hialeah, Fla.).

Iodination of Pinocytic Vesicles within Intact Cells

J774 cells were suspended in PBS-G ($5–15 \times 10^6$ ml) containing LPO (0.1–2.0 mg/ml) and glucose oxidase (GO, EC 1.1.3.4, Sigma type IV, 10–250 μg/ml) for 3–5 min at 37°C with constant agitation. Cells were then layered on ice-cold 3% Metrizamide and washed as described above. The washed cells were suspended at 4–20 × 10^6/ml on ice in PBS containing 20 mM glucose. Carrier free ^{125}I-Na (Amersham Corp., Arlington Heights, Ill.) was added at 0.08–0.4 mCi/ml and the cells maintained for 12 min with frequent shaking on ice. Iodination was terminated by diluting the suspension with 10 ml of cold, serum-free MEM. The cells were pelleted by centrifugation (750 g, 2.5 min) at 4°C and washed twice more with cold MEM. Cell viability remained >95% as assessed by trypan blue exclusion, and the cells would reattach to culture dishes and resume normal growth.

Incorporation of ^{125}I into TCA-precipitable material was quantified by spotting small aliquots (≤20 μl) of Nonidet-P40 (NP-40, 0.5%, Bethesda Research Laboratories, Rockville, Md.)-lysed cell pellets on glass fiber filters (GF/B, Whatman), followed by washing with fresh, cold 10% TCA containing 0.1 M KI on a vacuum manifold or by batch as described (10). Radioactivity was determined in a Packard Auto Gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

To assess the extent to which lipid was iodinated by this procedure, total cell lipids were extracted according to the method of Folch et al. (6) and analyzed by two-dimensional thin-layer chromatography as described previously (18). This chromatographic system allowed separation of most free iodine and iodide from lipid.

Cell Surface Iodination

Externally disposed PM proteins were iodinated using a slight modification of the LPO-GO method of Hubbard and Cohn (9, 10). Cell viability was not altered by this procedure.

Quantitative Electron Microscope Autoradiography (EM-ARG)

PVs or PMs of J774 cells were iodinated as described above. Glutaraldehyde-fixed cells were incubated overnight at 4°C in 0.1 M NaI to exchange unincorporated ^{125}I before further processing. The flat substrate method of Salpeter and Bachman (27) was employed to prepare the autoradiographs. Thin (~1,000 Å) sections were transferred to collodian-coated glass slides, covered with a layer (~1,000 Å) of Ilford L-4 emulsion (Polysciences, Inc., Warrington, Pa.), stored at −20°C for 1–3 wk, and developed in Kodak D-19. Sections were stained in lead citrate and uranyl acetate and examined at final magnification of 34,000 using a Siemens Elmiskop 1 (calibrated with a reference grid, 2,160 lines/mm; Ernest F. Fullam, Inc., Schenectady, N. Y.). To maximize the amount of peripheral cytoplasm examined and hence maximize the average distance between PM and PV compartments, only cell profiles showing little or no nucleus were analyzed. Every such cell profile was photographed and the distribution of silver grains quantified essentially as described by Carpentier et al. (4). The shortest distance in millimeters between the midpoint of the longest axis of each grain and the PM was measured. These distances were then converted to nanometers and plotted as histograms relating frequency of grains as a function of grain half-distance (HD, see references, 28 and 29) from the PM. The HD assumed was 100 nm and is defined as the distance from a given radioactive source compartment within which there is a 50% probability a silver grain will be observed. Approximately 300 grains were scored for each sample.

Preparation of Monoclonal Antibodies

Anti-J774 antibodies were produced and isolated as described previously (36). Briefly, a female Sprague-Dawley rat was immunized twice with J774 and/or

another mouse macrophage cell line, P388D1, and its spleen cells were hybridized with the mouse myeloma line P3U1 (7, 14). Hybrids were screened for the secretion of anti-J774 antibodies by immunofluorescence (36). Positive colonies were cloned twice on agar and grown as ascites in CD₂F₁ mice (Flow Laboratories, Inc.). Antibodies were purified from ascitic fluid by precipitation in 45% saturated ammonium sulfate followed by chromatography on DEAE-cellulose (36). Six monoclonal antibodies were obtained, each of which recognized a different PM polypeptide(s) ranging from 20,000 to 180,000 mol wt. Antibody 2.4G2 has been shown to be directed against the mouse trypsin-resistant Fc receptor for IgG2b complexes (36). Antibody 1.21J is almost certainly identical to the macrophage-specific monoclonal antibody "Mac-1" described by Springer et al. (31).

A monoclonal antibody directed against the H-2D^d mouse histocompatibility alloantigen was obtained from a rat spleen cell × P3U1 hybridoma derived from a rat immunized with a subpopulation of mouse spleen cells as described (23). The antimacrophage antibody F480 was a gift from Siamon Gordon (Oxford University).

Preparation of Rabbit Anti-Rat Sepharose

To facilitate the use of monoclonal anti-J774 antibodies for immune precipitation, we employed an immunoadsorbent consisting of F(ab')₂ fragments of affinity-purified rabbit anti-rat (Rαrat) coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.). To prepare this reagent, we first purified rat serum IgG by ammonium sulfate precipitation (45% saturated) and DEAE-cellulose chromatography (36). The IgG fraction was coupled to CNBr-activated Sepharose 4B (5 mg IgG/ml packed resin, as described by Pharmacia) and the beads were treated with 3 M ammonium thiocyanate just before use to remove any noncovalently bound immunoglobulin. Rαrat IgG (N. L. Cappell Laboratories Inc., Cochransville, Pa.) was then purified by affinity chromatography (in PBS) on the rat IgG-Sepharose column and eluted with 3 M ammonium thiocyanate. After exhaustive dialysis first against PBS and then against 0.1 M sodium acetate (pH 4.5), the antibody was concentrated to >5 mg/ml and digested with pepsin (1% wt/wt; Sigma Chemical Co.) for 15 h at 37°C (22). Digests were neutralized and F(ab')₂ fragments isolated by a second passage on the rat IgG-Sepharose column. After dialysis against 0.1 M sodium bicarbonate (pH 8.3) containing 0.5 M NaCl, the antibody was coupled to CNBr-activated Sepharose 4B at 2 mg antibody protein/ml packed resin (as described by Pharmacia) and stored for up to 3 mo in PBS containing 0.02% sodium azide at 4°C.

