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The Membrane Polypeptides of the Vacuolar System: Composition and Recycling

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THE MEMBRANE POLYPEPTIDES OF THE VACUOLAR SYSTEM: COMPOSITION AND RECYCLING

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by

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ABBREVIATIONS

ARG	autoradiography
BSA	bovine serum albumin
CMC	1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate
CM-latex	carboxylate-modified polystyrene latex spheres
d	day(s)
DAB	3,3'-diaminobenzidine tetrahydrochloride
DIT	di-iodotyrosine
EM-ARG	autoradiography at the electron microscope level
FCS	fetal calf serum
GO	glucose oxidase
h, hr	hour(s)
HD	half distance
HRP	horseradish peroxidase
i.p.	intraperitoneal
LDL	low density lipoprotein
LM-ARG	autoradiography at the light microscope level
LPO	lactoperoxidase
LPO-latex	lactoperoxidase covalently coupled to carboxylate modified polystyrene latex spheres
min	minute(s)
MIT	mono-iodotyrosine
NCS	Nelson-Collins strain of Swiss white mice
NHS	*N*-hydroxysuccinimide
PBS	Dulbecco's phosphate buffered saline (with divalent cations)
PD	Dulbecco's phosphate buffered saline (without divalent cations)
PL	phagolysosome(s)
PM	plasma membrane

PMSF	phenylmethyl sulfonyl fluoride
PS-latex	polystyrene latex spheres
PV	pinocytic vacuole(s)
s, sec	second(s)
S-B latex	styrene-butadiene latex spheres
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SW	Swiss/Webster strain of Swiss white mice.
TCA	trichloroacetic acid

I. SUMMARY

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 pre-existing 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; 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.

When macrophages iodinated intralysosomally by LPO-latex were returned to culture at 37°, TCA-precipitable radioactivity was lost from

cells with biphasic kinetics. 20-50% of the cell-associated radiolabel was rapidly digested ($t_{1/2} \tilde{=}$ 1 h) and recovered in the culture medium as monoiodotyrosine. 50-80% of the label was lost slowly from cells ($t_{1/2} \tilde{=}$ 24-30h). Quantitative analysis of gel autoradiograms showed that all radiolabeled proteins were lost at the same rate in both the rapid and slow phases of digestion.

Within 15-30 min after labeling of the PL membrane, EM autoradiography revealed that the majority of the cell-associated grains, which at time 0 were associated with PL, were now randomly dispersed over the plasmalemma. At this time, analysis of PM captured by a second phagocytic load revealed the presence of all labeled species originally present in the PL membrane. This demonstrated the rapid, synchronous centrifugal flow of PL polypeptides to the cell surface.

Evidence was also obtained for the continuous influx of representative samples of the PM into the PL compartment by way of pinocytic vesicles. This was based on the constant flow of fluid phase markers into latex-containing PL and on the internalization of all iodinatable PM polypeptides into this locus.

These observations provide evidence for the continuous, bidirectional flow of membrane polypeptides between the PM and the secondary lysosome and represent an example of a membrane flow and recycling mechanism.

II. MATERIALS

Female mice weighing 25-30 g were used for these experiments. The Nelson-Collins strain (NCS) was obtained from The Rockefeller University breeding colony. The Swiss-Webster (SW) strain was obtained from Taconic Farms, Germantown, N. Y. Cells of the J774 macrophage line were a gift from Dr. Jay C. 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 from Grand Island Biological Co., Grand Island, N. Y. Other materials and their abbreviations and sources were: carboxylate-modified polystyrene latex spheres (CM-latex) 0.86 μm in diameter and styrene-butadiene latex spheres (S-B latex) 0.527 μm in diameter, purchased from Dow Diagnostics, Inc., Indianapolis, Ind.; *N*-hydroxy-succinimide (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) and chymotrypsin 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 and chloroquine diphosphate 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., Bellefone, Pa.; TCA,potassium iodide (KI), sucrose, hydrogen peroxide (Superoxol), from Mallinckrodt Chemical Works, St. Louis, Mo.; carrier-free $Na^{125}I$ and aquasol from New England Nuclear, Boston, Mass., ^{3}H-AMP from Amersham-Searle. All other chemicals were reagent grade or better.

III. METHODS

A. 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.

B. Covalent Coupling of LPO to CM-Latex

The coupling reaction is outlined schematically in Figure 1. The reaction was routinely carried out in a 1.5 ml plastic microfuge tube. Plastic pipettes were used to handle and transfer latex, since it sticks more to the walls of glass vessels.

The stock suspension of CM-latex (10% w/v) was resuspended well by vortexing the bottle. 150 microliters was withdrawn and transferred to the microfuge tube. This was washed three times in 0.2M acetate buffer pH 5.4 by sequentially adding 1 ml of buffer, centrifuging at 12,000 rpm for 5 min in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, N. Y.) at 4°, carefully decanting the supernate and resuspending the latex pellet in 1 ml of buffer by vigorous vortexing or gentle sonication (2-3 sec at setting 2 on Branson Cell Sonifier, using the microtip attachment).

After the final wash, the beads were resuspended to 1.5% w/v in 1 ml acetate buffer. A clean ⅛" magnetic stirring bar was placed in the

