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 with 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 trypsin blue stain were purchased.
from Grand Island Biological Co., Grand Island, N. Y. Other materials and their abbreviations and sources were: carboxylate-modified polysyntere latex spheres 0.86 μm in diameter from Dow Diagnostics, Inc., Indianapolis, Ind.; N-hydroxy-
ysuccinimide (NHS) and 1-cyclohexyl-3-[(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC) from Pierce Chemical Co., Rockford, Ill.; lactoperoxidase, purified grade (LPO) and peroxide, 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'-diamino-
benzidine 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 Suptelicco, 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""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 x 10^6 cells were plated in 16-mm-diameter flat-bottom wells (Costar,
Data Packaging, Cambridge, Mass.); for experiments involving electron micros-
copy, 2 x 10^6 cells were plated in 35-mm plastic dishes (NuncIon Delta, Kamstrup,
Roskilde, Denmark); and for cell fractionation, 4.5 x 10^6 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 supernatants 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 0.064 by weight (OD_500 = 0.03–1.5). Absolute latex numbers were also counted directly in some experiments in a hemocytometer at x 240
with bright-field optics. LPO-latex uptake into cells was measured by direct
were also counted directly in some experiments in a hemocytometer at x240
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
carefully. The beads were vortexed quickly into 0.5 ml of LPO (II U/ml in
Westbury, N. Y.) for 5 min at 12,000 rpm at 4°C and the bead pellet rinsed

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 ethanolns, removed from
the culture dish with propylene oxide, and embedded in Epon. DAB cytochem-
istry (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 A) were mounted on Formvar-coated grids and stained with
uranyl acetate and lead citrate. They were examined in a Siemens Elmiskop 1
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 sedimenta-
tion 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

LIPIDE: Macrophages on a 35-mm dish were iodinated intracellularly, washed in PBS, and scraped from the dish in PD containing 5 mM sodium thioulate to retard oxidation of iodide (3). To these cells were added 2 x 10^6
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

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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 \(^{14}C\)-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 promase (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 promase 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-\(\mu\)m Millipore filter (Millipore Corp., Bedford, Mass.), lyophilized, and chromato-graphed on a Sephadex G-25 column (16 x 1-cm) in 1 M acetic acid (8) at a flow rate of 4 ml/h. The radioactivity of the filters was determined by overlaying each autoradiogram with a grid comprised. This was determined by overlaying each autoradiogram with a grid.

**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 \(^{14}C\), lIfford L4 emulsion diluted 1:1 with water, exposed for 1-7 d, and developed in Kodak D-19.

**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-Latex.

**Intracellular Iodination of Macrophages**

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.

**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% \(\beta\) 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), \(\beta\)/galactosidase (135,000), phospholipase A (93,000), LPO (78,000), bovine serum albumin (BSA) (68,000), myoglobin G (Gg) 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% ethanol (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), \(\beta\)/galactosidase (135,000), phospholipase A (93,000), LPO (78,000), bovine serum albumin (BSA) (68,000), myoglobin G (Gg) 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.

**Protocol for ingestion of LPO-Latex and intracellular iodination.**

- 1. WASH cells 4 x with cold PBS.
- 2. ADD LPO-late in PBS.
- 3. CENTRIFUGE at 1,000 g for 2 min at 4°C.
- 4. DECAPTATE supernate.
- 5. REPLACE with warm PBS or culture medium.
- 6. INOCULATE at 37°C to allow ingestion (15-30 min).
- 7. TRYPsinize to remove uningested latex (5 min at 37°C; 200 \(\mu\)g/ml). (Unnecessary with dense cell monolayers)
- 8. WASH cells 4 x 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 mM/mg GO.
- 11. WASH with KCl in PBS, then with PBS.
- 12. TEST VIABILITY by trypsin blue dye exclusion.
- 13. WASH with PBS.
- 14. FURTHER PROCESSING, i.e., lysis for TCA precipitation, fixatation for ARG, homogenization for cell fractionation.
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-H₂O₂ reaction product localized LPO enzymatic activity to the rim of each latex sphere (Fig. 3a). Reaction product was absent if H₂O₂ or DAB were omitted.

**Figure 3** The distribution and properties of LPO-latex within cultured macrophages. (a) DAB-H₂O₂ 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. X 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-H₂O₂. 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. X 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. X 16,000.