Immune Precipitation

Procedures were developed to quantify the amount of a given iodinated antigen present on the cell surface as well as that present intracellularly in PVs. To determine surface antigen, 5 × 10⁶ iodinated J774 cells were suspended on ice in 0.5 ml of cold PBS containing 1 mg/ml bovine plasma albumin (BPA, fraction V, Armour Pharmaceuticals, Phoenix, Ariz.) and 0.02% sodium azide. 2.5 μg of purified monoclonal antibody was added, and the intact cells were incubated at 0°C for 30 min. Unbound antibody was then removed by pelleting the cells in 3 ml of cold PBS-BPA-azide three times at 750 g for 2.5 min. Washed cells were lysed in 0.2 ml of lysis buffer (0.5% NP-40, 100 U/ml aprotinin, 1 mM phenyl-methylsulfonyl fluoride in PBS) and centrifuged at 45,000 g for 15 min at 4°C. 50 μl of a 50% suspension of F(ab')₂ Rαrat-Sepharose was added to the clarified supernate which was then incubated at 4°C for 60 min with constant agitation. The immunoadsorbent was collected by centrifugation in an Eppendorf micro-fuge for 1 min, washed twice in SA buffer (0.3 M NaCl, 0.0125 M potassium phosphate, pH 7.4, 0.02% NaN₃), twice in a mixed detergent solution (13; footnote 2) (0.1% SDS, 0.05% NP-40, 10 mM Tris-HCl, pH 8.6, 0.3 M NaCl), and once again in SA buffer. Antigen-antibody complexes were dissociated by adding 40 μl of electrophoresis sample buffer (2% SDS, 0.01% bromphenol blue, 12% sucrose, 50 mM dithiothreitol, 50 mM Na₂CO₃, pH 8.5). Samples were stored at −20°C until use.

To measure total cellular iodinated antigen, i.e., cell surface and intracellular antigen, immune precipitation was performed by adding antibody to lysates rather than to intact cells. 5 × 10⁶ J774 cells were lysed in 0.2 ml of lysis buffer, centrifuged as described above, and incubated with 1 μg of monoclonal antibody for 1 h on ice before the addition of the F(ab')₂ Rαrat Sepharose.

The amount of ¹²⁵I precipitated was determined by counting the final washed Sepharose pellet and/or by excising and counting the radioactive bands identified after SDS PAGE. Background radioactivity was assessed in each experiment by omitting the monoclonal antibody. This procedure was judged to be quantitative

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² Kaplan, G., H. Plutner, I. Mellman, and J. C. Unkeless. Isolation of externally disposed plasma membrane proteins; trinitrobenzene sulfonate derivatization and immune precipitation. Manuscript submitted for publication.

because sequential immune precipitations from the same cell lysate indicated that >80% of the labeled antigen recognized by a given antibody was removed initially.

SDS PAGE

SDS PAGE was performed according to Neville (21) using 1 mm thick 4–11% polyacrylamide slab gels. To analyze total iodinated proteins, cell pellets were lysed in lysis buffer at 0°C, centrifuged at 40,000 g at 4°C for 15 min, and the supernates were added to electrophoresis sample buffer. Approximately 5 × 10⁴ TCA-precipitable cpm were applied to each lane. To analyze immunoprecipitated samples, isolated antigens were dissociated in sample buffer, the Sepharose was removed by centrifugation, and the supernates were applied to the gel. Samples were heated to either 37° or 100°C for 5 min before electrophoresis, with identical results. Gels were dried onto filter paper and autoradiograms developed at −70°C using Kodak X-O-Mat or DuPont Cronex ray film with image intensifier screens (Lightning Plus, DuPont Instruments, Wilmington, Del.). Proteins used as relative molecular weight standards were: soybean trypsin inhibitor (21,500); concanavalin A (26,000); ovalbumin (43,000); BPA (68,000); E. coli RNA polymerase (40,000, 90,000, 160,000); myosin (220,000).

Subcellular Fractionation

J774 cells were exposed to 2 mg/ml HRP (in PBS-G) for 5 min at 37°C, washed extensively in the cold, and subjected to cell surface iodination to provide for a PM marker. 6 × 10⁷ cells were then incubated in 1.5 ml of a hypotonic solution (0.09% NaCl, 80 μM MgCl₂, 550 μM CaCl₂) for 10 min at 0°C. 1.5 ml of 84% (wt/vol) Metrizamide containing 4 mM EDTA (pH 7.5) were added and the cells were disrupted immediately thereafter in a Dounce homogenizer (Kontes Co., Vineland, N. J.). The homogenate was centrifuged at 1,200 g for 12 min to remove nuclei and debris. 1 ml was then layered under a linear gradient of Metrizamide (10–30%, in HEPES-saline) and centrifuged at 108,000 g for 2.5 h (4°C) using an SW50.1 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) and a Beckman L5-65 ultracentrifuge. 0.3-ml fractions were collected and assayed for TCA-precipitable ¹²⁵I and peroxidase activity using o-dianisidine (Sigma Chemical Co.) as described previously (33, 34, 36). Latency of HRP activity in both homogenates and gradient fractions was assessed by performing the enzyme assay in the presence and absence of 0.05% Triton X-100. This detergent was found not to affect HRP activity itself.

RESULTS

Quantitative Studies of LPO Uptake by J774 Cells

It was first necessary to demonstrate that the uptake of LPO by J774 cells occurred by means of fluid-phase pinocytosis (30). Therefore, we performed a series of experiments analogous to those previously used to demonstrate the fluid-phase uptake of soluble HRP by cultured mouse cells (33, 34). LPO uptake during a 2-h exposure at 37°C was determined and was found to be linear with respect to extracellular LPO concentration over a wide range, 0.01–0.75 mg/ml (Fig. 1). As in previous studies (33, 34), the amount of cell-associated enzyme represented only a minute percentage of the total administered load per milliliter of culture medium, specifically 0.011%/h per 10⁶ J774 cells. Similar data (not shown) were obtained when the uptake of HRP by J774 cells was studied.

In addition, cell-associated LPO activity was found to increase linearly with increasing lengths of exposure to the enzyme (at time intervals between 0 and 2 h; not shown). The enzymatic assay employed was sensitive enough to detect LPO activity associated with cells incubated for as little as 5 min in 2 mg/ml LPO. In general, identical results were obtained with or without serum supplementation for both monolayer and suspension cultures.