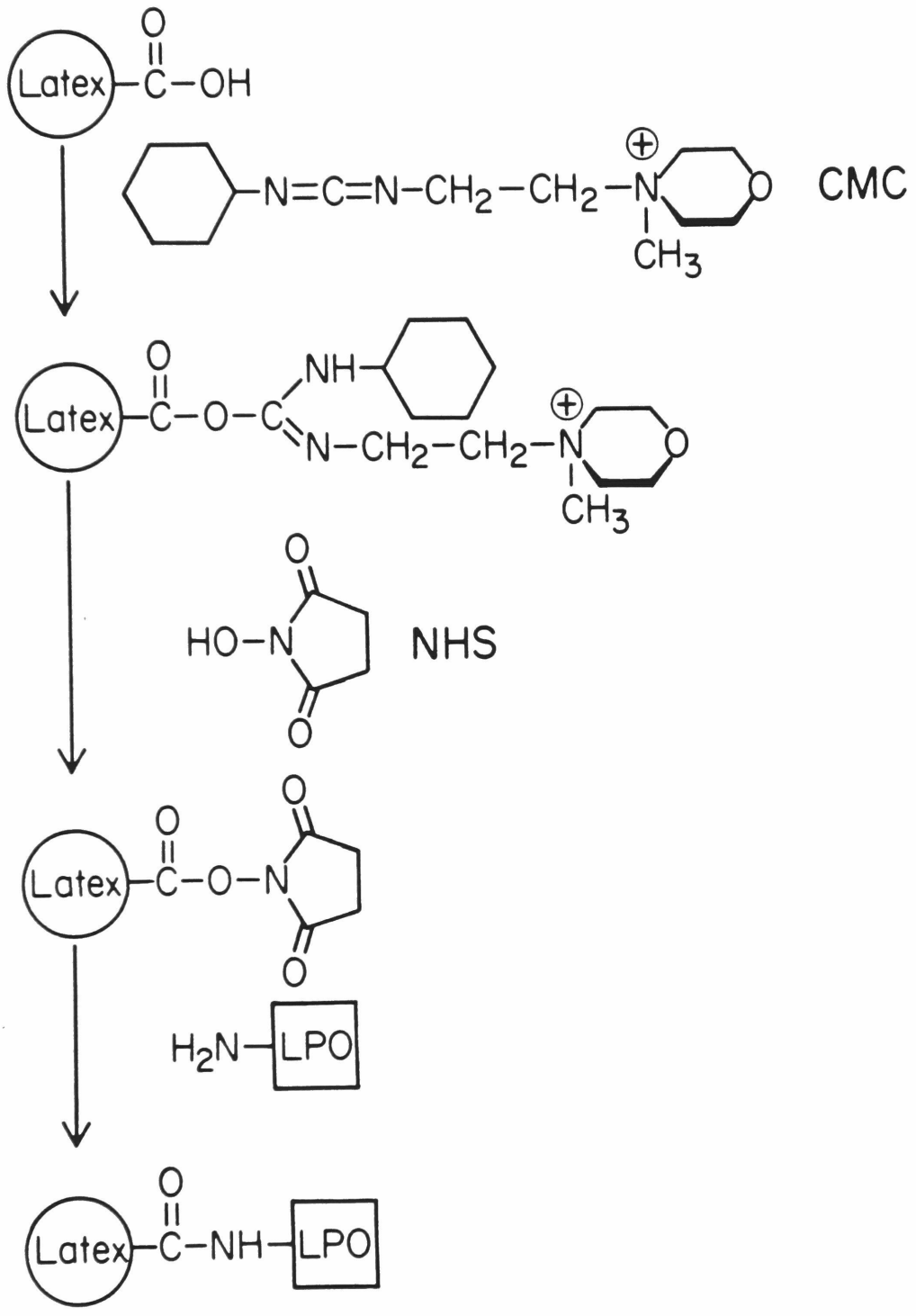


FIGURE 1. The covalent coupling of lactoperoxidase to carboxylated latex spheres by means of carbodiimide.

tube. 11.5 mg of crystalline *N*-hydroxysuccinimide (NHS) was added to the tube (final concentration = 0.1m). This was vortexed for several seconds to dissolve the NHS. Immediately following, 42.36 mg of crystalline 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMS) was added to the tube (final concentration = 0.1M) which was then vortexed.

The reactants were placed on a magnetic stirrer for 10 hrs at room temperature. Stirring kept the latex in suspension. During this step the microfuge tube was held upright within a flat-bottomed glass vial. To insulate the reactants from the heat developed by the magnetic stirrer, a foam pad was placed underneath the glass vial.

The activated latex beads were pelleted in the microfuge as before. The bead pellet was rinsed carefully in acetate buffer and resuspended rapidly into 0.5 ml lactoperoxidase (11 U/ml in carbonate-bicarbonate buffer, pH 9.6) at 4°. This solution was routinely made by injecting 0.5 ml of buffer into a vial of purified grade lactoperoxidase (Calbiochem catalogue # 427488 100 I.U. per vial, which has 5.5 units per vial as assayed by o-dianisidine (section III. C.1). The resuspended latex was stirred magnetically for 30 min at 4°. Crystalline glycine was then added to a final concentration of 1M to quench unreacted ester bonds, and the mixture was stirred an additional 15-30 min at room temperature. The latex beads with covalently bound LPO (LPO-latex) were pelleted and washed four to five times in PBS until two successive supernates showed no LPO activity by the *o*-dianisidine assay (section III. C.1). The beads were then suspended in 1 ml of 50% glycerol and stored at -20°C.

C. Enzymatic and Chemical Assays

1. LPO Activity

Lactoperoxidase activity was measured using o-dianisidine as described (54) but at pH 6. To measure the activity of LPO-latex preparations, the stock was first diluted 1:100 to 1:200 in buffer. LPO-latex settles very slowly and didn't interfere with the spectro-

photometric measurements at these dilutions. To measure LPO-latex uptake into cell cultures, the more sensitive triiodide assay (Worthington Biochemical Co. Enzyme Manual) was performed on latex-containing cell lysates.

2. HRP Activity

HRP activity was assayed by o-dianisidine at pH 5 as described (54).

3. Latex Concentration

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) (Figure 2).

Absolute latex numbers were counted in some experiments in a hemocytometer at x240 bright field optics.

4. Lysosomal Hydrolases

Lysosomal acid hydrolase activities were measured using the assay of Peters *et al.*(42). 4-Methylumbelliferyl-β-D-glucuronide trihydrate, 4-methylumbelliferyl-β-D-galactopyranoside monohydrate and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside substrates (Koch Light Laboratories, Colnbrook Buckinghamshire, England) were the generous gift of Dr. Stanley Fowler of The Rockefeller University.

For measurements of enzyme latency the incubations were carried out for 10 min at 37°. Total enzyme activity was measured in the presence of 0.1% Triton X-100.

5. 5'-Nucleotidase

5'-Nucleotidase activity of macrophage lysates was measured by the assay of Avruch and Wallach (8). Macrophage cultures in 16 mm Costar wells were lysed in 0.4 M 0.05% Triton X-100. 100 µl from each well was assayed. Assays were run in triplicate for each variable tested. 200 µl from each well was set aside for determination of total cell protein.