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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. 3b). 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. 3c). 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 absence of exogenous GO was mediated by the H$_2$O$_2$ that was generated by cells during phagocytosis (13). Several sensitive assays showed that there was no H$_2$O$_2$ 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$_2$O$_2$ 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 deiodination 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).
nucleus. Latex labeling was best visualized in cells flattened by containing areas and were absent or diminished over the radiolabeled (Fig. 4a). Grains were concentrated over latex-leveled. Cell monolayers on glass coverslips when exposed for as means of autoradiography at the light and electron microscopezymatic iodination of cellular lipids was negligible. Because the lipid extractable counts were only lipids well separated from the former contained only traces of well represent contamination from I- and I2, because phospho-iodine regions. The counts recovered in these lipid spots could associated with three spots that ran very close to the free iodide and free iodide or iodine. Most of the remaining label was associated with less than half of the TCA-precipitable counts, little if any radioactivity. Because the lipid extractable counts were only 5% of the total radioactivity of the cell lysate, and equivalent radioactivity. Because the lipid extractable counts were only 5% of the total radioactivity of the cell lysate, and equivalent to less than half of the TCA-precipitable counts, little if any, 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— ARGLight 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$_2$O$_2$ 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

| Compartments | 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) | 0.206 | 0.478 | 0.320 | 0.240 |
| | 5.977 (Rim) |

* 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. 5a). SDS-PAGE analysis revealed that the same polypeptides were labeled in all fractions (Fig. 6b).

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 μm² to 0.748 μm². 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 (125I)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. 6a), nor those of the culture medium (BSA and IgG) (Fig. 7a) 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 H₂O₂ 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

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**Table V**

Subcellular Fractionation of Macrophages

| Fraction | Not iodinated | Intracellular iodination | Surface iodination after the ingestion of unmodified latex |
|----------|---------------|--------------------------|---------------------------------------------------------|
|          | Latex         | NAGase*                  | Latex         | Total NAGase* | TCA | Latex | TCA |
|          | × 10⁶         | μU                       | × 10⁹         | μU           | cpm | μU   | cpm |
| 10%      | —             | —                        | —             | —            | —   | —    | —   |
| 10/25% IF| 95.55         | 37.7                     | 116.03        | 33.0         | 44,948 | 0.21 | 2,478 |
| (75.8)§  | (3.1)         | (80.9)                   | (2.1)         | (30.4)       | (67.7) | (1.4) |
| 25%      | —             | —                        | —             | —            | —    | —    | —   |
| 25/35% IF| 4.41          | 100.4                    | 3.99          | 70.0         | 35,160 | —   | 24,830 |
| (3.5)    | (8.2)         | (2.8)                    | (4.5)         | (23.8)       | (14.5) |
| 35%      | —             | 219.6                    | 275.2         | 22,660       | —    | 36,660 |
| (18.0)   | (17.9)        | (15.3)                   | (21.4)        |               |      |
| Pellet   | 26.1          | 851.6                    | 23.4          | 1,157.8      | 44,880 | 0.10 | 107,500 |
| (20.7)   | (70.6)        | (16.3)                   | (75.4)        | (30.4)       | (32.3) | (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 (OD₆₀₀).

* 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 (125I) 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 is 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     | Total/Free | Percent of latent activity* |
|----------------------|--------------|------------|----------------------------|
|                      | µU           | Total      |                             |
| 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 |

* Percent of latent activity = total/free

Portions of the 10/25% interface were incubated with the 4-methylumbelliferone-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).

TABLE VII
Distribution of Radiolabeled Protein within the PL

| Acid hydrolyase activity (in relative fluorescence units) | Acetylglycosaminidase | β-Glucuronidase | TCA-precipitable 125I |
|----------------------------------------------------------|-----------------------|-----------------|-----------------------|
| N-Acetyl glucosaminidase                                 | 95.7                  | 53              | 6,665                 |
| β-Glucuronidase                                          | 72.4                  | 43.2            | 150                   |
| Control supernate                                        | 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%.

intracellularly was also unchanged if the cells were maintained at 37°C for 1 h after latex uptake and then iodinated (Fig. 7a, lane 1).

When macrophage PM was iodinated and unmodified latex...
spheres were ingested thereafter, phagosomes isolated from these cells yielded exactly the same autoradiographic pattern as the whole cell homogenate (Fig. 7b). Therefore, it is unlikely that the differences in labeling intensity seen in Fig. 7a 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. 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 H₂O₂ 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-
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. 6b). 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 labeling 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. 7b). 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 125I and 35S. 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 cytoidal 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 macropage and fibroblast endocytosis (26). The results of such studies are found in the following papers.

We express our sincere thanks to Richard Hunley and Ms. Judy Adams for expert technical assistance. We are grateful to Dr. Ann L. Hubbard for teaching us the flat substrate method of autoradiography, to Dr. Marilyn G. Farquhar for advice on analysis of autoradiograms, to Drs. Stanley Fowler, Nancy Haley, and Judi Butler for the gift of 4-methylumbelliferyl sulfates and advice on the assays, to Drs. William A. Scott and Eileen Mahoney for help with the lipid analysis, and to Miss Betty Bryles for typing the manuscript.

The work was performed while W. A. Muller held a predoctoral traineeship awarded under National Institute of General Medical Sciences grant no. 5 TOS GM02243, R. M. Steinman is an Irma T. Hirschl Fellow of The Rockefeller University; Z. A. Cohn is the recipient of U. S. Public Health Service grant no. AI07012.

Received for publication 7 December 1979, and in revised form 22 February 1980.

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