Uptake of LPO was also strictly temperature dependent. Binding of enzyme by either monolayer or suspension cultures was not detectable at 0°C, even after incubations of up to 2 h with 2 mg/ml LPO. At 23°C, uptake was five- to six-fold less than that observed at 37°C.
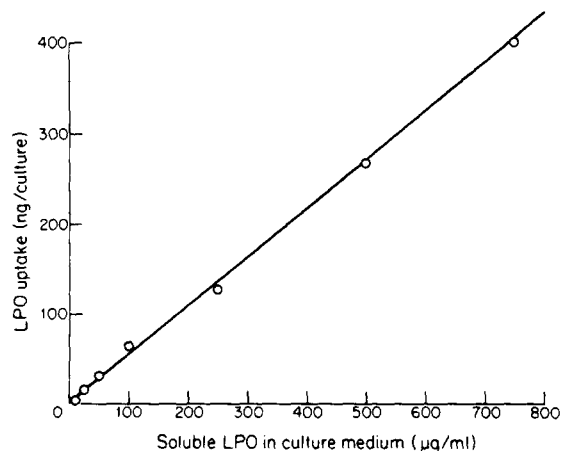
FIGURE 1   LPO uptake by J774 cells. 1 × 10⁶ J774 cells were plated in duplicate 35-mm tissue culture dishes and exposed to the indicated concentrations of LPO in complete medium for 2 h at 37°C. After extensive washing, monolayers were lysed in 0.15% Triton X-100 and LPO activity determined spectrophotometrically. A standard curve of LPO activity was obtained for each experiment. See text for details.

Cytochemical Localization of LPO

After a 3- to 5-min exposure to 2 mg/ml at 37°C, DAB-H₂O₂ reaction product was detected uniquely in PVs, that is, electron-lucent vesicles distributed mainly in the peripheral cytoplasm (Fig. 2). The PVs were heterogeneous in size and typically ranged from 0.2 to 1.5 μm in diameter. Reaction product was not observed on the PM, or in membrane-bounded, dense granules (lysosomes). The latter did accumulate LPO after longer exposures to enzyme (20–60 min). DAB-H₂O₂ reaction product could not be visualized in J774 cells exposed to LPO at 4°C. Similar cytochemical findings were made with HRP as the marker solute, except that HRP reaction product was more abundant in the individual PVs. We conclude from these quantitative and cytochemical data that LPO is pinocytosed in the fluid phase, without prior binding to the PM, and is then delivered to typical lysosomes.

Subcellular Localization of Internalized Peroxidase Activity

Corroborative evidence for the vesicular localization of the internalized peroxidase came from cell fractionation studies. For this purpose we utilized the more active HRP molecule and examined the buoyant density and latency of the intracytoplasmic organelles with which it was associated (see reference 35). 5 min after uptake, cells were homogenized and organelles floated in a Metrizamide gradient. As shown in Fig. 3, ~75% of the peroxidase activity banded in a single peak with a density of ρ = 1.13 and the remainder was found free in the load volume. The peroxidase activity of the peak was increased six to eightfold after Triton lysis, indicating its latency. The vesicle fraction containing HRP was not appreciably contaminated with iodinated plasma membrane (Fig. 3).

Surface Area of Incoming PVs

The fractional surface area of peroxidase-labeled PVs relative to PM was measured by stereology (32). After a 5-min exposure to enzyme (in this case HRP), the area of PV membrane associated with DAB reaction product was 23% of PM

surface area. Because the absolute volume of J774 cells was 1,662 μm³, we could calculate from stereological measurements (32) that the cell surface area of these cells was ~3,925 μm². Therefore, every 5 min at least 902 μm² of PM is interiorized as PVs. This is only a minimal estimate as the HRP-labeled PV space may saturate between 3 and 5 min (32). In any event, it is clear that J774 cells, like cultured peritoneal macrophages, interiorize an area of membrane equivalent to their entire cell surface at least twice an hour.

Iodination of Pinocytic Vesicles from within Intact Cells

Because a 5-min exposure to LPO appeared to deliver the enzyme uniquely to PVs, conditions were established to use this internalized enzyme to mediate the radioiodination of PV

FIGURE 2   Cytochemical localization of LPO in J774 cells. J774 cells were exposed to LPO at 2 mg/ml in complete medium for 5 min at 37°C. After several rinses with ice-cold PBS, cells were fixed in glutaraldehyde and peroxidase activity was visualized using DAB-H₂O₂. Upon examination of unstained thin sections in the EM, DAB reaction product was uniquely observed in electron-lucent PVs and was absent from both the cell surface and membrane-bounded dense granules (lysosomes). Bar, 1 μm. × 20,000.
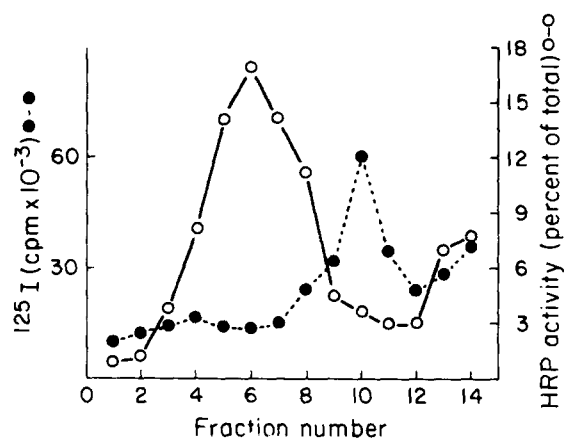

FIGURE 3   Subcellular fractionation of peroxidase-labeled J774 cells. J774 cells were exposed to HRP for 5 min at 37°C, washed in the cold, and then subjected to PM iodination. After homogenization in 42% Metrizamide, a sample was layered under a 3.5 ml 10–30% linear Metrizamide gradient and centrifuged 2.5 h at 108,000 g. Gradient fractions (0.3 ml) were analyzed for HRP activity (in the presence of Triton X-100) (O) and TCA-precipitable ¹²⁵I (●). Approximately 100% of both the HRP activity and ¹²⁵I applied to the gradient was recovered in the fractions.

membrane proteins from within live cells. In early experiments, we found that the efficiency of iodination was significantly increased by allowing the simultaneous pinocytosis of the complete iodination system, LPO and GO (the enzymatic source of H_2O_2), followed by extensive washing at 0°C to remove extracellular enzyme (see Table I for details). Using this procedure, up to 5×10^5 cpm/10^6 cells were incorporated into TCA-precipitable material (Table II). The cells remained fully viable (see Materials and Methods).[3] Less than 5% of this level of iodination was observed if either enzyme was omitted from the initial 5-min pinocytosis step at 37°C. Iodination was similarly reduced if the cells were exposed to both enzymes at 0°C, under which conditions pinocytosis is severely inhibited (33, 34). This observation is in agreement with the enzymatic measurements to LPO uptake (see above) and indicates that the enzyme does not adsorb appreciably to the PM. Thus, incorporation of ^{125}I into TCA-precipitable material was totally enzyme dependent and required the pinocytic uptake of both LPO and GO.

To assess the extent of lipid labeling, lipids from iodinated cells were extracted according to Folch et al. (6) and analyzed by thin-layer chromatography (18). Less than 5% of the TCA-precipitable radioactivity was found to be associated with cellular lipids.