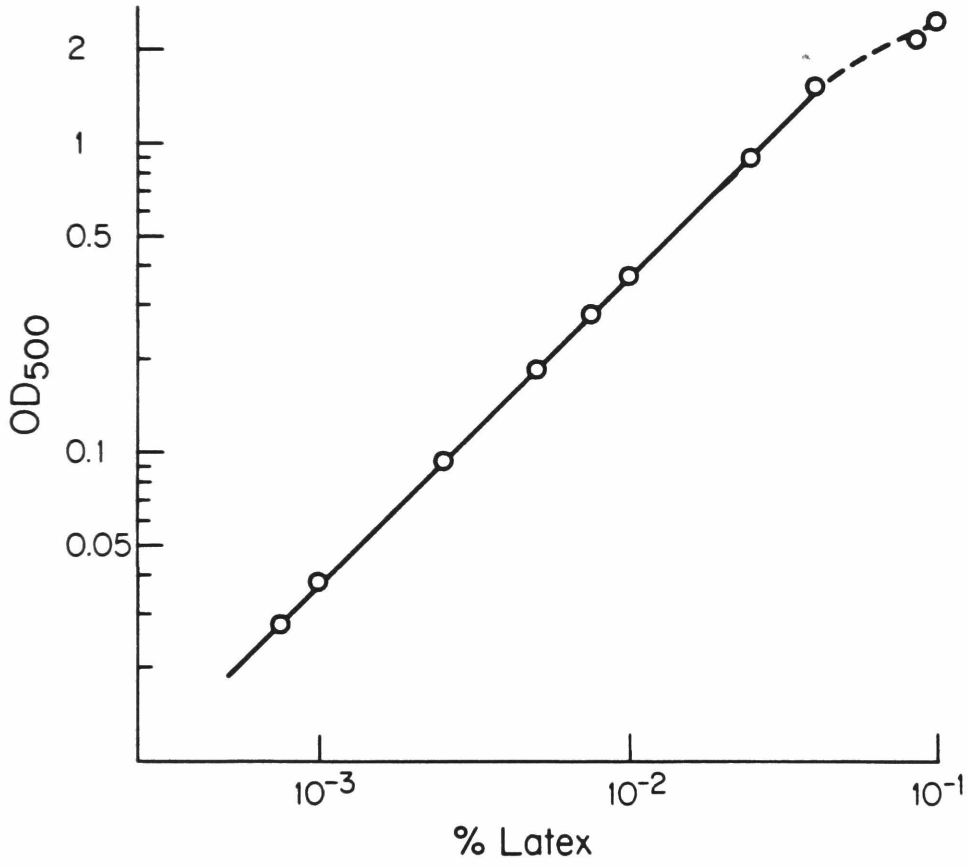


FIGURE 2. Measurement of latex bead concentration by light scattering. The 10% stock suspension of CM-latex was diluted in 2% SDS. Absorbance measurements were made at 500 nm.

6. Protein

Protein was measured by the method of Lowry *et al.* (30) using lysozyme as a standard. Samples containing latex beads were centrifuged for 5 min at 1,000xg to remove these beads before measuring the absorbance of the supernate.

7. Quantitation of Incorporated ^{125}I in Soluble Samples

For determination of radioactivity 10-100 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 hr 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;

the pellets were counted in a Packard Auto-Gamma Scintillation Spectrometer model 5220 (Packard Instrument Company, Downers Grove, Ill.).

8. Quantitation of Incorporated ^{125}I from Gels

To determine the exact amount of radioactivity in a particular polypeptide in an SDS-polyacrylamide gel, a well-exposed autoradiogram film of the gel was properly aligned over the original dried gel. The band was traced from the film onto the gel via an intervening piece of carbon paper. This traced area was then cut from the gel and counted in a Gamma Scintillation Spectrometer. Similar sized pieces of the gel were cut from areas (usually the same lane) which did not detectably expose the X-ray film to determine the background radioactivity.

D. Uptake of LPO-Latex by Macrophages

1. Direct Microscopic Examination

Macrophages were cultured on glass coverslips (Gold Seal 12.5 mm) for two days. The coverslips were transferred to 16 mm Costar wells and washed in PBS. 0.4 ml of LPO-latex stock diluted to various degrees in PBS was centrifuged down upon the cell monolayers (section V. B). Coverslips were washed and returned to the 37° incubator for 15 min in warm

PBS. Monolayers were then trypsinized for 5 min and washed extensively. This treatment was empirically found to remove extracellular latex bound to dish, but did not remove beads bound to cells.

Duplicate coverslips were prepared for each dose of latex and fixed in 2.5% glutaraldehyde. One coverslip that had received the 1:800 dilution of latex was returned to culture for 51 hr before fixation.

Coverslips were examined by phase-contrast microscopy at 1.000x under oil. Ingested latex spheres were counted for each latex dose in fifty cells (25 from each coverslip) chosen at random.

2. Uptake of LPO Enzymatic Activity

Once it was established (see section V. B) that the ingestion process resulted in no loss of LPO activity from LPO-latex,cell-associated LPO activity could be used to monitor latex uptake. The procedure was similar to that described above, except that cells were cultured directly on 16 mm Costar wells. Following ingestion cells were lysed in 0.2 ml Triton X-100. 100 µl was taken from each well for assay of activity by triiodide production (section III. C.1.). Assays were run in triplicate each bead dose.

E. 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 hr, 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 (20) was performed on 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.* (39) 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.

F. LPO-Catalyzed Iodination of Macrophages

The procedure for iodinating from within the PL is described in detail in section VI. A. Volumes of LPO-latex suspension added to cell cultures were: for 16 mm Costar wells 0.4 ml, for 35 mm dishes 1.5 ml, and for 60 mm dishes, 5 ml.

1. Plasma Membrane

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 (23). 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 (56) 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 surfaces 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 500g.

2. Controls for intracellular iodination

a) Iodination of LPO on LPO-latex.

[^{125}I] LPO-latex of high specific activity was made by exposing concentrated suspensions (1:40 dilution of stock) of LPO-latex in PBS containing 400 μCi/ml $^{125}I^-$ and 300 μM H_2O_2 for 10 minutes at room temperature. H_2O_2 was added in four aliquots at 2.5 minute intervals. The reaction was terminated by the addition of NaN_3 to a final concentration of 0.02%. The [^{125}I]LPO-latex beads were washed several times by centrifugation in the presence of 100 mM KI. The beads were then diluted in PBS and fed to cells as usual. Incorporated radioactivity was determined by TCA precipitation of the bead suspension. This served mainly to remove residual noncovalently bound ^{125}I.

b) Mode of delivery of LPO-latex.

Macrophages cultured for two days received a 1:50 suspension of LPO-latex in culture medium. This was allowed to settle on top of the monolayer for 1½ hr. At the end of this time, the culture was washed extensively to remove beads that had not settled, and was returned to the incubator for 10 min to allow ingestion to be completed. An identical macrophage culture received the standard 1:800 LPO-latex dose. Following centrifugation of latex as usual, ingestion proceeded for 30 min at 37° in culture medium. Both cultures were iodinated on ice (see Section VI. A), then lysed and prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE; see Section III.K.).

