The Membrane Proteins of the Vacuolar System

I. Analysis by a Novel Method of Intralysosomal Iodination

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ABSTRACT A method has been developed to deliver an iodinating system into the confines of the phagolysosome, allowing us to study the nature of the phagolysosomal membrane. Lactoperoxidase (LPO) is covalently coupled to carboxylated latex spheres (LPO-latex) in a stable, enzymatically active form. The addition of LPO-latex to cultured macrophages leads to their rapid attachment, ingestion, and enclosure in a plasma membrane-derived phagocytic vacuole. These organelles rapidly fuse with preexisting lysosomes and are converted to phagolysosomes (PL) that demonstrate both acid phosphatase and lactoperoxidase activities. The exposure of LPO-latex containing cells to $^{125}I^-$ and an extracellular peroxide-generating system, glucose oxidase-glucose, at 4°C leads to incorporation of label into TCA-precipitable material. The incorporated cell-associated label was present as monoiodotyrosine, and negligible amounts were found in lipids. Cell viability remained >99%.

Autoradiography at both the light and EM level revealed that >97% of the cells were labeled, and quantitative analysis demonstrated the localization of grains to LPO-latex containing PL. PL were separated on sucrose gradients, and their radiolabel was confined almost exclusively to the membrane rather than soluble contents.

SDS-polyacrylamide gel electrophoretic analysis of the peptides iodinated from within PL demonstrated at least 24 species with molecular weights ranging from 12,000 to 250,000. A very similar group of proteins was identified on the plasma membrane (PM) after surface iodination, and on latex phagosomes derived from iodinated PM. No novel proteins were detected in PL, either immediately after phagosome-lysosome fusion or after 1 h of intracytoplasmic residence.

We conclude that the membrane proteins accessible to LPO-catalyzed iodination on the luminal surface of the PL and on the external face of the PM are similar, if not identical.

Our knowledge of the vacuolar apparatus is largely based upon static ultrastructural, cytochemical, and biochemical analysis of its components. Much of this information has been concerned with the matrix polypeptides and enzymes, their localization, fluctuation, substrate specificity, and ability to degrade complex biological macromolecules. Much less is known, however, about the membranes of these organelles and their vectorial flow and interactions. We have in the past approached one aspect of this flow and quantitated the steady-state interiorization of plasma membrane in the form of pinocytic vesicles (26). This analysis based upon cytochemical and stereological information strongly suggested that much of the extensive influx of plasma membrane was balanced by a recycling process in which plasma membrane was returned to the cell surface and reused.

To examine the influx, efflux, mixing, and composition of plasma and lysosomal membrane in more detail, a selective labeling system was required. In this paper we report on the use of lactoperoxidase (LPO) covalently coupled to carboxylated polystyrene latex spheres. These particles are readily ingested by macrophages and rapidly established within the phagolysosomal (PL) compartment. Here, in the presence of H_2O_2 and $^{125}I^-$, the enzyme predominantly labels accessible membrane polypeptides rather than contents of the organelle. The labeled polypeptides of the PL and plasma membrane were virtually indistinguishable by gel electrophoresis. In the accompanying report we examine the directional flow and fate of labeled PL membrane polypeptides.

MATERIALS AND METHODS

Materials

Female mice of the Nelson-Collins strain (NCS) weighing 25–30 g were obtained from The Rockefeller University breeding colony. Cells of the J774 macrophage line were a gift from Dr. Jay Unkeless of The Rockefeller University. Medium 199, fetal calf serum (FCS), phosphate-buffered saline with (PBS) and without (PD) magnesium and calcium, and trypan blue stain were purchased

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from Grand Island Biological Co., Grand Island, N. Y. Other materials and their abbreviations and sources were: carboxylate-modified polystyrene latex spheres 0.86 μm in diameter from Dow Diagnostics, Inc., Indianapolis, Ind.; *N*-hydroxysuccinimide (NHS) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) from Pierce Chemical Co., Rockford, Ill.; lactoperoxidase, purified grade (LPO) and pronase, B grade from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; trypsin (TRL3) from Worthington Biochemical Corporation, Freehold, N. J.; glucose oxidase, type VI (GO), horseradish peroxidase, type II (HRP), beef liver catalase, 3,3'-diaminobenzidine tetrahydrochloride grade II (DAB), glycine, phenylmethyl sulfonyl fluoride (PMSF), aprotinin, SDS, cytidine monophosphate, sodium salt from Sigma chemical Co., St. Louis, Mo.; colloidal thorium dioxide (Thorotrast) from Fellows Testagar, Anaheim, Calif.; Sephadex G-25 from Pharmacia Fine Chemicals, Uppsala, Sweden; silica gel plates from Supelco, Inc., Bellefonte, Pa.; TCA, potassium iodide (KI), sucrose, hydrogen peroxide (Superoxol), and all other salts and solvents from Mallinckrodt Chemical Works, St. Louis, Mo.; carrier-free $Na^{125}I$ from New England Nuclear, Boston, Mass.

Cell Cultures

Resident peritoneal macrophages were lavaged from female NCS mice, using PD, and cultured for 2 d in medium 199 containing 10% FCS and 100 U/ml penicillin G. Culture medium was replaced daily. To achieve nearly confluent macrophage monolayers, the following numbers of cells were plated: for routine iodination, 3×10^6 cells were plated in 16-mm-diameter flat-bottom wells (Costar, Data Packaging, Cambridge, Mass.); for experiments involving electron microscopy, 2×10^7 cells were plated in 35-mm plastic dishes (Nunclon Delta, Kamstrup, Roskilde, Denmark); and for cell fractionation, $4\text{–}5 \times 10^7$ cells were plated in 60-mm Nunc dishes.

Covalent Coupling of LPO to CM-Latex

The coupling reaction is outlined schematically in Fig. 1. CM-latex spheres were washed several times in 0.2 M acetate buffer, pH 5.4, and brought to 1.5% wt/vol in 1 ml of buffer in a microfuge tube. Crystalline NHS and CMC were added successively to a final concentration of 0.1 M each, and the reactants were mixed with a small magnetic flea for 10 h at room temperature. The activated beads were pelleted in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, N. Y.) for 5 min at 12,000 rpm at 4°C and the bead pellet rinsed carefully. The beads were vortexed quickly into 0.5 ml of LPO (11 U/ml in carbonate-bicarbonate buffer, pH 9.6) and the mixture stirred magnetically at 4°C for 30 min. Crystalline glycine was then added to 1 M to quench unreacted ester bonds, and the mixture was stirred an additional 15–30 min. The latex beads with covalently bound LPO (LPO-latex) were pelleted and washed four to five times in cold PBS until two successive supernates showed no LPO activity by the *o*-dianisidine assay (see below). The beads were then suspended in 1 ml 50% glycerol and stored at −20°C. This procedure reproducibly coupled some 20% of the LPO enzymatic activity and 20% of the total protein originally added to the reaction mixture. The enzyme-bead complex was completely stable for at least 6 mo at −20°C.

Enzymatic and Chemical Assays

LPO activity was measured with *o*-dianisidine as described (25), but at pH 6. Relative latex concentrations were determined by light scattering at 500 nm, using samples boiled in 2% SDS. Absorbance was linear with latex concentration from 0.00075 to 0.04% by weight (OD_{500} = 0.03–1.5). Absolute latex numbers were also counted directly in some experiments in a hemocytometer at × 240 with bright-field optics. LPO-latex uptake into cells was measured by direct counts with phase contrast optics at × 1,000 or by assaying the cell lysates for LPO activity using the triiodide assay (Worthington Biochemical Co. Enzyme Manual). Lysosomal acid hydrolase activities were measured using the assay of Peters et al. (20). 4-Methylumbelliferyl-β-D-glucuronide trihydrate, 4-methylumbelliferyl-β-D-galactopyranoside monohydrate, and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glycopyranoside substrates (Koch-Light Laboratories, Colnbrook, Buckinghamshire, England) were the generous gift of Dr. Stanley Fowler of The Rockefeller University. Protein was measured by the method of Lowry et al. (10). For determination of radioactivity, 50-μl aliquots of sample were transferred to 1.5-ml microfuge tubes containing 5 μl of FCS as carrier protein. Cell monolayers had been lysed in 0.05% Triton X-100 (0.4 ml for 16-mm wells); other samples were transferred directly. After addition of ice-cold 10% TCA containing 100 mM KI, the tubes were placed in the cold for at least 1 h, then spun for 5 min in a microfuge at 12,000 rpm. The pellets were washed twice, then the bottoms of the tubes containing the pellets were cut off with a razor blade; counting was done and the pellets were counted in a Packard Auto-Gamma Scintillation Spectrometer model 5220 (Packard Instrument Company, Downers Grove, Ill.).