The amount of ^{125}I incorporated after the uptake of LPO and GO ("PV iodination") was also found to be similar to that observed when the standard PM iodination procedure (LPO and GO supplied extracellularly at 0°C) was performed at identical concentrations of ^{125}I-NA (Table II). However, these values cannot be compared directly because the LPO and GO concentrations used for PV iodination (and presumably the intravesicular enzyme concentrations as well) are 20- and over 1,000-fold greater than those employed for cell surface labeling (9, 10). If cells were allowed to take up LPO at concentrations less than 0.5 mg/ml, the extent of iodination decreased proportionately.

Autoradiographic Localization of Incorporated [125]I

Although it seemed likely that most of the radioactivity incorporated after the pinocytosis of LPO and GO was localized to PV membrane polypeptides (as opposed to cell surface proteins), it was necessary to demonstrate directly the subcellular distribution of [125]I. To this end, we employed autoradiographic techniques to compare the localization of radiolabel in intact cells iodinated according to the PM (extracellular LPO, GO, and ^{125}I on ice) or PV (pinocytosis of LPO and GO at 37°C, wash at 0°C, ^{125}I at 0°C) protocols. Light microscope autoradiography of thick (1 µm) sections showed that most cell profiles were similarly labeled. However, it was impossible to distinguish differences between the PM and PV labeled samples.

Iodinated cells were then processed for EM-ARG using the flat substrate technique (reference 28; see Materials and Methods). After PM iodination, silver grains were associated closely with the cell surface and distributed in a uniform fashion (Fig. 4a). On the other hand, cells subjected to the PV iodination procedure, had a different pattern of grain distribution (Fig. 4b). In these profiles, a greater percentage of grains was located

[3] It should be pointed out that J774 cells are very resistant to killing by H_2O_2 (20) which may account for their tolerance of the relatively high concentrations of GO employed.

TABLE I

Iodination of PV Membrane in Intact Cells

1. J774 macrophages harvested from suspension culture and washed with PBS-glucose three times.
2. Cells suspended (~2 × 10^7/ml) in PBS-glucose containing LPO (0.75 mg/ml) and GO (50-100 µg/ml).
3. Cell suspension incubated for 5 min at 37°C to permit pinocytosis of LPO and GO.
4. After cooling to 4°C, extracellular LPO and GO are washed away by centrifuging cells through a cushion of 3% Metrizamide followed by three sequential washes with PBS-glucose.
5. Cells are suspended (~2 × 10^7/ml) in PBS-glucose on ice and carrier-free ^{125}I-Na (0.08-0.4 mCi/ml) is added, iodination of pinocytic vesicle membrane is catalyzed by LPO sequestered uniquely within vesicles. Incubation is continued for 15 min at 0°C.
6. Iodination terminated by dilution and repeated washing with serum-free MEM at 4°C.
7. Cell viability assessed by trypan blue exclusion.

TABLE II

Conditions for PV Iodination

Conditions	TCA-precipitable [125]I
	cpm/10^6 cells
LPO + GO* 37°C, 5 min	4.81 × 10^5
LPO + GO 4°C, 5 min	0.13 × 10^5
LPO only 37°C, 5 min	0.13 × 10^5
GO only 37°C, 5 min	0.05 × 10^5
No addition	0.01 × 10^5
Surface iodination‡	1-5 × 10^5

* J774 cells were exposed to LPO (0.75 mg/ml) and GO (0.05 mg/ml) for 5 min at 37°C, washed extensively in the cold, and ^{125}I-Na added (0.4 mCi/ml) at 0°C. Iodination was terminated after 12 min as detailed in Table I.
‡ Typical results given for purposes of comparison.

over the peripheral cytoplasm, often in small clusters. Because a significant number of grains was also found at or near the PM, we quantified grain distribution by measuring the distance of individual grains from the cell surface (as described in Materials and Methods). The results obtained for several hundred silver grains are illustrated in Fig. 5. After a PM iodination, grains were distributed symmetrically around the PM, as would be expected from a "line source" of radioactivity (28, 29). Grain distribution was quite different after a PV label. Most (~65%) of the grains were displaced two or more HDs intracellularly from the PM (Fig. 5). This indicates that the bulk of the radioactivity was caused by some source of ^{125}I in the peripheral cytoplasm. It seems likely that the grains emanated from PVs because the quantitative and cytochemical evidence discussed above indicate that these organelles uniquely contained the iodinating system. In addition, LPO could not be visualized on the PM nor were silver grains seen when the pinocytosis of LPO-GO was blocked by low temperature.

Immune Precipitation Analysis of PV and PM Iodinated Cells

The results of the autoradiography experiments strongly support the concept that most of the incorporated ^{125}I after the pinocytosis of LPO and GO was localized to PVs. Because peroxidase-labeled PVs were often observed within two HDs of the PM, the resulting distribution of silver grains might not be totally distinguishable from that caused by some residual
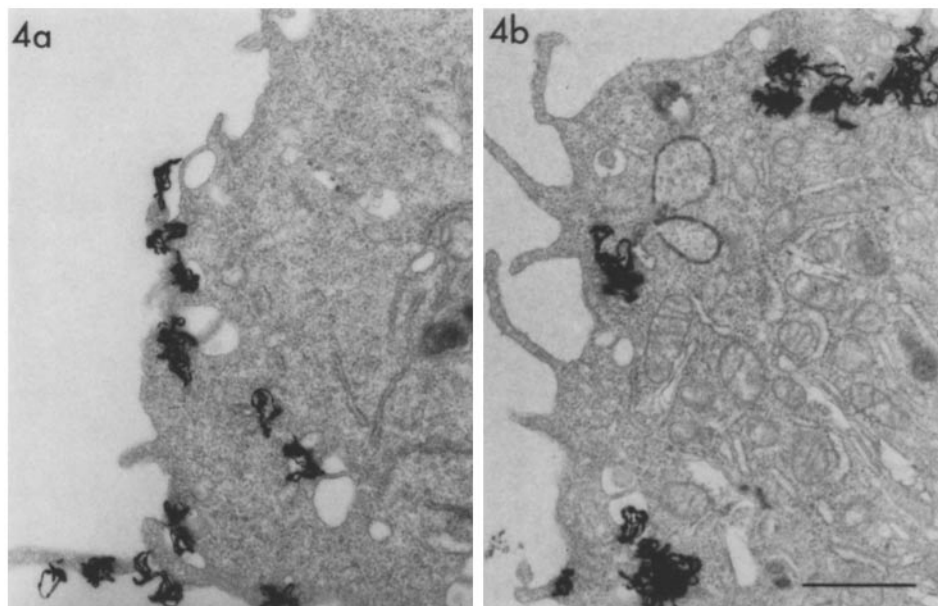
FIGURE 4 Localization of incorporated ^{125}I by EM-ARG. J774 cells were iodinated according to either the PM- or PV-labeling protocol, fixed in glutaraldehyde, and processed for EM-ARG using the flat substrate method (37). TCA-precipitable radioactivity in each sample was ~1 × 10^6 cpm/10^6 cells, and the autoradiographs shown were exposed for 14 d at −20°C. (a) PM iodination: silver grains were closely associated with the cell surface (including microvilli) and were distributed around the perimeter of each cell. (b) PV iodination: most grains were observed over the peripheral cytoplasm, often in clusters of two or more. In general, all cells were labeled to comparable extents and silver grains occurred throughout the peripheral cytoplasm. (a and b) Bar, 1 μm. × 14,200.