3. Iodination of a lysosome content marker

One 35 mm dish of macrophages cultured 1½ days was incubated overnight in medium 199 with 10% normal mouse serum. After extensive washing, the cells were given LPO-latex as usual and returned to culture in medium 199 + 10% FCS. Two other macrophage cultures, identical to the first except for the overnight incubation in mouse serum, received the same LPO-latex dose and were returned to culture; one in 199 with 10% FCS and the other in 199 with 10% normal mouse serum. Following ingestion of latex,cells were washed extensively and iodinated on ice. The cells were then lysed in 300 µl of 0.5% NP-40 with protease inhibitors. 50 µl was prepared for SDS-PAGE and two 10 µl samples were assayed for TCA precipitable radioactivity. 200 µl was shaken for 1 hr at 4°C with 50 µg of an $F(ab')_2$ fragment of rabbit antibody recognizing rat IgG. (The $F(ab')_2$ had previously been coupled to sepharose 4B, and was the generous gift of Dr. Ira Mellman.) This antibody also recognizes determinants on mouse IgG. The sepharose beads bearing immune complexes were pelleted in a microfuge. The supernate was processed for SDS-PAGE and TCA precipitation as above. The pelleted sepharose beads were washed by the procedure of Kaplan *et al.* for immune complexes bound to *S. aureus* (26) and the washed beads boiled in detergent for SDS-PAGE.

G. Chemical Analysis of Radioiodinated Material

1. 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 (13). To these cells were added 2 x 10^8 J774 cells to provide carrier lipid. The cell suspension was extracted twice in chloroform-methanol by the method of Bligh and Dyer (9). 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.

2. 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 (23) and placed in glass vials with two drops of 1 N NaOH to bring the pH close 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 hrs, the supernates were removed and frozen. An additional 1 ml of fresh pronase solution was added for an additional 24 hrs. 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 x 1 cm) in 1 M acetic acid (25) at a flow rate of 4 ml/h. Spectrophotometrically detectable quantitites 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.

H. Autoradiography (ARG)

1. Light Microscope Level (LM-ARG)

Samples of isolated phagolysosomes were prepared by placing 5 µl aliquots of phagolysosome suspension in the center of a glass coverslip and rapidly drying the sample by placing the coverslip face up on an electric hot plate. 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.

2. Electron Microscope Level (EM-ARG)

The flat substrate method of Salpeter and Bachman (48) 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.

I. Analysis of EM Autoradiograms

The probability circle method was used to localize the source of radiolabel corresponding to particular silver grains (49). 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 x the half-distance (HD) of 800 Å (50). 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.* (17). 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.

J. Cell Fractionation

We used a modified version of the discontinuous sucrose gradient first described by Wetzel and Korn (60). Cell monolayers were washed extensively in cold PBS, then scraped in 1.5 ml of isotonic sucrose containing 1 mM EDTA. Cells were 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 hr 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 $\geq$ 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 (12).

K. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

5-15% gradient slab gels (23) or 4-11% slab gels (38) 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 Du Pont Cronex or Kodak XR-1 x-ray film. When an enhancing screen (Du Pont Lightning Plus, E. I. Du Pont de Nemours & Co., Wilmington, Del.) was used, the film was presensitized by the method of Laskey and Mills (29).

L. Return of Iodinated Cells to Culture

Following iodination, cells labeled either intracellularly or extracellularly were washed three times in ice-cold phosphate-buffered saline

solution (PBS) containing KI, then at least three times in PBS, and returned to the incubator in fresh medium which had been warmed to 37°C. Upon removal from culture, they were always washed at least three times with PBS.

M. Proteolytic Treatment Following Iodination

At various times after return to culture, cells were washed in PBS containing 100 μg/ml chymotrypsin. (These solutions had been warmed to 37°C before being added to the cells.) The supernates were collected, passed through a Millex filter (Millipore Corp., Bedford, Mass.) to remove any detached cells (<< 1% of the total number), and lyophilized. The freeze-dried material was redissolved in 1 M acetic acid and chromatographed on Sephadex G-25. The cell monolayers were lysed in 0.05% Triton X-100 and precipitated with 10% TCA containing 100 mM KI. (See sections III. G.2 and III. C.7).

N. Isolation of Plasma Membrane

The following method was devised to retrieve representative samples of radiolabeled plasma membrane (PM) polypeptides at any given time. Its success depends upon the density differences between LPO-latex phagolysosomes (PL) and styrene-butadiene latex phagolysosomes (S-B latex PL), and upon the use of a large dose of S-B latex to incorporate a large area of PM into phagocytic vesicles within a short time. Although the membrane retrieved in this manner is truly phagosome membrane, we found that this procedure isolated a representative sample of labeled proteins following extracellular iodination of PM at 4° by soluble LPO. (See Fig. 12).

Immediately after intracellular iodination with LPO-latex beads or 30 min after return to culture, cells were washed and a 1:50 dilution of the 10% stock of low density (ρ = 1.027) S-B latex particles was centrifuged down upon the monolayer at 1,000 g for 5 min at 4°C. The cultures were washed with PBS and warmed to 37°C for 5 min to allow ingestion of the S-B latex. Each cell ingested several hundred beads during this time, compared to the several dozen LPO-latex beads already present within the cells.

The cells were then scraped from the plates and homogenized in isotonic sucrose. 2.5 ml of the homogenate was brought to 35% sucrose (wt/wt) and overlaid first with 6 ml of 15% sucrose and then 2.5 ml of 5% sucrose. All sucrose solutions contained the protease inhibitors phenylmethyl sulfonyl fluoride (PMSF) and aprotinin. The discontinuous gradient was centrifuged at 100,000 g for 1 hr at 4°C. Fractions were collected and processed for TCA precipitation, latex quantitation, and SDS-PAGE. LPO activity was assayed by triiodide production.

Microscopic examination (x 1,000) and enzymatic assay of the fractions showed that the 5/15% interface contained about two-thirds of the total S-B latex but no detectable LPO-latex. The 15/35% interface contained most of the LPO-latex and a few S-B latex. The pellet fraction also contained both latex types. Therefore, the material in the 5/15% interface represented plasma membrane as it existed at the time of S-B latex addition as well as membrane that joined the plasmalemma or S-B latex phagosome during the 5-min ingestion period.

O. Fusion of Pinocytic Vesicles with LPO-Latex Phagolysosomes

Three approaches were used to document fusion of pinocytic vesicles with phagolysosomes (PL).

1. Accumulation of a Particulate Marker

Macrophage monolayers were allowed to ingest LPO-latex as usual, washed, and returned to culture for 9 hrs in fresh medium containing 0.5% colloidal thorium dioxide (Thorotrast). Cells were washed, fixed, exposed to 3,3'-diaminobenzidine tetrahydrochloride (DAB) H_2O_2 to visualize LPO (20) and further processed for transmission EM to examine the Thorotrast accumulation in LPO-latex PL.