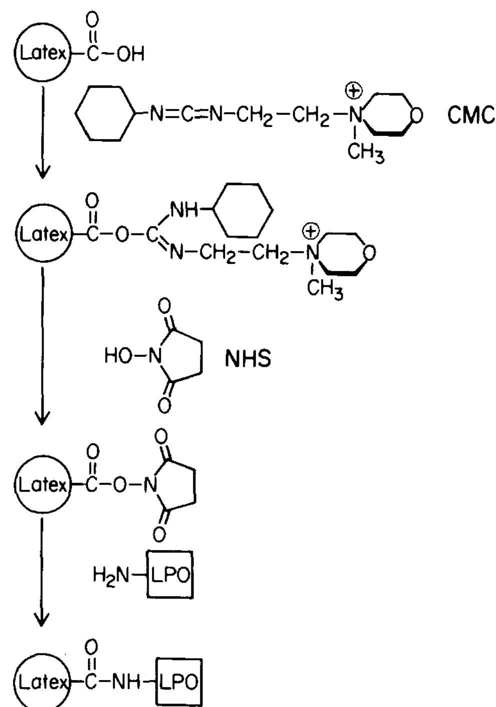

FIGURE 1 The covalent coupling of lactoperoxidase to carboxylated latex spheres by means of carbodiimide.

Electron Microscopy

Monolayer cultures were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, stained for cytochemistry when desired (see below), postfixed in 1% osmium tetroxide on ice for 1 h, and stained en bloc with 0.1% uranyl acetate for 30 min. The monolayers were dehydrated in graded ethanols, removed from the culture dish with propylene oxide, and embedded in Epon. DAB cytochemistry (5) was performed in cells fixed for 10 min and exposed to the reaction mixture for 15 min at room temperature. Acid phosphatase was detected by the method of Novikoff et al. (18) but with cytidine monophosphate as the substrate. Thin sections (1,000 Å) were mounted on Formvar-coated grids and stained with uranyl acetate and lead citrate. They were examined in a Siemens Elmiskop I electron microscope operating at 80 kV.

LPO-catalyzed Iodination of Macrophages

The procedure for iodinating from within the PL is described in detail under Results. To label the plasma membrane, freshly isolated peritoneal cells were washed and iodinated in suspension at 4°C by the method of Hubbard and Cohn (6). Macrophages were purified from this mixture either by adherence to a culture dish for 30 min at 37°C in the presence of FCS or by rosetting the macrophages with opsonized erythrocytes (27) and collecting the rosettes by velocity sedimentation through a continuous 5–14% gradient of bovine plasma albumin (fraction V, Armour Pharmaceuticals, Phoenix, Ariz.). In some experiments, we wished to iodinate only the surface of cells after they had ingested unmodified latex. To prepare these macrophages, 1 ml of 1:100 CM-latex stock was injected i.p. into each mouse. Peritoneal cells were harvested 30 min later and separated from uningested latex by centrifugation at 500 *g*.

Chemical Analysis of Radioiodinated Material

LIPIDS: Macrophages on a 35-mm dish were iodinated intracellularly, washed in PBS, and scraped from the dish in PD containing 5 mM sodium thiosulfate to retard oxidation of iodide (3). To these cells were added 2×10^8 J774 cells to provide carrier lipid. The cell suspension was extracted twice in chloroform-methanol by the method of Bligh and Dyer (1). The latex dissolved by these solvents was concentrated at the interface of the aqueous and organic phases. The organic phase was concentrated under nitrogen. The component lipids were resolved by two-dimensional, thin-layer chromatography on silica gel

plates. The solvent system for the first dimension was chloroform: methanol: ammonium hydroxide (65:25:5); for the second dimension, chloroform: acetone: methanol: acetic acid: water (30:40:10:10:5). Iodine and $^{125}I^-$, extracted and processed as were the cells, were run concurrently on a separate plate. Lipids were visualized under UV light after the plate was sprayed with 0.2% 2,7-dichlorofluorescein (Aldrich Chemical Co., Milwaukee, Wis.) in ethanol.

PROTEIN: Following intracellular iodination and washing, cells were scraped in a small (0.1–0.5 ml) volume of PBS and either lyophilized immediately or dried on glass-fiber filters (Whatman Inc., Clifton, N. J., GF/C). The filters were incubated in cold 10% TCA (6) and placed in glass vials with two drops of 1 N NaOH to bring the pH to neutrality. 1 ml of pronase (1 mg/ml in 0.1 M borate buffer, pH 7.6) was added and the vials were incubated at 37°C with gentle shaking. After 24 h, the supernates were removed and frozen. An additional 1 ml of fresh pronase solution was added for an additional 24 h. At this time, the two supernates for each filter were combined. Hydrolysis was stopped by the addition of TCA to a final concentration of 10%. The mixture was filtered through a 0.45-μm Millex filter (Millipore Corp., Bedford, Mass.), lyophilized, and chromatographed on a Sephadex G-25 column (16 × 1 cm) in 1 M acetic acid (8) at a flow rate of 4 ml/h. Spectrophotometrically detectable quantities of protein (FCS), $^{127}I^-$, monoiodotyrosine (MIT) and diiodotyrosine (DIT) (both from Nutritional Biochemicals, Cleveland, Ohio) were mixed with the radiolabeled samples on each run to serve as carriers and internal standards.

Autoradiography (ARG)

LIGHT MICROSCOPE LEVEL (LM-ARG): Coverslips bearing cell monolayers or subcellular fractions were fixed either in glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.4) or by rapid drying with a hot-air blower, followed by immersion in absolute methanol. The coverslips were dipped in Ilford L4 emulsion diluted 1:1 with water, exposed for 1–7 d, and developed in Kodak D-19.

ELECTRON MICROSCOPE LEVEL (EM-ARG): The flat substrate method of Salpeter and Bachman (22) was employed. Thin sections on collodion-coated slides were coated with a crystalline monolayer of Ilford L4 emulsion (purple interference color), exposed at 4°C, and developed with either D-19 or Microdol X (Eastman-Kodak, Rochester, N. Y.). D-19 was used for statistical analyses because of its greater sensitivity.

Analysis of EM Autoradiograms

The probability circle method was used to localize the source of radiolabel corresponding to particular silver grains (23). In this analysis, a circle is drawn around each grain center that has a 50% probability of containing the source of the grain. For ^{125}I, Ilford L4 emulsion, and 1,000 Å sections, the radius of this circle is 1.73 × the half-distance (HD) of 800 Å (24). Every organelle completely or partially within that circle is recorded. The number of grains assigned to each organelle was normalized for the relative area of the cell profile that the organelle comprised. This was determined by overlaying each autoradiogram with a grid of random points. A 50% probability circle was drawn around each point, and any organelle completely or partially within the circle was recorded.

Scattering of grains from a heavily labeled compartment into adjacent areas will falsely elevate the apparent degree of labeling of these other areas. To correct for this "cross fire," we employed the method of Farquhar et al. (4). In this analysis, the density of grains associated with an organelle when it is within 2 HD of the suspected heavily labeled compartment is corrected by adjusting for the difference in grain density of that organelle when it is near as opposed to far (>2 HD) from that compartment.