cell surface associated radioactivity (see Fig. 5). Therefore, we developed procedures for immune precipitation using monoclonal anti-J774 antibodies which provided an independent measure of the subcellular localization of incorporated ^{125}I. Iodinated cell surface antigen was quantitatively measured by incubating intact, viable J774 cells at 4°C with a given monoclonal antibody; under these conditions, only antigen present on the PM would be accessible to the antibody. Subsequently, unbound antibody was washed away and the cells lysed in NP-40. On the other hand, total ^{125}I-antigen (cell surface and intracellular) was determined by adding monoclonal antibody directly to detergent lysates. In both cases, antigen-antibody complexes were isolated using an immunoadsorbant consisting of affinity-purifed Rαrat F(ab′)$_2$ fragments coupled to Sepharose. Nonspecifically bound proteins were removed by washing the Sepharose with a novel detergent mixture,[2] 0.1% SDS-0.05% NP-40.

We first tested this approach on surface-iodinated J774 cells. In this case, virtually 100% of each ^{125}I-antigen should be accessible on the cell surface. Representative results obtained with two monoclonal antibodies are illustrated in Fig. 6a. As expected, identical amounts of ^{125}I-antigen were isolated both from intact cells (lanes 1 and 3) and from lysed cells (lanes 2 and 4). However, an entirely different result was obtained when this analysis was performed on PV-labeled cells. As shown in Fig. 6b, only a small fraction of the total ^{125}I-antigen was accessible at the cell surface.

These data were then quantified by excising and counting the radioactive bands. By dividing the amount of ^{125}I-antigen isolated from intact cells by that from lysates, we could estimate the percentage of total labeled antigen that is present on the cell surface. Although this was close to 100% for J774 cells subjected to surface iodination, <25% of the ^{125}I incorporated in PV-labeled cells was precipitated by antibody added to

intact cells (Table III). Accordingly, at least 75% of the radioactivity incorporated in these cells was associated with internalized (i.e., PV) membrane.

Polypeptide Composition of the PV Membrane

Having established by several criteria that pinocytosed LPO-GO was selectively iodinating intracellularly, presumably only in PVs, we were in a position to compare the iodinated polypeptides of PM and PV membrane. As shown in Fig. 7, one-dimensional SDS PAGE autoradiograms indicated that the patterns of labeled proteins were strikingly similar after PV or PM iodination. Most of the differences in band intensity were quite variable except for those in the low molecular weight (<40,000) region, where the indicated differences were consistently obtained. In addition, we often observed that a 310,000-dalton protein was more heavily labeled after PV iodination. Perhaps the major and most consistent difference between the two electrophoretic profiles was an 80,000-dalton protein in the PV-labeled lysate. This band comigrated with authentic LPO and almost certainly represents self-iodinated LPO trapped intracellularly within PVs.

As expected when only membrane proteins are iodinated (12), the spectrum of labeled polypeptides after PM or PV iodination differed markedly from the total cell lysate spectrum, identified by either Coomasie Blue staining or autoradiograms of ^{35}S-methionine-labeled J774 cells (Fig. 7). In particular, actin was not iodinated as a consequence of either regimen.

SDS PAGE allowed us to compare qualitatively ~20 major iodinated proteins on PM and PV membrane. Using our monoclonal antibody panel, we next quantitatively examined how 11 individual polypeptides were distributed between surface and internalized membrane by determining the extent to which
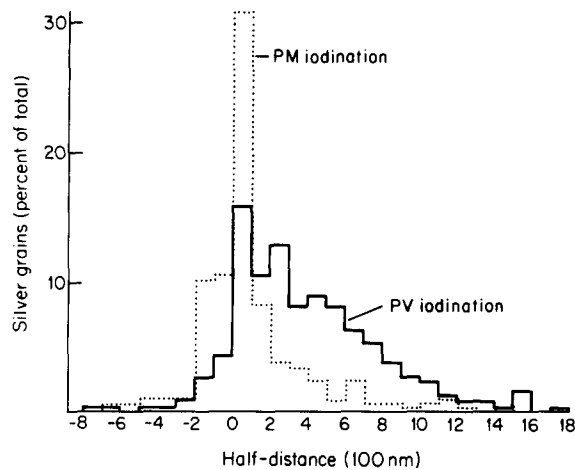
FIGURE 5 Quantitative analysis of silver grain distribution. The distribution of silver grains with respect to the PM was quantified in autoradiographs of both cell surface and PV-iodinated cells. For each sample, a series of 30 micrographs was printed at a final magnification of 32,000 and the shortest distance between the midpoints of individual grains and the PM was measured (see Materials and Methods). Data obtained from scoring several hundred grains in both PM (···) and PV (—) labeled cells are displayed as histograms relating distance from the PM (in HD, assumed to be 100 nm) to the percentage of grains observed. The position of the PM is defined by "0"; positive and negative HDs refer to distance from the PM in the intracellular and extracellular directions, respectively.

these antigens were iodinated after both PV and PM labeling. After immune precipitation from NP-40 lysates of iodinated J774 cells, ^{125}I was quantified both by direct measurement of radioactivity bound to the Rαrat-Sepharose and by excision and counting the radioactive bands identified after SDS PAGE. Representative results are illustrated in Fig. 8. In this autoradiogram, it is possible to rank each antigen in terms of its intensity of labeling. The relative amounts of radioactivity precipitated after PM iodination yielded 2D2C > F480 > 25-1 > 2F44 > 2.4G2 and 2.6. Roughly the same order was obtained when PV-iodinated cells were employed, with 2.4G2 and 2.6 reversed in this experiment.

The quantitative representation of these data pooled from four experiments is given in Table IV. A series of eight monoclonal antibodies was used which effected the isolation of 10–11 distinct polypeptides (ranging from <20,000 to 180,000 in mol wt). Because antibody 2D2C precipitated the most radiolabel, the amounts of ^{125}I immune precipitated by the other antibodies were normalized against it. All of these ^{125}I-antigens were isolated in roughly the same relative amounts from both PV- and PM-labeled lysates. Antigen 2.6, however, did consistently appear to be somewhat more heavily iodinated as a result of the PV-labeling procedure. In general, these data supply quantitative evidence that none of these PM antigens were either preferentially included in, or excluded from, PV membrane.