2. Accumulation of Fluid Marker

Macrophage monolayer cultures were allowed to ingest a dose of unmodified polystyrene latex spheres (PS-latex) equivalent to the usual LPO-latex load. The cells were then returned to culture with 1 mg/ml

horseradish peroxidase (HRP) in the medium. After various times of incubation, the cells were washed extensively and returned to culture for 20 min in medium without HRP. The cells were then homogenized and fractionated on a 10/25/35% sucrose gradient. Peroxidase enzymatic activities of the whole-cell homogenate and the purified PL fraction (10/25% interface) were determined by the *o*-dianisidine assay. at pH 5. Latex was quantitated by light scattering and protein by the method of Lowry *et al.* (Section II. C.6).

3. Accumulation of Radiolabeled Plasma Membrane

Macrophage monolayers were allowed to ingest unmodified PS-latex as above. The cells were then chilled, and their external surfaces were iodinated on ice for 10 min, using soluble LPO (40 mU/ml) and glucose oxidase (GO) (10 mU/ml). The cells were washed and either homogenized immediately or returned to culture for 30 min before homogenization. The homogenate was fractionated on a 10/25/35% sucrose gradient.

P. Two Dimensional Gel Electrophoresis

The procedure used was essentially that of Ames and Nikaido (6) as modified by Piperno *et al.* (43). Iodinated macrophages were lysed in 2% SDS, 5% βME and boiled. The lysate was then centrifuged to remove latex and mixed with ½ volume NP-40 and 2 volumes of sample buffer (43). Sixty microliters were first subjected to isoelectric focusing, run with electrodes reversed (*i. e.* anode at the origin), at 2 mA. Six-mm strips were then cut from this gel and annealed to a 4-11% gradient gel for SDS-PAGE in the second dimension. This was carried out at 37.5 mA for 6 hr.

PART ONE

INTRACELLULAR IODINATION OF MACROPHAGE PHAGOLYSOSOME MEMBRANE

IV. INTRODUCTION

Our knowledge of the vacuolar apparatus is largedly 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. Steinman *et al.* approached one aspect of this flow and quantitated the steady-state interiorization of plasma membrane in the form of pinocytic vesicles (55). 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 Part One 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.

V. SELECTIVE DELIVERY OF ACTIVE LACTOPEROXIDASE TO SECONDARY LYSOSOMES

A. Covalent Coupling of LPO to CM-Latex

The coupling procedure was designed to take advantage of the manipulatability of the isoluble carboxylate-modified polystyrene latex beads (Fig. 1). The carboxyl group of the beads is activated using a water-soluble carbodiimide; the carboxyl group is then susceptible to nucleophilic attack by *N*-hydroxysuccinimide, which displaces the carbodiimide and forms an ester. The esterified latex is then centrifuged away from the soluble reagents, and resuspended in a solution of lactoperoxidase. In this way, the LPO is not exposed to the carbodiimide. At the high coupling pH (9.6) nonprotonated ε-amino groups of lysine residues readily attack the ester bond on the latex, displacing *N*-hydroxysuccinimide and forming a more stable amide linkage.

This procedure routinely coupled 15-20% of the original LPO activity and 15-20% of the original protein to the latex spheres (Table I). The specific activity of the lactoperoxidase was unchanged by this procedure.

Over the course of these investigations, many different batches of LPO-latex were made using 0.15 ml of a 10% (w/v) suspension of CM-latex and one vial of purified lactoperoxidase dissolved in 0.5 ml buffer. (The LPO, Calbiochem catalogue number 427488 contained 100 I.U. of activity by their assay, and about 5.5 Units by our *o*-dianisdine assay at 25°). The various batches of LPO-latex all had similar enzymatic activities (about 1 Unit/ml, Table II). Light scattering measurements confirmed that the various batches had the same latex concentration.

Enzymatic activity was stable for at least 11 months when stored in 50% glycerol at -20°C (Table II).

When LPO was exposed to CM-latex which had not been "activated" by the carbodiimide step, substantial enzymatic activity was still

TABLE I

EFFICIENCY OF COUPLING OF LACTOPEROXIDASE TO CARBOXYLATE-MODIFIED POLYSTYRENE LATEX

	Total LPO Activity (mU)	%Total LPO Activity	Total Protein (μg)	%Total Protein	Specific Activity (mU/μg)
LPO* Solution	5,480	100	667	100	8.2
1st Supernate	3,980	72.6	≠		
2nd Supernate	150	2.7	19.2	2.8	7.8
3rd Supernate	50	0.9	1.4	0.2	0.2
4th Supernate	-§		-		
5th Supernate	-		-		
LPO-Latex	830	15.1	111	16.6	7.5

To assess the efficiency of the coupling procedure, the original LPO solution, the supernates from the washing steps and the final LPO-latex suspension were assayed for LPO activity and protein content (see Materials and Methods).

* LPO solution = the solution added to the activated beads in the coupling procedure.

≠ The 1M glycine in this supernate interferes with the protein assay and prevents determination of LPO protein in this sample.

§ - = Not detectable.

TABLE II

REPRODUCIBILITY OF COUPLING PROCEDURE AND STABILITY OF LPO-LATEX AT -20°C

Date Made	Date Assayed	Elapsed Time	Activity (Units/ml)
7/13/77	7/13/77	-	1.04
	8/5/77	4 wk	1.07
	12/5/77	5 mos	1.14
8/8/77	8/8/77	-	1.05
4/18/78	4/18/78	-	1.06
	5/30/78	6 wk	1.00
7/31/78	7/31/78	-	1.10
11/8/78	11/8/78	-	0.96
	12/23/78	1½ mos	0.95
2/4/79	2/4/79	-	0.97
	2/11/79	1 wk	1.07
	4/14/80	2½ mos	0.85
5/11/79	5/11/79	-	1.25
	4/14/80	11 mos	1.40
11/18/79	11/18/79	-	0.9
	1/22/80	2 mos	1.4
9/17/80	9/17/80	-	1.02
12/16/80	12/16/80	-	1.04

Various batches of LPO-latex made over the course of this study were assayed using *o*-dianisidine (see section III.C.1) at the time of coupling and after storage in 50% glycerol at -20°C.

bound to the beads after several washes with PBS. Unlike the covalently coupled LPO, however, the activity continued to elute with further saline washes.

This activity could be removed by washing the beads with a nonionic detergent indicating that it was bound to the latex by hydrophobic interaction. This same procedure removed no detectable activity from LPO-latex made by the standard procedure.

B. Delivery of LPO-Latex to Secondary Lysosomes

A protocol was designed to deliver LPO-latex rapidly and selectively to macrophage phagolysosomes (PL) (Fig. 3). Since latex spheres settle very slowly out of suspension, 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 the dish.

Latex beads on cells sat on the plasma membrane and were not depressed into the cell. The monolayers were then brought to 37°C for 15-30 min, resulting in a rapid and synchronous wave of phagocytosis of the LPO-latex.