Cell Fractionation

We used a modified version of the discontinuous sucrose gradient first described by Wetzel and Korn (28). Cell monolayers were washed extensively in cold PBS, then scraped in 1.5 ml of isotonic sucrose containing 1 mM EDTA. Cells werre homogenized on ice to ~90% breakage in a loose-fitting Dounce homogenizer. The homogenate was brought to 35% sucrose by adding a 60% solution. 0.5 ml was taken for analysis and the remaining 2.5 ml was transferred to a nitrocellulose centrifuge tube. This was overlaid with 6 ml of 25% sucrose and then 2.5 ml of 10% sucrose. The middle layer must be 25% sucrose because LPO-latex has a higher density than plain polystyrene latex (ρ = 1.066 vs. 1.050). All sucrose solutions are expressed as wt/wt percentages. All contained the protease inhibitors aprotinin (1%) and PMSF (1 mM). The pH was held at 7–7.4 by the addition of 5 mM sodium phosphate buffer.

The discontinuous gradient was centrifuged for 1 h at 100,000 g in a Beckman L5-65 ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) at 4°C using the SW-41 rotor. Fractions were harvested with a bent Pasteur pipette.

To assure ourselves that lysosomal hydrolases were present in the presumptive purified PL fraction (10/25% interface) because of phagosome-lysosome fusion, we examined the behavior of the exogenous lysosome marker HRP in our gradients. When HRP-loaded cells were fed latex beads, the marker was present in the 10/25% interface. In contrast, no HRP floated if we homogenized a mixture of two cell populations, one exposed to latex only, and the other to HRP only.

To concentrate latex-containing fractions for gel electrophoresis, aliquots ≥0.4 ml were expelled through a syringe onto a 13-mm-diameter Millipore filter (0.45-μm pore size). The filter containing the sample was boiled in 2% SDS, 5% β-mercaptoethanol, for 2 min; then the latex was removed by centrifugation. Control experiments showed that no radiolabeled bands were depleted or lost from these samples.

To examine the contents of the 10/25% interface fraction morphologically, the sample was diluted in PBS and pelleted at 12,000 rpm for 10 min in a microfuge. The pellet was fixed in glutaraldehyde, stained with DAB, postfixed in osmium tetroxide, dehydrated, and embedded in the microfuge tube. Sections of the embedded pellet were cut parallel to and perpendicular to the direction of centrifugation (2).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

5–15% gradient slab gels (6) or 4–11% slab gels (17) 15 cm long and 1 mm thick were used. Samples were boiled for 2–3 min in 2% SDS, 5% β-mercaptoethanol (final concentration), before being loaded onto the gel. The samples contained the protease inhibitors PMSF and aprotinin. Proteins of known molecular weight (monomeric molecular weights in parentheses) were run as standards. These were myosin heavy chain (220,000), β-galactosidase (135,000), phosphorylase b (93,000), LPO (78,000), bovine serum albumin (BSA) (68,000), immunoglobulin G (IgG) heavy chain (50,000), ovalbumin (43,000) concanavalin A (26,000), soybean trypsin inhibitor (22,000), and cytochrome c (12,000). At least four standards were run with every gel.

After electrophoresis at constant current (usually 20–30 mA for 5–15% gels and 37.5 mA for 4–11% gels), the gels were fixed in 7.5% acetic acid, 30% methanol, stained with Coomassie blue, destained in acetic acid/methanol, and dried under vacuum. Autoradiograms of the dried gels were made on DuPont Cronex or Kodak XR-1 x-ray film. When an enhancing screen (DuPont Lightning Plus, E. I. DuPont de Nemours & Co., Wilmington, Del.) was used, the film was presensitized by the method of Laskey and Mills (9).

RESULTS

Delivery of LPO-Latex to Secondary PL

A protocol was designed to deliver LPO-latex rapidly and selectively to macrophage PL (Fig. 2). Dilute suspensions of LPO-latex were centrifuged onto macrophage monolayers at 4°C. Scanning EM showed that particles were attached to the surfaces of the cells and to the dish. Latex beads on cells sat on the plasma membrane and were not depressed into the cell. The cells were then brought to 37°C for 15–30 min, resulting in a rapid and synchronous wave of phagocytosis of the LPO-

Intracellular Iodination of Macrophages

1. WASH cells 4 × with cold PBS.
2. ADD LPO-latex in PBS.
3. CENTRIFUGE at 1,000 g for 2 min at 4°C.
4. DECANT supernate.
5. REPLACE with warm PBS or culture medium.
6. INCUBATE at 37°C to allow ingestion (15–30 min).
7. TRYPSINIZE to remove uningested latex (5 min at 37°C; 200 μg/ml). (Unnecessary with dense cell monolayers)
8. WASH cells 4 × with cold PBS.
9. CHILL cells on ice-water bath.
10. IODINATE cells on ice (4°C) in PBS containing carrier-free ^{125}I, 20 mM glucose, and 0.24 mU/ml GO.
11. WASH with $K^{127}I$ in PBS, then with PBS.
12. TEST VIABILITY by trypan blue dye exclusion.
13. WASH with PBS.
14. FURTHER PROCESSING, i.e., lysis for TCA precipitation, fixation for ARG, homogenization for cell fractionation.

FIGURE 2 Protocol for ingestion of LPO-latex and intracellular iodination.

latex. Ingestion was proportional to bead dose over a range corresponding to the uptake of 10–100 beads per cell. Two methods were employed to eliminate extracellular and dish-bound latex. In one, confluent monolayers were established so that dish-bound latex was effectively cleared by the cells themselves. At lower cell densities, brief trypsinization (200 μg/ml for 5 min) removed the vast majority of the extracellular beads. In separate experiments we found that trypsin neither inactivated nor released LPO activity from LPO-latex beads. The same results were obtained with either ingestion protocol, with one exception. There were a few plasma membrane polypeptides that were trypsin sensitive and whose intensities were altered in SDS-PAGE autoradiograms. All of the gels presented in this and the accompanying paper (11) show cells that had not been trypsinized.

After the phagocytic pulse, all beads had been ingested and were situated in typical membrane-bounded vacuoles. Scanning electron microscope examination of >1,000 cells showed that all latex had been completely internalized. Transmission EM examination of the DAB-H_2O_2 reaction product localized LPO enzymatic activity to the rim of each latex sphere (Fig. 3 *a*). Reaction product was absent if H_2O_2 or DAB were omitted