DISCUSSION

Selective Iodination of PVs within Living Cells

Subcellular fractionation has yet to provide purified PV fractions for the study of this important organelle. Consequently, there exists little direct biochemical information on the composition of PV membrane. Direct data on its structural

relationship to the PM or its metabolic fate are not available. In the present paper, we first describe a method that permits the specific LPO-catalyzed iodination of luminally disposed PV membrane proteins in intact viable cells. We then used this approach to analyze the polypeptide composition of internalized membrane and to compare it with that of the cell surface.

The utility of soluble LPO for the iodination of PV membrane was based largely on the demonstration that the enzyme is pinocytosed by J774 cells in the fluid phase, i.e., without adsorbing to the PM. Uptake was found to be both time and temperature dependent, and was directly proportional to the concentration of enzyme in the culture medium over a wide range. These characteristics are virtually identical to those exhibited by the extensively studied marker of fluid-phase pinocytosis HRP (33, 34) as well as polyvinylpyrrolidone, sucrose, and inulin (3, 37, 38). In a further analogy with HRP, the subcellular localization of cell-associated LPO could be determined by the Graham and Karnovsky cytochemical technique for peroxidase. Although the reaction product caused by LPO was significantly weaker than HRP, the enzyme was readily and selectively detected in typical PVs (0.2–1.5 μm in diameter) after a 5-min pulse at 37°C. Enzymatic and cytochemical assays gave identical results when J774 cells were treated with HRP in lieu of LPO.

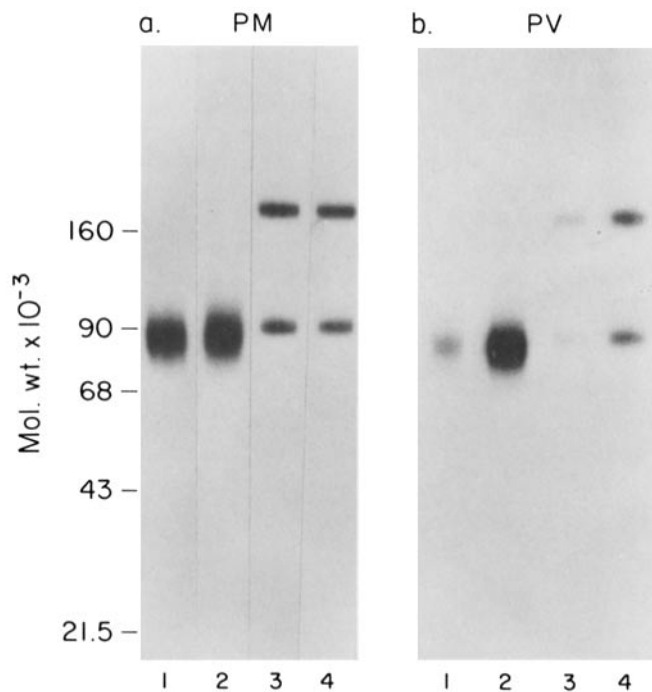With soluble LPO sequestered uniquely in PVs, conditions

FIGURE 6 Selective determination of iodinated PM antigens present in the cell surface or on intracellular membranes by immune precipitation. To detect labeled cell surface antigen, intact viable J774 cells, iodinated according to either the PM or PV iodination protocols, were incubated at 0°C with one of two anti-J774 monoclonal antibodies (2D2C, lanes 1 and 2; 1.21J, lanes 3 and 4). After several washes to remove unbound antibody, the cells were lysed in NP-40 and antigen-antibody complexes isolated using Rαrat-Sepharose. Total labeled antigen (cell surface and intracellular) was determined by adding antibody directly to cell lysates. Immune precipitates were analyzed by SDS PAGE and autoradiography. Lanes 1 and 3: iodinated antigen isolated from 5 × 10⁶ intact cells. Lanes 2 and 4: iodinated antigen isolated from NP-40 lysates of 5 × 10⁶ cells.

TABLE III

Subcellular Distribution of Labeled Membrane Antigens after LPO-Catalyzed PM or PV Iodination

Monoclonal antibody	Fraction of total ^{125}I-antigen accessible to antibody on the cell surface*	
	PM iodination	PV iodination
2D2C	0.94	0.24
1.21J	0.95	0.33
2E2A	1.02	0.20
25-1	0.93	0.20
2.4G2	0.93	ND‡
2F44	0.82	ND
Mean ± SD§	0.94 ± 0.2	0.24 ± 0.11

* Immune precipitations were carried out using PM- or PV-iodinated J774 cells. In paired experiments, monoclonal antibody was added either to intact cells (to detect cell surface associated antigen) or to cell lysates (to detect total labeled antigen, i.e., both cell surface and intracellular). The fraction of labeled antigen accessible to antibody on the cell surface was obtained by dividing the amount of radioactivity immune precipitated from intact cells by that immune precipitated from lysed cells. Data are pooled from two to four experiments.
‡ ND, not determined.
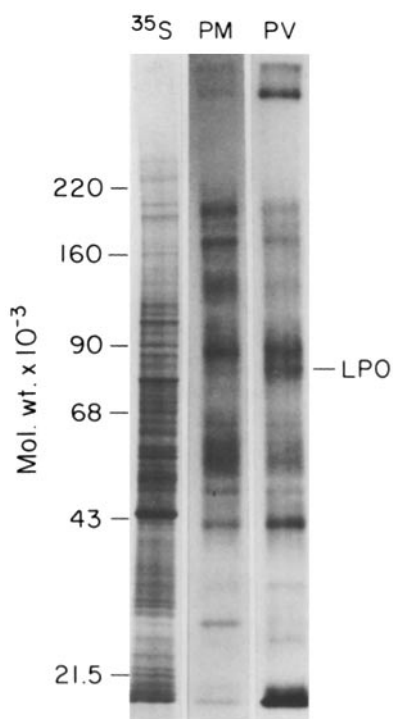§ Means (and standard deviations) were determined by pooling all data from individual experiments.