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) was empirically found to remove the vast majority of the extracellular beads. Trypsin neither inactivates nor releases LPO activity from LPO-latex heads (Table III). The same results were obtained with either ingestion protocol, with one exception. There were two major plasma membrane polypeptides which were trypsin sensitive and whose intensities were altered in SDS-PAGE autoradiograms. All of the gels presented in this study are of cells which had not been trypinized.

After the phagocytic pulse, all beads had been ingested. Scanning electron microscopic examination of > 1,000 cells showed that all latex had been completely internalized. Ingestion of LPO-latex

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 3. Protocol for ingestion of LPO-latex and intracellular iodination.

TABLE III

TRYPSINIZATION DOES NOT AFFECT LPO-LATEX ACTIVITY

Sample	Trypsin	Units ml
1:100 LPO-Latex	-	1.371
		1.327
1:100 LPO-Latex	+	1.345
		1.327
Supernate	-	0.000
		0.000
Supernate	+	0.000
		0.000
Pellet	-	1.106
		1.062
Pellet	+	1.194
		1.150

LPO-latex beads were diluted 1:100 in PBS or PBS containing 500 µg/ml trypsin.

The suspension was incubated for 10 min at 37° before assaying. An aliquot of each sample was then centrifuged at 20,000 RPM for 10 min and the supernate and pelleted latex (resuspended to the original volume) were assayed separately.

Only 85% of the original LPO activity is recovered in the resuspended pellets because latex beads stick to the plastic centrifuge tube and are not easily removed.

did not alter its enzymatic activity within 30 min (Table IV and Figure 4 compare curves —0— and —X—). Ingestion was proportional to bead dose over a wide range (corresponding to the uptake of 10-100 beads per cell) as assayed by LPO enzymatic activity or by direct counting of latex in cells (Fig. 4). To be certain that all beads were entirely intracellular, we generally used a bead dilution of 1:800 (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 when cells are grown on plastic, and 39 $\pm$ 12 beads per cell when grown on glass (Fig. 4).

Ingested LPO-latex spheres are situated in typical membrane bounded vacuoles. Transmission EM examination of the DAB-H_2O_2 reaction product localized LPO enzymatic activity to the rim of each latex sphere (Fig.5a). Reaction product was absent if H_2O_2 or DAB were omitted 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. 5b). 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. 5c). We conclude that LPO can be introduced selectively into typical PL in active form.

C. Fate of LPO-Latex Following Ingestion

Phagocytosed LPO-latex beads remained in secondary lysosomes and were found predominantly in the perinuclear region. Cells remained alive (> 99% excluded trypan blue), adherent and well-spread. They were morphologically indistinguishable from cells which had ingested unmodified latex. Latex beads were not exocytosed. Free latex was never seen on the dish surface or suspended in the culture media once ingestion had been completed. Quantitative examination of cultures 5 hrs after ingestion showed that the standard deviation in the number of beads per cell as well as the mean number was unchanged (Fig. 4).

TABLE IV

INGESTION OF LPO-LATEX DOES NOT INACTIVATE

ENZYMATIC ACTIVITY

Sample	$\frac{\Delta A_{350}/min}{A_{500}}$
1:800 LPO-Latex	1.23
Monolayer 1	1.05
2	1.23 Avg = 1.16 $\pm$ 0.1
3	1.19

0.4 ml of a 1:800 dilution of LPO-latex in PBS was centrifuged onto peritoneal macrophages cultured in 16 mm Costar wells. Ingestion at 37° proceeded for 20 min at which time phase-contrast microscopy showed that all latex had been internalized. The wells were washed in cold PBS and lysed in 0.4 ml of 0.05% Triton X-100. LPO activity was assayed by the triiodide assay (ΔA_{350}/min) and normalized for the amount of latex recovered in each lysate. LPO-latex stock was diluted 1:800 in 0.05% Triton X-100 for the enzymatic assay.