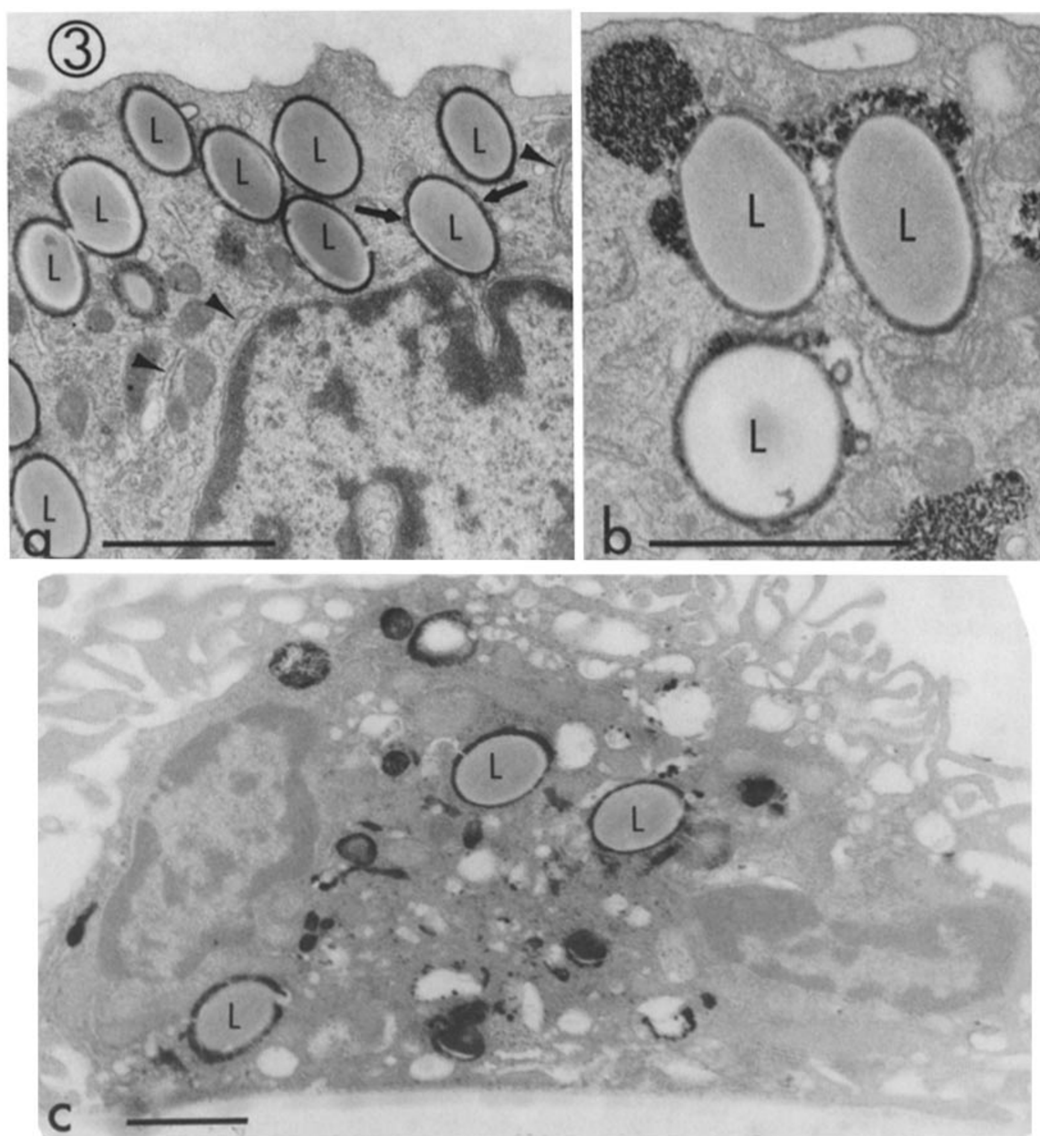

FIGURE 3 The distribution and properties of LPO-latex within cultured macrophages. (*a*) DAB-H_2O_2 cytochemistry to visualize peroxidase. Reaction product surrounds each sphere (*L*). In some instances, the PL membrane is apparent (arrows). Under the conditions employed, only exogenous (i.e., LPO) peroxidatic activity is visualized, and the endogenous activity of the endoplasmic reticulum (arrowheads) is not seen. Bar, 1 μm. × 23,000. (*b*) Thorotrast-containing PL. The lysosomes of macrophages were first loaded with the exogenous marker, Thorotrast (0.5% suspension in culture medium for 9 h). The LPO-latex was administered for 20 min at 37°C, and the cultures were fixed and stained with DAB-H_2O_2. The electron-dense colloid particles are present in the LPO-latex vacuoles (*L*), indicating that fusion of 2° (secondary) lysosomes with latex has occurred. Under these conditions, the lysosomes are swollen with Thorotrast and the usual tight apposition of PL membrane around the entire bead is not always seen. Compare to *a* and *c*. Bar, 1 μm. × 35,500. (*c*) Fusion of latex with lysosomes is also demonstrable with acid phosphatase cytochemistry. Dense reaction product is visualized around all LPO-latex spheres (*L*), as well as within many other lysosomes. This cell is typical of several hundred sampled in three different experiments. A similar result was obtained after administration of nonmodified CM-latex as well. Bar, 1 μm. × 16,000.

TABLE I

Variables Affecting Intracellular Iodination at 4°C

Experimental variable	Peritoneal cells plated per culture*	^{125}I	LPO-latex (dilution)	GO	cpm/Culture ± SD of triplicate measurements
		μCi/ml		*mU/ml*	
GO concentration	2.6×10^6	40	1:800	0.0	10,112 ± 224
				0.24	40,596 ± 6,604
				0.72	56,784 ± 1,432
				2.4	61,204 ± 3,060
				7.2	64,456 ± 3,680
				24.0‡	72,364 ± 9,572
LPO	2.6×10^6	40	1:800	0.0	11,458 ± 1,058
				0.24	27,552 ± 1,764
				0.24 + 6mM NaN_3	160 ± 5
			1:800 CM-latex	0.24	202 ± 49
Dose of LPO-latex	1.3×10^6	40	1:2,400	0.24	6,999 ± 833
			1:1,200		11,594 ± 4,058
			1:600		31,399 ± 2,527
			1:300		115,555 ± 12,722
$^{125}I^-$ concentration	2.2×10^6	40	1:800	0.24	21,976 ± 1,923
		80			46,676 ± 2,155

* Resident peritoneal cells were cultured for 2 d in 16-mm Costar wells in 1 ml of medium. Nonadherent cells were removed by washing. After ingestion of latex, trypsination, and chilling, cells were incubated at 4°C for 30 min in the presence of the stated reagents. The monolayers were then washed and lysed in Triton X-100, and 50-μl aliquots were taken for quantitation of TCA-precipitable radioactivity.

‡ Viability was >99% by trypan blue dye exclusion in all cases, except at 24 mU/ml GO, where viability was 90-95%.

from the reaction, or if CM-latex lacking LPO had been ingested. If macrophage lysosomes were loaded with Thorotrast before LPO-latex ingestion, all the vacuoles containing DAB-positive beads also exhibited colloid particles, indicating that phagosome-lysosome fusion had taken place (Fig. 3*b*). Vacuoles were examined for the endogenous lysosomal marker acid phosphatase, and all were surrounded by reaction product within 20 min of the start of ingestion (Fig. 3*c*). We conclude that LPO can be introduced selectively into typical PL.

Radioiodination with Intralysosomal LPO-Latex

The procedure employed for intracellular iodination is outlined in Fig. 2. After ingestion of LPO-latex, monolayers were washed in cold PBS and maintained at 4°C on an ice-water bath to inhibit pinocytosis. ^{125}I was added in cold PBS containing 20 mM glucose and was incorporated linearly for ~20 min into TCA-precipitable counts. Iodination was enhanced 3- to 6-fold by the addition of low concentrations of GO to the medium (Table I). We presume that iodination occurring in the absene of exogenous GO was mediated by the H_2O_2 that was generated by cells during phagocytosis (13). Several sensitive assays showed that there was no H_2O_2 contaminating our reagents. To show that iodination was catalyzed by LPO, we blocked LPO activity by the addition of 0.02% (6 mM) sodium azide, or omitted it completely (cells ingested unmodified latex). Under these conditions no radioactivity was incorporated (Table I).

The effect of varying the concentration of the different reactants was assessed. Iodination increased with increasing numbers of LPO-latex beads within the phagocytic capacity of the cells (Table I). To be certain that all beads were entirely intracellular, we generally used a bead dilution of 1;8,000 (A_{500} = 0.33 from our LPO-latex stock [~1.5% latex by weight]), which scanning and transmission EM had shown to be completely phagocytosed within 15 min. This dose results in an uptake of 24–36 beads per cell. Further increases in exogenous GO above our standard dose of 0.24 mU/ml resulted in little increase in iodination (Table I) and could be toxic to the macrophages. In contrast, iodination varied linearly with the concentration of ^{125}I (Table I) within the range generally employed.

We conclude that macrophages that have interiorized LPO-latex will incorporate iodide into macromolecular products. Incorporation is most efficient with the exogenous generation of H_2O_2 and does not alter the viability of the cell.[1]

Biochemical Characterization of the Cell-associated Radiolabel

PROTEIN: After intracellular iodination, the vast majority of the radiolabel associated with the cell monolayer was unreacted $^{125}I^-$. Even extensive washing with carrier iodide failed to remove this label, which eluted relatively slowly from cells. The remainder of the radiolabel eluted exclusively in the void volume of the Sephadex G-25 column. (Table II).

TCA precipitation removed most of this free iodide from acid-insoluble cellular material. When this material was subjected to extensive proteolysis, most of the radiolabel co-chromatographed with MIT. A substantial portion ran with free iodide, but much of this may have been the result of de-iodination of MIT during the procedure. No radiolabel was ever detected as DIT.