FIGURE 7 Comparison of the iodinated polypeptides of PV membrane and PM by SDS PAGE. J774 cells were iodinated according to either the cell surface or PV-labeling protocol and lysed in NP-40. Similar amounts of TCA-precipitable radioactivity were subjected to electrophoresis in 4–11% polyacrylamide gradient SDS gels. The resulting autoradiographs are illustrated. *LPO* marks the migration of authentic LPO (~80,000 daltons); a corresponding band of radioactivity was consistently detected in the PV-labeled lysate but not in the PM-labeled lysate. In addition, PV iodination labels only a minor and restricted subset of J774 proteins. As indicated by a comparison of iodinated polypeptides with total J774 protein labeled by overnight incubation of cells in ^{35}S-methionine, none of the major J774 cytoplasmic proteins (e.g., actin, ~43,000 daltons) was found to be iodinated.
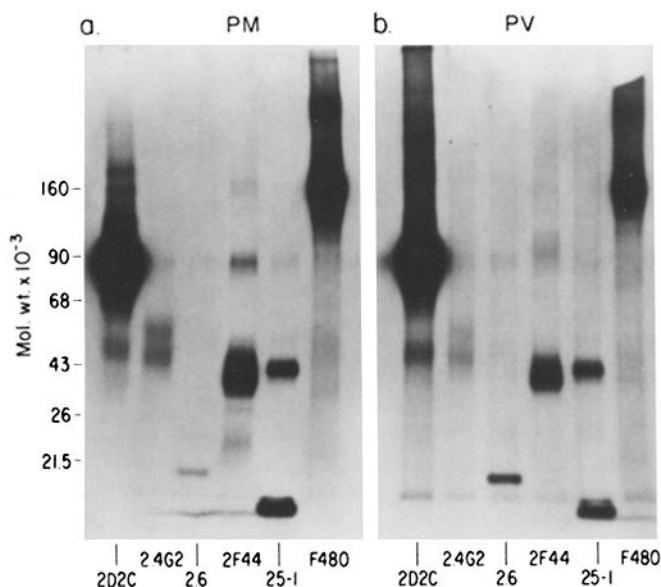

FIGURE 8 Comparison of the iodination of six membrane antigens in J774 cells subjected to either PM or PV iodination. Lysates of iodinated cells were analyzed by quantitative immune precipitation (see Materials and Methods) using 6 anti-J774 monoclonal antibodies. Isolated antigens were separated by SDS PAGE and visualized by autoradiography at a single development time (4 d). The autoradiograph shown is overexposed to permit detection of poorly-labeled antigens. The amount of radioactivity immune precipitated by each antibody was determined directly to enable a quantitative representation of these results (Table IV). Antibody 2D2C precipitated 1.27×10^5 and 0.85×10^5 cpm from PM and PV iodinated cells, respectively.

were derived to allow it to catalyze the iodination of PV proteins. It was found optimal to include GO along with LPO during the uptake period at 37°C. Presumably the GO and LPO are sequestered in the same PVs. ^{125}I-Na, which diffuses freely across biological membranes (26), can then be added extracellularly at 0°C without loss in cell viability. The resulting incorporation of radioactivity into TCA-precipitable material was shown to be dependent upon the presence of both enzymes during the 5-min pinocytosis step, and most of the label was incorporated into protein. Iodination was also observed if GO was supplied extracellularly (at 0°C) after LPO uptake; but incorporation was some 10-fold less.

By way of analogy to the case of LPO-GO catalyzed cell surface iodination (9, 10), it can be assumed that the iodination of PV membrane is limited to proteins exposed on the luminal aspect of the PV, and that the active species of ^{125}I does not cross biological membranes (12). In fact, major cytoplasmic proteins were not iodinated, as indicated by a comparison by SDS PAGE of PV-labeled cell lysates with those metabolically labeled with ^{35}S-methionine.

It was necessary, however, to demonstrate directly that the ^{125}I incorporated after pinocytosis of LPO and GO was indeed localized in PVs as opposed to PM. The possibility remained that a minute amount of LPO, enzymatically and cytochemically undetectable, was adsorbing to the PM and causing the preferential iodination of cell surface proteins. Four independent lines of evidence were obtained to confirm the intracellular localization of label: (a) Incubation of J774 cells with LPO and GO at 0°C resulted in only 5% of the iodination observed after incubation at 37°C. Because little enzyme can be pinocytosed

TABLE IV

Composition of PM and PV Membrane as Determined by the Relative Labeling of Eight Membrane Antigens

Monoclonal antibody	Relative ^{125}I isolated by immune precipitation*	
	PM iodination	PV iodination
2D2C (90,000)‡	1.000§	1.000‖
1.21J (94,000, 180,000)	0.552	0.452
2E2A (82,000)	0.150	0.166
F480 (150,000)	0.170	0.092
2F44 (42,000)	0.035	0.024
25-1 (44,000, 12,000)	0.065	0.041
2.4G2 (60,000, 47,000)	0.022	0.026
2.6 (20,000)	0.008	0.017

^{125}I was determined both by excising and counting the radioactive band identified after SDS PAGE and by counting the final washed pellet of Rαrat-Sepharose. Although the latter procedure resulted in ~40% higher values, both methods gave identical results with respect to the relative amounts of ^{125}I detected for each antigen. The values given were obtained by counting the Sepharose pellets directly (see Materials and Methods). Iodinated 2D2C accounted for ~10% of the total TCA-precipitable ^{125}I present in cell lysates (see Discussion).
* Representative data from four paired experiments.
‡ Numbers in parentheses indicate the relative molecular weights of the polypeptide(s) precipitated by each antibody.
§ 7,744 cpm (exp 1); 127,781 cpm (exp 2); 3,691 cpm (exp 3); 55,045 cpm (exp 4).
‖ 11,341 cpm (exp 1); 84,900 cpm (exp 2); 4,712 cpm (exp 3); 59,848 cpm (exp 4).

at 0°C, this experiment provides an approximation of the iodination caused by PM-adsorbed enzyme. (*b*) Self-iodinated LPO was detected by SDS PAGE after PV labeling, but not after cell surface labeling. This indicates that LPO was sequestered intracellularly and consequently was not washed away after iodination. (*c*) EM-ARG showed that cell-associated ^{125}I emanated from a source in the peripheral cytoplasm after PV iodination, whereas the silver grains detected in micrographs of surface-labeled cells were distributed symmetrically around the PM. (*d*) Using anti-J774 monoclonal antibodies and a quantitative immune precipitation technique, we found that only a small fraction of the total ^{125}I incorporated onto several different antigens was accessible to antibody when added at 4°C to intact PV-iodinated cells. In contrast, virtually 100% of the ^{125}I associated with these antigens was accessible to antibody at the cell surface after PM iodination. From these data, we calculated that at least 75% of the radioactivity incorporated into membrane antigens as a result of the PV-labeling procedure was intracellular, i.e., in PV membrane. It is likely that the 75% value is at best a minimum estimate. We are currently considering several factors which might act to artifactually increase the amount of radioactivity detectable as cell surface associated. For example, pinocytosis may not be completely inhibited during the 30 min that the cells were exposed to antibody and washed since low temperature could not be rigorously controlled; thus it is possible that a small percentage of the antibody actually gained access to iodinated antigens in internalized membrane. Similarly, a portion of the iodinated PV membrane proteins may have been recycled to the cell surface during this time interval. In addition, the monoclonal antibodies we employed may have a slow but finite rate of dissociation from their respective antigens. Consequently it is possible that a portion of the PM-bound antibody may have been dissociated during or after NP-40 lysis, thus becoming available to rebind labeled PV polypeptides. Finally, because the intact cells were incubated with saturating concentrations

of antibody, the attachment of most of the IgG would be monovalent. As a result, the second antigen-combining site on these molecules could conceivably bind an iodinated PV polypeptide after solutization of the cells by NP-40.