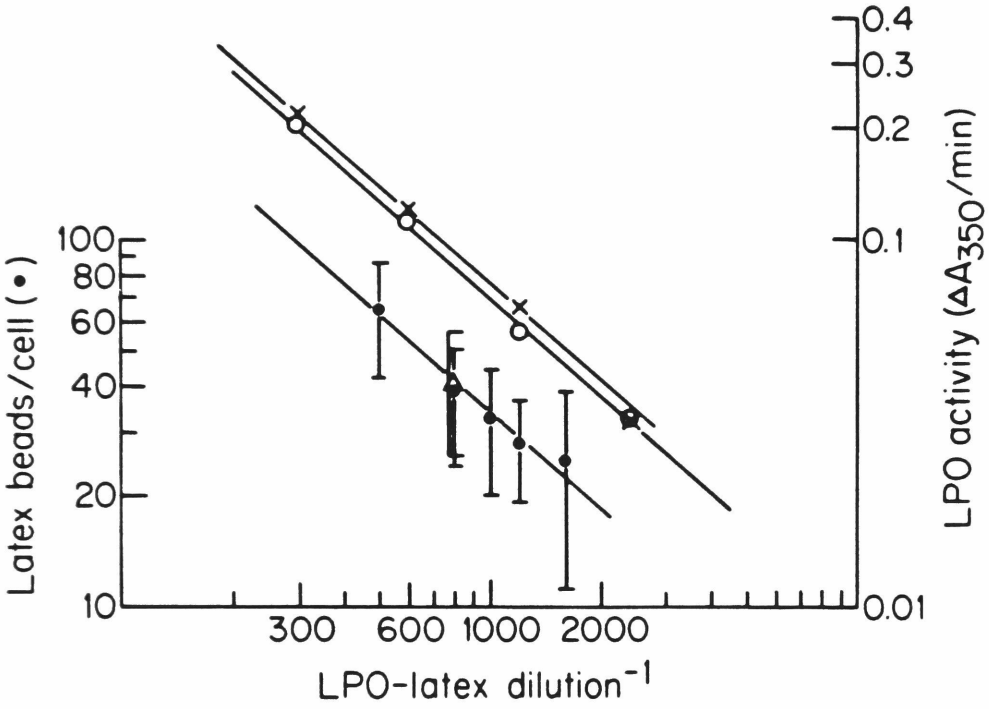
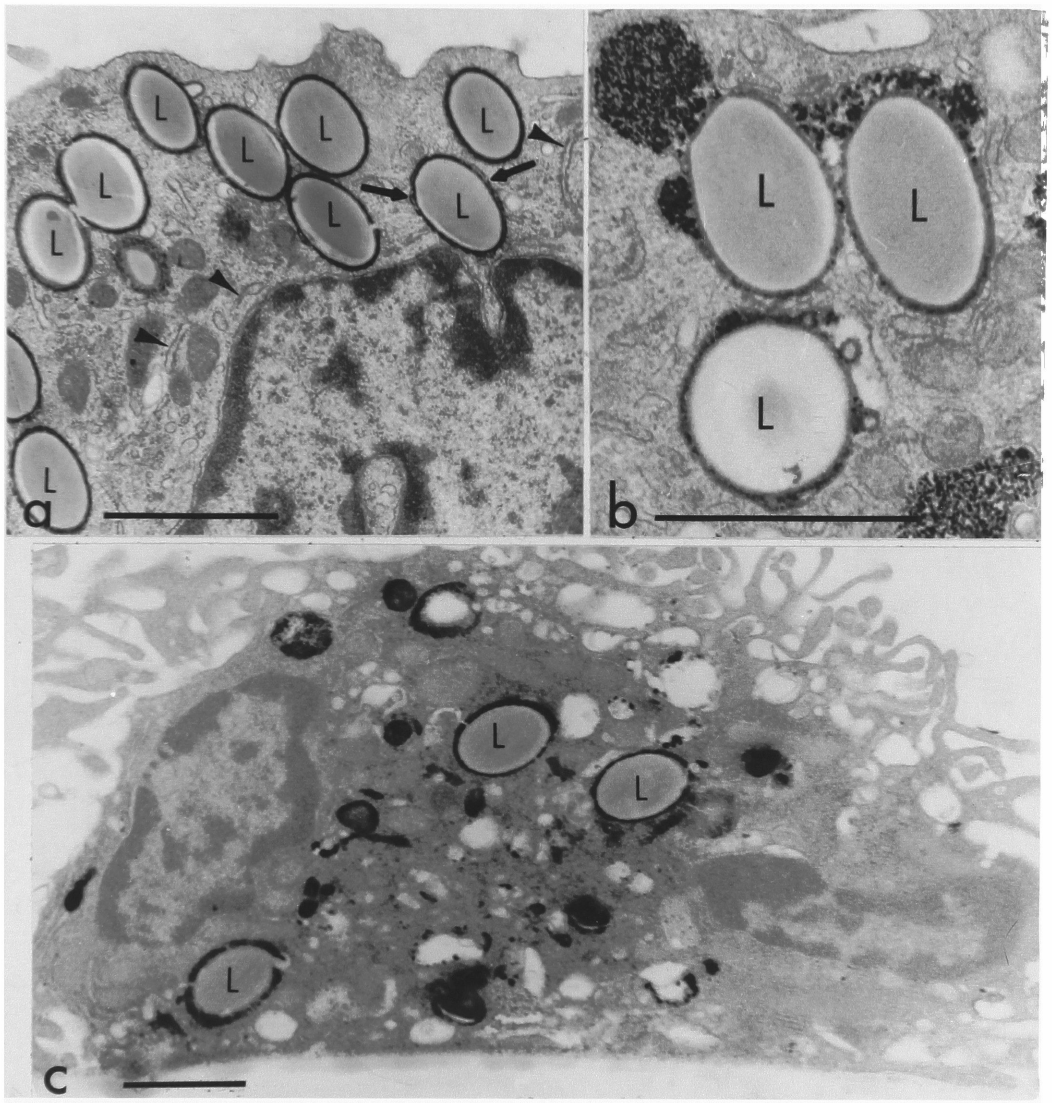


FIGURE 4. Ingestion of LPO-latex as a function of particle dose.

Two separate assays are shown here. Direct bead counts and LPO enzymatic assays were performed as described in Materials and Methods. Latex counts were performed on each of 50 cells chosen at random (25 from each of two coverslips). Standard deviations are indicated by the vertical bars. One coverslip at the 1:800 dose was kept in culture for 51 hours before counting (Δ) .

LPO assays were performed on lysates of cell monolayers either immediately following trypsinization to remove extracellular latex (0) or after returning cells to culture for 30 min following trypsinization (X). Triplicate measurements were made at each latex dilution. The standard deviations were all less than 10% of the indicated mean.

FIGURE 5. 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. 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-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 1μm. 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.


a
b
c
L

The enzymatic activity of the LPO was stable for at least 5 hrs, as detected by the triiodide assay. Activity then decreased over the course of two days. In cells which had harbored LPO-latex for 24 hrs, diaminobenzidine reaction product appeared somewhat reduced compared to time zero cells. Importantly, however, reaction product was still restricted to the rims of the latex beads.

VI. INTRACELLULAR IODINATION: CHARACTERIZATION OF RADIOLABEL

A. Radioiodination with Intralysosomal LPO-Latex

The procedure employed for intracellular iodination is outlined in Fig. 3. After ingestion of LPO-latex, monolayers were washed in cold PBS and maintained at 2°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 V). We presume that iodination occurring in the absence of exogenous GO was mediated by the H_2O_2 that was generated by cells during phagocytosis (34). 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 V).

The effect of varying the concentration of the different reactants was assessed. Iodination increased with increasing numbers of LPO-latex beads (Table V). Further increases in exogenous GO above our standard dose of 0.24 mU/ml resulted in little increase in iodination (Table V) and could be toxic to the macrophages. In contrast, iodination varied linearly with the concentration of ^{125}I (Table V) 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.

Table V

VARIABLES AFFECTING INTRACELLULAR IODINATION AT 4°C

Experimental Variable	Peritoneal Cells Plated Per Culture*	^{125}I (μCi/ml)	LPO-Latex (Dilution)	Glucose Oxidase (mU/ml)	CPM/Culture ± SD of Triplicate Measurements
Glucose Oxidase 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	1:2400	0.24	6,999 ± 833
			1:1200		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 days in 16 mm Costar wells in 1 ml of medium. Nonadherent cells were removed by washing. Following ingestion of latex, trypsination and chilling cells were incubated at 4° 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 taken for quantitation of TCA precipitable radioactivity.

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

B. Biochemical Characterization of the Cell-Associated Radiolabel

1. 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 VI).

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 of MIT during the procedure. No radiolabel was ever detected as DIT.

2. 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 VII shows that at least 93% of the lipid-extractable material was 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 lysate, 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.

Table VI

PRONASE DIGESTION AND IDENTIFICATION OF MOLECULAR SPECIES IODINATED INTRACELLULARLY

Sample	Percent of total label in cell lysate				
	Void Volume (Protein)	Poly-Peptide (<5,000)	Free Iodide	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.

Table VII

ANALYSIS OF LIPID EXTRACT OF MACROPHAGES IODINATED INTRACELLULARLY

Spot	Identification of Spot on TLC*	CPM	% of Lipid Extract
SF - I	Free iodide at solvent front	1034	6.9
I^-	Free iodide main spot	4363	29.2
I_2	Iodine	8462	56.7
$1^{\pm}$	Neutral lipid	194	1.3
$2^{\mp}$	Diphosphatidyl glycerol	305	2.0
$3^{\mp}$	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 following 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. Following 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.