LIPIDS: A chloroform:methanol extract of iodinated macrophages was subjected to two-dimensional, thin-layer chromatography on silica gel under conditions that were found to separate I^- and I_2 from neutral lipids and phospholipids. Table III shows that at least 93% of the lipid-extractable material was

[1] These cells are morphologically indistinguishable from control cells when returned to culture, as we report in the accompanying paper (11).

TABLE II

Pronase Digestion and Identification of Molecular Species Iodinated Intracellularly

Sample	Percent of total label in cell lysate				
	Vol*	Pep‡	I^-	MIT	DIT
Whole cell lysate	1.5	0	98.5	0	0
TCA-precipitated and pronase-digested whole cell lysate	0	2	34	64	0

Lysates of macrophages iodinated intracellularly were chromatographed on Sephadex G-25 before and after TCA precipitation and subsequent pronase digestion. See Materials and Methods.
* *Vol*, void volume (protein).
‡ *Pep*, small polypeptides (mol wt <5,000).

TABLE III

Analysis of Lipid Extract of Macrophages Iodinated Intracellularly

Spot	Identification of spot on TLC*	cpm	Percent of lipid extract
SF-I	Free iodide at solvent front	1,034	6.9
I^-	Free iodide main spot	4,363	29.2
I_2	Iodine	8,462	56.7
1‡	Neutral lipid	194	1.3
2‡	Diphosphatidyl glycerol	305	2.0
3‡	Phosphatidyl ethanolamine	185	1.2
4	Phosphatidyl choline	80	0.5
5	Phosphatidyl inositol	110	0.7
6	Phosphatidyl inositol	101	0.67
7	Phosphatidyl serine	11	0.07
8	Phosphatidic acid	12	0.08
0	Origin	69	0.5

* A chloroform methanol extract of iodinated cells was resolved by two-dimensional thin-layer chromatography (*TLC*) on silica gel plates. Lipids were visualized under UV light after staining with dichlorofluorescein and identified by their positions on the plate. The locations of iodine and iodide were determined from standards run on identical plates in the same solvent tank. After identification of spots, these regions were carefully scraped off the plate for quantitation of radioactivity.
‡ These spots were located very close to the SF-I and I_2 spots.

free iodide or iodine. Most of the remaining label was associated with three spots that ran very close to the free iodide and iodine regions. The counts recovered in these lipid spots could well represent contamination from I^- and I_2, because phospholipids well separated from the former contained only traces of radioactivity. Because the lipid extractable counts were only 5% of the total radioactivity of the cell lystate, and equivalent to less than half of the TCA-precipitable counts, little if any ^{125}I was incorporated into lipid.

We conclude that LPO-latex-mediated intracellular iodination exclusively labeled polypeptides (MIT) and that nonenzymatic iodination of cellular lipids was negligible.

The Localization of Incorporated Radiolabel—ARG

The localization of incorporated iodide was examined by means of autoradiography at the light and electron microscope levels. Cell monolayers on glass coverslips when exposed for as little as 18 h showed that >95% of the macrophages were radiolabeled (Fig. 4*a*). Grains were concentrated over latex-containing areas and were absent or diminished over the nucleus. Latex labeling was best visualized in cells flattened by air-drying and was noted to be heterogeneous, with some profiles being more heavily labeled than others (Fig. 4*b*).

EM autoradiograms were prepared by the flat substrate method of Salpeter and Bachman (22). The cells had been stained with DAB to unambiguously identify the LPO-latex; however, an identical labeling pattern was obtained in unstained cells. Radiolabel was associated with the periphery of the beads (Fig. 5). In this experiment, 45% of the beads bore grains after 3.5 d of exposure. About 5% of the latex profiles were heavily labeled (Fig. 5*a*). These were often seen side by side with lightly labeled beads. Occasionally the grains would obscure the bead, so that alternate serial sections had to be processed to ensure that cells containing "hot spots" actually had an interiorized bead in these locations. A substantial proportion of the incorporated label appeared to be associated with the heavily labeled beads, although the grains themselves could not be accurately counted. The distribution of the remaining label was further analyzed statistically by the probability circle method (23). In an analysis of 553 grains in 36 random cell profiles, the density of grains over LPO-latex was at least 10 × greater than over any other compartment, including plasma membrane (Table IV).

We conclude that PL are being iodinated within live cells at 4°C and that the labeling intensity varies considerably from one bead to another.

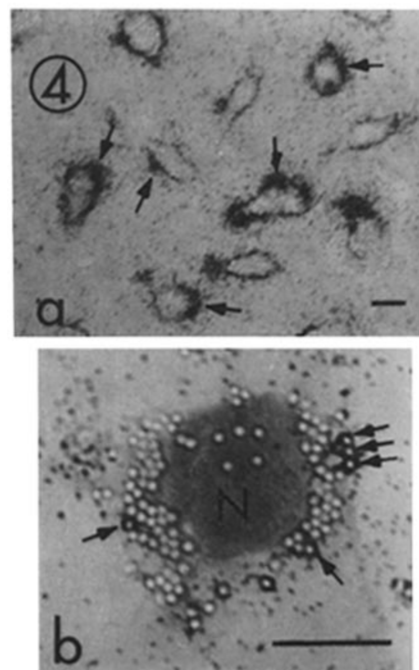

FIGURE 4 Light microscope autoradiography of macrophage monolayers iodinated intracellularly. For illustrative purposes, the macrophages were allowed to ingest large doses of LPO-latex relative to those generally employed. (*a*) Low-power, bright-field microscopy of 12 macrophages fixed in glutaraldehyde and exposed to Ilford L4 emulsion for 1 d. The black silver grains outline the perimeter of each cell (arrows). The central pale nuclear region of each cell profile exhibits background radioactivity. Bar, 10 μm. × 480. (*b*) High-power view of a single macrophage that was flattened by air-drying, fixed in methanol, exposed to Ilford L4 emulsion for 1 d, and stained with azure II-methylene blue. The nucleus (*N*) and many ingested, refractile, LPO-latex spheres are evident, but the remainder of the cytoplasm is otherwise unstained. A subpopulation of latex beads is clearly surrounded by dark rims of dense silver grains (arrows) Bar, 10 μm. × 1,600.