Polypeptide Composition of PV Membrane

The most important conclusions of the present study relate to the nature of the PV membrane and its derivation from the cell surface. Comparisons of NP-40 lysates of PV- and PM-iodinated J774 cells by SDS PAGE revealed a striking similarity between their respective polypeptide constituents. The only consistent qualitative differences involved a series of three low molecular weight (<30,000) proteins. We have not been able to exclude proteolytic cleavage as an explanation for these alterations in band intensity. It is also possible that the accessibility of the polypeptides to LPO-catalyzed iodination was altered after interiorization. In general, other differences in band intensity were variable from experiment to experiment so that no particular significance could be attributed to them. However, a high molecular weight (310,000) band was often found to be more heavily labeled in the PV-iodinated lysate. Given that at least 75% of the radiolabel was incorporated in internalized membrane after PV iodination, it is clear that any significant differences between the iodinated polypeptide compositions of PM and PV membrane would be readily identifiable as bands of consistently obvious differences in intensity.

Because SDS PAGE of cell lysates only allowed the analysis of 15-20 of the major iodinated membrane proteins, we also examined the relative distribution of a number of individual polypeptides. Using a panel of anti-J774 monoclonal antibodies, we measured the extent to which the series of corresponding antigens was labeled after PV and PM iodination. Significantly, each antigen appeared to occur in proportionately the same amount in both membranes. The one consistent exception to this observation was the low molecular weight antigen recognized by antibody 2.6 which appeared to be more intensely labeled after PV iodination. Because the measurements were made by determining the actual amount of ^{125}I in discrete polypeptides, they provide more highly specific and quantitative data than that obtained by conventional autoradiography of dried SDS polyacrylamide gels.

Both major and minor iodinatable polypeptides were included in this analysis. For example, the radioactivity associated with antigen 2D2C was found to account for 10-15% of the total TCA-precipitable ^{125}I in cell lysates. It would thus be expected to constitute a substantial portion of the radioactive 90,000 band observed after SDS PAGE of total cell lysates (Fig. 7). Similarly, the 180,000 band visualized in these gels (Fig. 7) is probably accounted for entirely by the higher molecular weight polypeptide of antigen 1.21J (~5% of the total ^{125}I). In contrast, the radioactivity caused by a relatively minor labeled polypeptide (e.g. 2F44, 0.25-0.35% of total ^{125}I) is not readily identifiable on lysate gels. Recent evidence[2] moreover suggests that cell surface iodination detects most major PM proteins. The spectrum of PM polypeptides labeled with [^{35}S]methionine (isolated after their haptenization with trinitrobenzene sulfonic acid) is similar to that obtained using LPO catalyzed iodination.

Taken together, these results indicate that PM is sequestered within cells during pinocytosis and that, at least at this level of resolution, the polypeptide composition of the PV closely reflects that of the PM.

Recycling of Internalized Membrane

Our stereological data on J774 cells, as well as data obtained using cultured mouse macrophages (32), show that an area of membrane equivalent to the entire cell surface is interiorized as PVs at least every 33 min. The fact that EM-ARG revealed that ^{125}I incorporated in PV-labeled cells was evenly distributed throughout the peripheral cytoplasm is strong evidence that iodination occurred in a large majority of these PVs. Therefore, it is apparent that the complete complement of PM polypeptides is internalized approximately twice every hour. Nevertheless, the PM proteins of J774 cells (unpublished data), thioglycollate-elicited (14), and resident (unpublished data) mouse peritoneal macrophages have been estimated to exhibit half-lives >25–30 h. Thus, the longevity of these polypeptides cannot be accounted for by their exclusion from PVs. Instead, the recycling of internalized membrane components back out to the cell surface must be invoked.

We have recently obtained evidence for a large and rapid return flow of internalized PM to the cell surface. Using a method analogous to the PV iodination scheme described here, Muller et al. (18, 19) have demonstrated the specific labeling of phagolysosome membrane after the ingestion of LPO-modified polystyrene latex beads. Analysis of iodinated macrophages by EM-ARG revealed a close association of silver grains with intracellular latex beads. When these cells were returned to culture, however, grains were associated predominantly with the PM. Assuming that the PV is the vector by which these movements are effected, it is clear that pinocytosis brings about a large flow of typical PM both into and out from the vacuolar apparatus. Using the iodination and immune precipitation procedures described in the present paper, we will be able to assess directly the fate (degradation vs. recycling) of PV membrane proteins.

Pinocytosis and Domains of the Plasma Membrane

The fact that typical PM proteins are being internalized at rapid rates argues against the existence of specialized domains of the PM within which pinocytic activity is exclusively limited. Nor does it appear likely that PM proteins must be clustered or aggregated by some extracellular force (lectin, multivalent antibody, specific ligand) before their internalization in PVs. Two antigens of particular interest that we have studied here are the mouse histocompatibility antigen (recognized by antibody 25-1) and the trypsin-resistant Fc receptor for IgG2b complexes (antibody 2.4G2; reference 36). Neither of these antigens appears to be preferentially included within or excluded from nascent PVs. We should now be able to determine whether the internalization and fate of these polypeptides is in any way modulated by the binding of such potential cross-linking agents as specific antibodies and/or immune complexes.

It is nevertheless possible that specialized domains of the PM exist which are involved in pinocytic activity distinct from that studied here. In particular, the contribution of coated PVs has not been evaluated directly. It is not clear whether coated vesicles contribute significantly to the uptake of a fluid-phase marker such as LPO and if so, whether their membrane polypeptides contribute a distinct set of proteins. That the compositions of PM and coated vesicle membrane might differ has been suggested by observations that these vesicles may have a

relatively low cholesterol content (16, 24). We are currently attempting to covalently couple LPO to ligands selectively internalized via coated vesicles (e.g., low density lipoprotein uptake by cultured fibroblasts, [1]) to determine if intracellular iodination can be used to analyze at least the polypeptide composition of their limiting membrane. If a biochemical distinction can be made between coated vesicle membrane and that of "uncoated" or fluid-phase PVs, some functional distinction between these two types of pinocytosis might follow.

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