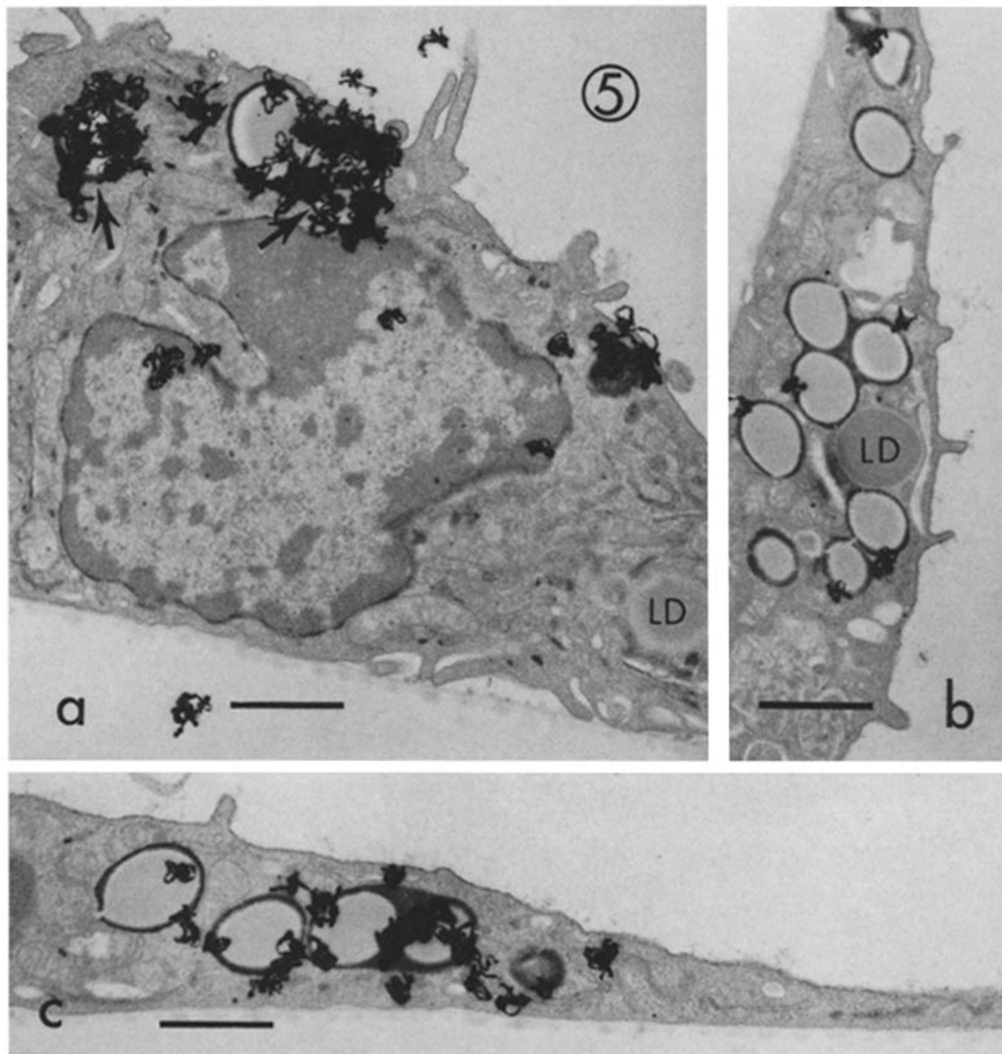

FIGURE 5 EM autoradiography of cells iodinated intracellularly by LPO-latex. Cells were stained with DAB-H_2O_2 to visualize the LPO. In this experiment longer staining visualized endogenous peroxidase activity as well. Fig. 5 *a* illustrates three heavily labeled phagolysosomes. The arrows point to rims of DAB reaction product, which for the most part are obscured by silver grains. This intense labeling is not typical for the exposure period we employed in these studies. Fig. 5 *b* and *c* shows more typical cell profiles containing unlabeled or lightly labeled phagolysosomes. The grains are clearly associated with the periphery of the LPO-latex beads. The cells in *a* and *b* contain spherical lipid droplets (*LD*) that lack rims of peroxidase activity. Ilford L4 emulsion, D-19 developer, 3.5 d of exposure. Bar, 1 μm. × 15,000.

TABLE IV
Distribution of Grains after Intracellular Iodination *

	Compartment				
	LPO-latex	Cytoplasm‡	PM	Nucleus	Mitochondria
Total grains	362	314	68	26	8
Corrected for cross fire from LPO-latex§	362 (76.5%)	49 (10.3%)	36 (7.6%)	23 (4.9%)	3 (0.6%)
Relative grain density‖	4.665 (Profile) 5.977 (Rim)	0.206	0.478	0.320	0.240

* When the probability circle around a grain fell over more than one compartment, the grain was assigned to each organelle falling completely or partially within the circle. (Of the total grains assigned to cytoplasm, 198 were shared with LPO-latex, of the total grains assigned to plasma membrane, 19 were shared with LPO-latex; of the total grains assigned to nucleus and mitochondria, 3 and 5, respectively, were shared with LPO-latex.)

‡ Cytoplasm includes structures not in separate categories, e.g., ground cytosol, RER, Golgi apparatus, vacuoles.

§ See Materials and Methods and reference 4.

‖ Percent of total grains in an organelle compartment divided by the percentage of cell area occupied by that organelle. For LPO-latex, the data were calculated for the total area of the latex profile (*Profile*) and the area within 1.73 HD from the rim of the bead (*Rim*).

Distribution of Radiolabel within PL—Cell Fractionation

The extent to which radiolabel was incorporated into the membrane or contents of PL was examined by first isolating a PL fraction from internally labeled cells. The distribution of latex and acid hydrolases in the gradient is shown in Table V and was unchanged by iodination. The percentage of total LPO enzymatic activity in each fraction matched the percentage of latex (not shown). Some 75–80% of the latex floated to the upper 10/25% interface and thin sections passing through the entire 10/25% interface fraction revealed a clean preparation of PL with virtually no contaminating mitochondria, rough microsomes, or Golgi saccules. The extent of plasma membrane contamination of this fraction was evaluated by iodinating the cell surface after the ingestion of CM-latex. After homogenization and fractionation, 1.4% of the radiolabel rose to the 10/25% interface (Table V).

Note (Table V) that the proportion of total radioactivity in the 25/35% interface and pellet was high relative to their latex content. Autoradiography of these fractions showed that they contained the subpopulation of heavily labeled PL visible in thin sections (Fig. 5*a*). SDS-PAGE analysis revealed that the same polypeptides were labeled in all fractions (Fig. 6*b*).

The relatively uncontaminated PL fraction (10/25% interface) contained acid hydrolases, and these enzymes demonstrated latency. Exposure to Triton X-100 (Table VI) increased enzymatic activity 6- to 9-fold and suggested the intact nature of these organelles.

The distribution of incorporated iodide in the PL was examined after separation of the matrix and membranes by means of three cycles of freezing and thawing. In the experiment shown in Table VII, only ~2% of the TCA-precipitable radioactivity was released by this procedure, whereas >75% of the acid hydrolase activity was solubilized. In three other experiments no detectable TCA-precipitable counts were released. We conclude that the internal labeling procedure preferentially labels the membrane of PL. With this information, we re-analyzed the EM autoradiograms, using the area within 1.73 HD of the PL membrane rather than the entire bead profile as the denominator for relative grain density. This reduced the area of the presumptive source compartment from 1.015 μm^2 to 0.748 μm^2. The labeling density of the LPO-latex PL membrane was 12.5 × greater than that of any other organelle (Table IV).

The Polypeptides of Internally Labeled PL Membrane

The spectrum of PL proteins iodinated intracellularly was analyzed by SDS-PAGE of cell lysates. 24 distinct bands ranging in apparent molecular weight from 250,000 to 12,000 were routinely visible in autoradiograms of the gels (Figs. 6 and 7). A band migrating slightly behind the LPO standard was routinely visible, but control experiments employing [^{125}I]LPO-latex fed to macrophages demonstrated that none of the bands corresponded to iodinated LPO or fragments thereof.

Neither the major cytoplasmic proteins actin and myosin (Fig. 6*a*), nor those of the culture medium (BSA and IgG) (Fig. 7*a*) were labeled under these conditions. In fact, the iodination pattern was identical whether beads were ingested in PBS or medium containing 10% FCS. The identical spectrum of iodinated polypeptides was obtained with endogenous H_2O_2, in the absence of GO. The restricted nature of the iodinated polypeptides is consistent with the selective labeling of PL membrane proteins.

Intracellularly iodinated PL proteins were then compared to plasma membrane (PM) proteins iodinated either by soluble LPO at 4°C or by LPO-latex spheres bound to the cell surface at 4°C. In both instances, the reduced temperature effectively

TABLE V

Subcellular Fractionation of Macrophages

Fraction	Not iodinated		Intracellular iodination			Surface iodination after the ingestion of unmodified latex	
	Latex	Total NAGase*	Latex	Total NAGase*	TCA	Latex	TCA
	× 10^6	μU	× 10^6	μU	cpm	OD_{500}	cpm
10%	—‡	—	—	—	—	—	—
10/25% IF	95.55 (75.8)§	37.7 (3.1)	116.03 (80.9)	33.0 (2.1)	44,948 (30.4)	0.21 (67.7)	2,478 (1.4)
25%	—	—	—	—	—	—	—
25/35% IF	4.41 (3.5)	100.4 (8.2)	3.99 (2.8)	70.0 (4.5)	35,160 (23.8)	—	24,830 (14.5)
35%	—	219.6 (18.0)	—	275.2 (17.9)	22,660 (15.3)	—	36,660 (21.4)
Pellet	26.1 (20.7)	851.6 (70.6)	23.4 (16.3)	1,157.8 (75.4)	44,880 (30.4)	0.10 (32.3)	107,500 (62.7)

Macrophages containing latex were homogenized and fractionated on discontinuous sucrose gradients. The gradient layers as well as the interfaces (*IF*) were assayed for *N*-acetyl glucosaminidase activity (*NAGase*), TCA-precipitable radioactivity (*TCA*) and latex, either by counting a suitable dilution in a hemocytometer chamber or by light scattering (OD_{500}).

* After addition of Triton X-100.

‡ Not detectable or ≪1% of total.

§ Numbers in parentheses are the percentage of the total recovered.

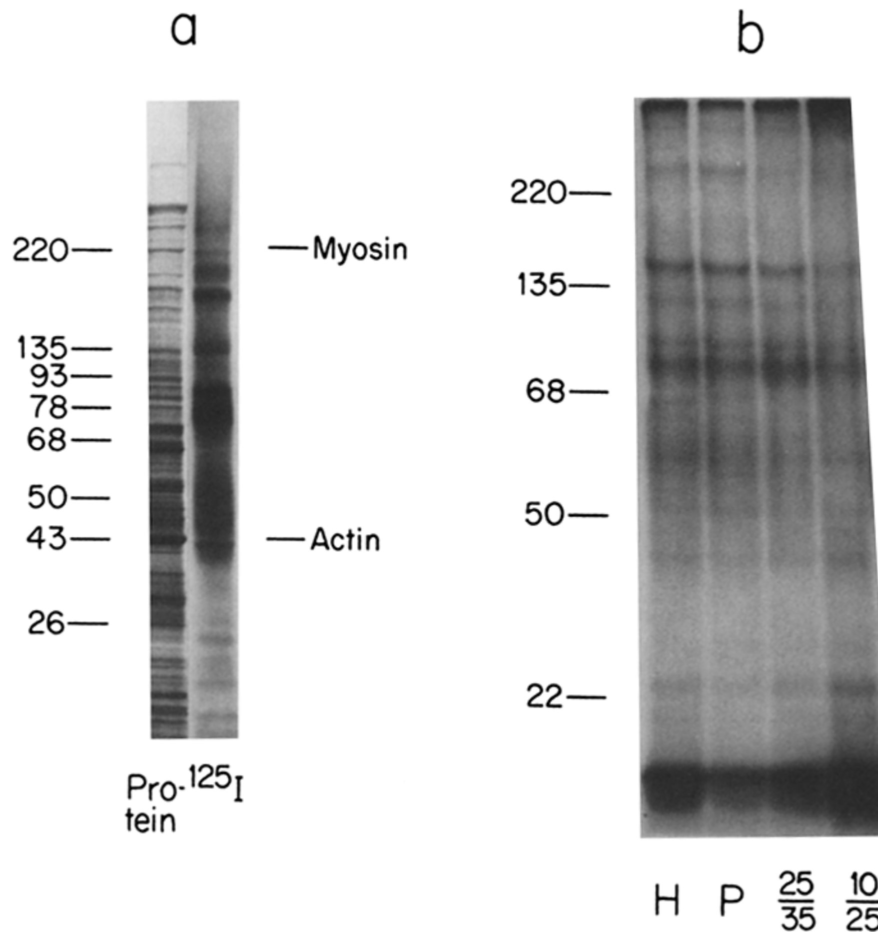

FIGURE 6 (*a*) LPO-latex mediates the iodination of a select group of cell proteins. Macrophages were iodinated from within the PL and cell lysates separated by SDS-PAGE with a 4–11% gradient gel. The gel was stained with Coomassie blue (*Protein*), dried, and exposed for autoradiography (^{125}I) on DuPont Cronex film for 4 d with an enhancing screen. Positions of the gel standards are indicated on the left, while the positions of the presumptive actin and myosin bands are on the right. (*b*) The spectrum of iodinated polypeptides in fractions of cell homogenates are similar. Macrophages were iodinated by phagocytosed LPO-latex. Cell homogenates were separated on discontinuous sucrose density gradients, and aliquots of each fraction containing equal numbers of TCA-precipitable counts were analyzed in autoradiograms of SDS-PAGE, using a 5–15% gradient gel. The spectrum of iodinated polypeptides is similar in all samples. Analysis of such autoradiograms from six separate experiments showed no consistent difference among the fractions. Homogenate (*H*), pellet (*P*), and 25/35% and 10/25% interfaces (see Table V).

TABLE VI

Latency of Acid Hydrolase Activity in LPO-latex PL

Enzyme	Activity: Free	Activity: Total	Total/free	Percent of latent activity*
	μU			
N-Acetyl glucosaminidase	8.04	48.43	6.02	83.4
β-Galactosidase	4.54	40.1	8.83	88.7
β-Glucuronidase	4.92	35.0	7.1	85.9

Portions of the 10/25% interface were incubated with the 4-methylumbelliferyl-derivatized substrates for 10 min at 37°C under isotonic conditions (*Activity—Free*) or in the presence of 0.1% Triton X-100 (*Activity—Total*).

* Percent of latent activity $= \frac{\text{total-free}}{\text{total}}$.

TABLE VII

Distribution of Radiolabeled Protein within the PL

	Acid hydrolase activity (in relative fluorescence units): N-Acetyl glucosaminidase	Acid hydrolase activity (in relative fluorescence units): β-Glucuronidase	TCA-precipitable ^{125}I
			cpm
Total sample	95.7	53	6,665
Supernate	72.4	43.2	150
% Released	75.6	81.5	2.2

Purified PL from cells iodinated intracellularly were retrieved from the 10/25% interfaces of discontinuous sucrose density gradients. The fraction was diluted 1:1 in isotonic sucrose and passed through three cycles of freezing and thawing to destroy latency. Samples for total activity were first taken and then the latex beads were pelleted in a microfuge to yield supernate (released) activities. All data are means of duplicate samples, which agreed within 5%.

blocked LPO uptake and iodination was restricted to the PM. The SDS-PAGE autoradiograms of PM and PL membranes were very similar (cf. lanes *2* and *3* of Fig. 7 *a*), except for minor differences in the intensity of a few high molecular weight bands (asterisks, Fig. 7 *a*). Control experiments in which phagocytosis was not dependent on centrifugation of latex gave identical results. The pattern of polypeptides radioiodinated intracellularly was also unchanged if the cells were maintained at 37°C for 1 h after latex uptake and then iodinated (Fig. 7 *a*, lane *1*).

When macrophage PM was iodinated and unmodified latex

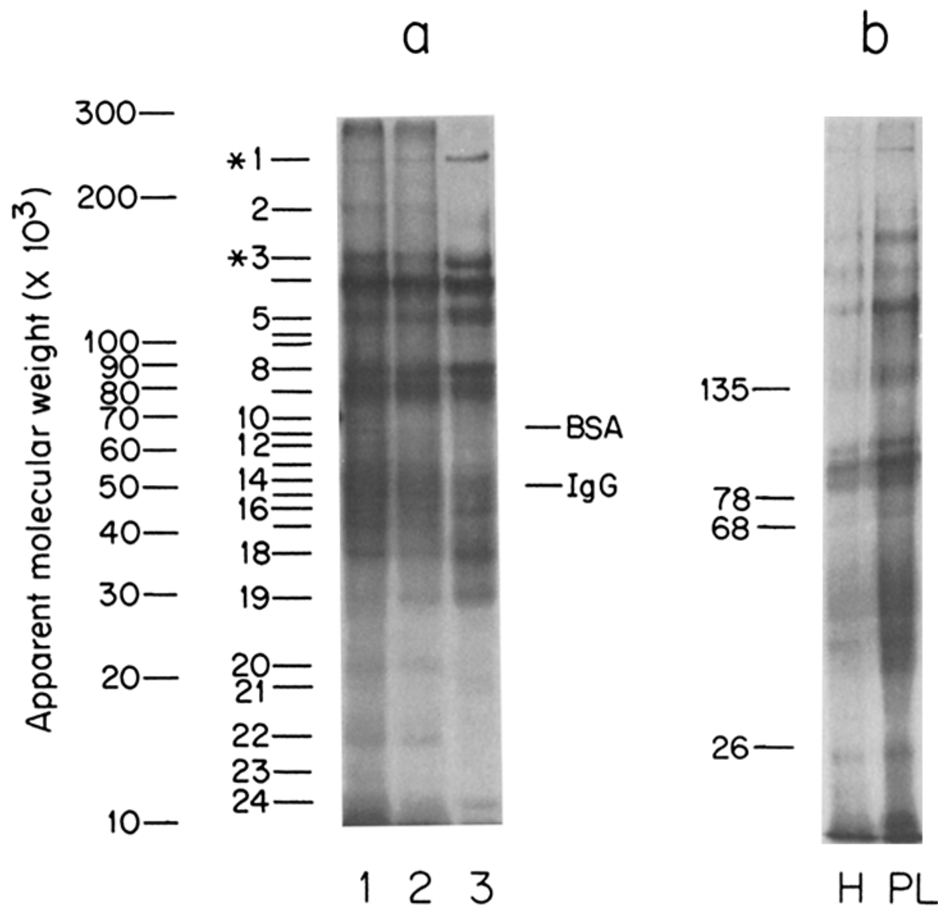

FIGURE 7 (*a*) Comparison of PL and PM iodinated polypeptides. Macrophages were iodinated under three different conditions, and equal numbers of TCA-precipitable counts were analyzed by autoradiography of 5–15% SDS-polyacrylamide gels. In lanes *1* and *2*, the cells were iodinated intracellularly by phagocytosed LPO-latex. In lane *2* the cells were iodinated immediately after phagocytosis (our standard conditions), whereas in lane *1*, the cells were cultured an additional 60 min at 37°C between the ingestion and iodination parts of the protocol. In lane *3*, the PM of resident peritoneal cells were iodinated in suspension at 4°C, and then the macrophages were purified by plastic adherence for 30 min at 37°C. 24 Distinct bands appear on the autoradiogram (indicated on the left). The radiolabeled bands are identical in lanes *1*, *2* (PL membrane), and *3* (PM), except for two bands (asterisks) that are consistently more heavily labeled after cell surface iodination. A scale of apparent molecular weights based on the position of gel standards is on the left. The positions of the two major iodinatable serum proteins, IgG and BSA, are indicated on the right. (*b*) Comparison of macrophage PM with PM interiorized during phagocytosis. This experiment is to be compared with *a*, in which PL membrane was labeled after rather than before phagocytosis. In *b*, the protocol was to label the surface of macrophage monolayers with soluble LPO at 4°C and then to drive some of the PM into the cell with a 5-min pulse of unmodified latex. Cells were then homogenized and a purified PL fraction was prepared on sucrose density gradients. The spectra of labeled bands in the total homogenate (*H*) and PL fraction (*PL*) are identical. These samples were run on a 4–11% SDS-polyacrylamide gel, which expands the high molecular weight end of the gel. Positions of gel standards (× 10^3) are indicated at the left.

spheres were ingested thereafter, phagosomes isolated from these cells yielded exactly the same autoradiographic pattern as the whole cell homogenate (Fig. 7 *b*). Therefore, it is unlikely that the differences in labeling intensity seen in Fig. 7 *a* were resulted from selective exclusion of these PM proteins from the phagosome. It is more likely that the differences represent altered susceptibility of these polypeptides to iodination when in the PL.[2] We conclude that the major iodinatable membrane proteins of the macrophage lysosome are the same as those on the PM. Fusion of lysosomes with phagocytic vacuoles imparts no unique proteins that can be detected by these techniques.

DISCUSSION

We have established an effective halogenating system within the lumen of secondary lysosomes that selectively iodinates the tyrosine groups of membrane polypeptides. To accomplish this, it was necessary to construct particles with covalently linked LPO, an enzyme that has a pH optimum in the acidic range and preferentially utilizes iodide rather than the other halides Cl^- and Br^-. In this locus, LPO maintains its enzymatic activity for prolonged periods of time, allowing iodination to be carried out sometime after the phagocytic event. Although the endogenous production of hydrogen peroxide allowed some iodination to take place (13), optimum values were obtained only after the extracellular generation of this reactant. This implies that a portion of H_2O_2 can traverse the PM, cytosol, and PL membrane and interact with the intravacuolar LPO. Similar conclusions have been reached by Reed (20) and Root (21) in their studies of granulocyte metabolism and microbicidal activities. Even less is known about the transport and compartmentalization of iodide in the macrophage, although this component must also enter the phagolysosome.

Some of the uncertainties concerning the intravacuolar con-

[2] When intralysosomal pH is raised during the iodination reaction by exposing the cells to 100 μM chloroquine at that time, the iodination pattern of the PL is exactly identical to that of the PM.

centrations of iodide and hydrogen peroxide may be reflected in the variation in labeling intensity of individual LPO-latex PL. Although the majority of PL contained label by EM autoradiography, a small percentage were heavily labeled. Similarly, cell fractionation studies revealed a small population of heavily labeled PL with increased buoyant density in sucrose gradients. The polypeptides labeled in this fraction, however, were identical to those labeled in the majority of PL (Fig. 6 *b*). What is most striking, however, is the selective iodination of the PL membrane as opposed to the matrix polypeptides. It is unlikely that this is related to the accessibility of tyrosine residues and probably represents steric influences associated with the tight apposition of latex beads to the PL membrane.

The Nature of the PL Membrane

The labeling of membrane proteins of the PL with the LPO-latex method imposes certain restraints in interpreting similarities and differences between PM and PL membranes. First, the particle-bound enzyme iodinates only the luminal surface, and label is incorporated only into available tyrosine residues. This membrane face is comparable to the outer surface of the PM, which, after endocytosis, now faces the lysosome matrix. With these reservations in mind, it is nevertheless striking to find that the iodinated polypeptides of the PM and PL are essentially identical except for rather minor differences in the intensity of a few bands. These differences were not attributable to selective exclusion of PM proteins from the PL. A truly representative sample of iodinated PM was internalized by latex phagocytosis (Fig. 7 *b*). A similar result has been obtained with L cells (7).

We were surprised by these results and had expected to be able to identify distinctive lysosomal membrane proteins coming from 1° (primary) lysosomes, Golgi apparatus, or GERL. This was, however, not the case, and both membranes exhibit striking similarities, if not identity, by these techniques. A final solution to this question will only come when we have more information concerning the total polypeptide composition of both membranes and a display of the cytoplasmic face. Preliminary evidence, however, obtained from SDS-PAGE gels of concentrated PL indicates that most Coomassie blue bands have a corresponding iodinated band on the autoradiogram, indicating that the LPO-latex technique is identifying a majority of PL proteins. Additional speculation concerning the extensive flow, fate, and origin of the polypeptides of these membranes are discussed in the accompanying paper.

Other Applications

It seems that the techniques outlined in this report would have application to other cells, other enzymes, and other problems in cell biology. Experiments are already under way to examine the polypeptides of the luminal and cytoplasmic faces of the PL membrane by labeling with both ^{125}I and ^{131}I. Such studies should allow a more detailed description of the transmembrane proteins of this organelle as well as the possible association of other cytosol polypeptides with the endocytic vacuole. Similar experiments might also be feasible with other endocytic cells and cell lines, including other mononuclear phagocytes, neutrophils, fibroblasts, and a variety of Protozoa.

Useful information may also be gained by covalently linking other enzymes to carboxylated polystyrene latex particles and delivering them to vacuolar apparatus. One could consider the use of specific lysosomal hydrolase inhibitors, the modification of metabolism, and perhaps cytocidal activities by utilizing a GO complex (12, 14–16). Effectiveness of such approaches would probably be highest with products that could diffuse readily through the lysosomal membrane, because latex is not delivered to each lysosome.

Perhaps the most interesting application of the LPO-latex technique will be to examine the process of membrane recycling—a concept raised some time ago from kinetic and stereological analyses of macrophage and fibroblast endocytosis (26). The results of such studies are found in the following paper.

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