VII. INTRACELLULAR IODINATION: LOCALIZATION OF INCORPORATED RADIOLABEL

A. The Localization of Incorporated Radiolabel by Autoradiography

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 hrs showed that > 95% of the macrophages were radiolabeled (Fig. 6a). 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. 6b).

EM autoradiograms were prepared by the flat substrate method of Salpeter and Bachman (48). 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. 7). 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. 7a). 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 (49). In an analysis of 553 grains in 36 random cell profiles, the density of grains over LPO-latex was at least 10 X greater than over any other compartments, including plasma membrane (Table VIII).

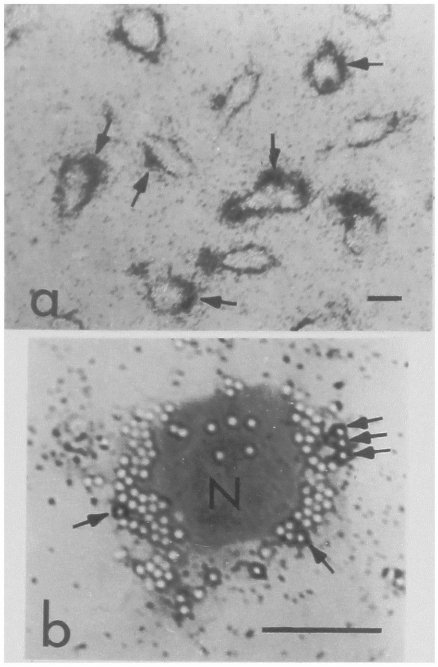


FIGURE 6. Light microscope autoradiography of macrophage monolayers iodinated intracellularly. For illustrative purposes, the macrophages were allowed to ingest large doses of LPO-latex relative to those generally employed. (a) Low-power, bright-field microscopy of 12 macrophages fixed in glutaraldehyde 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. X 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. X 1,600.

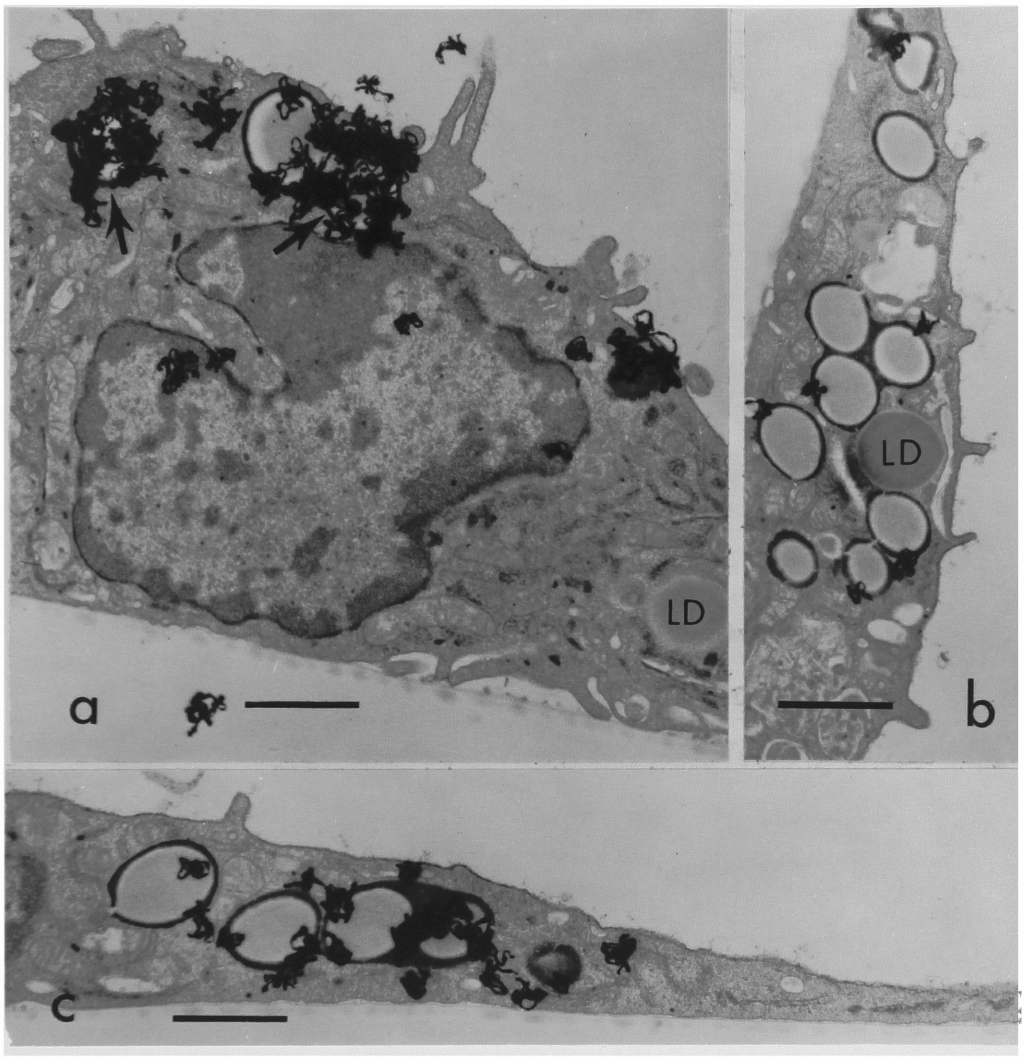


FIGURE 7. EM autoradiography of cells iodinated intracellularly by LPO-latex. Cells were stained with DAB-H_2O_2 to visualize the LPO. In this experiment longer staining visualized endogenous peroxidase activity as well. Fig. 5a illustrates three heavily labeled phagolysosomes. The arrows point to rims 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 show 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. X 15,000.

Table VIII

DISTRIBUTION OF GRAINS FOLLOWING INTRACELLULAR IODINATION*

	Compartment				
	LPO-Latex	Cytoplasm‡	Plasma Membrane	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, vacuoles.

§ See Materials and Methods and Reference (17).

‖ 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).

B. Distribution of Radiolabel within Phagolysosomes - 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 IX and was unchanged by iodination. The percentage of total LPO enzymatic activity in each fraction matched the percentage of latex (now 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 (Fig. 8). 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 IX).

The relatively uncontaminated PL fraction (10/25% interface) contained acid hydrolases, and these enzymes demonstrated latency. Exposure to Triton X-100 (Table X) 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 XI , only $\sim$ 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 X greater than that of any other organelle (Table VIII).

Table IX

SUBCELLULAR FRACTIONATION OF MACROPHAGES

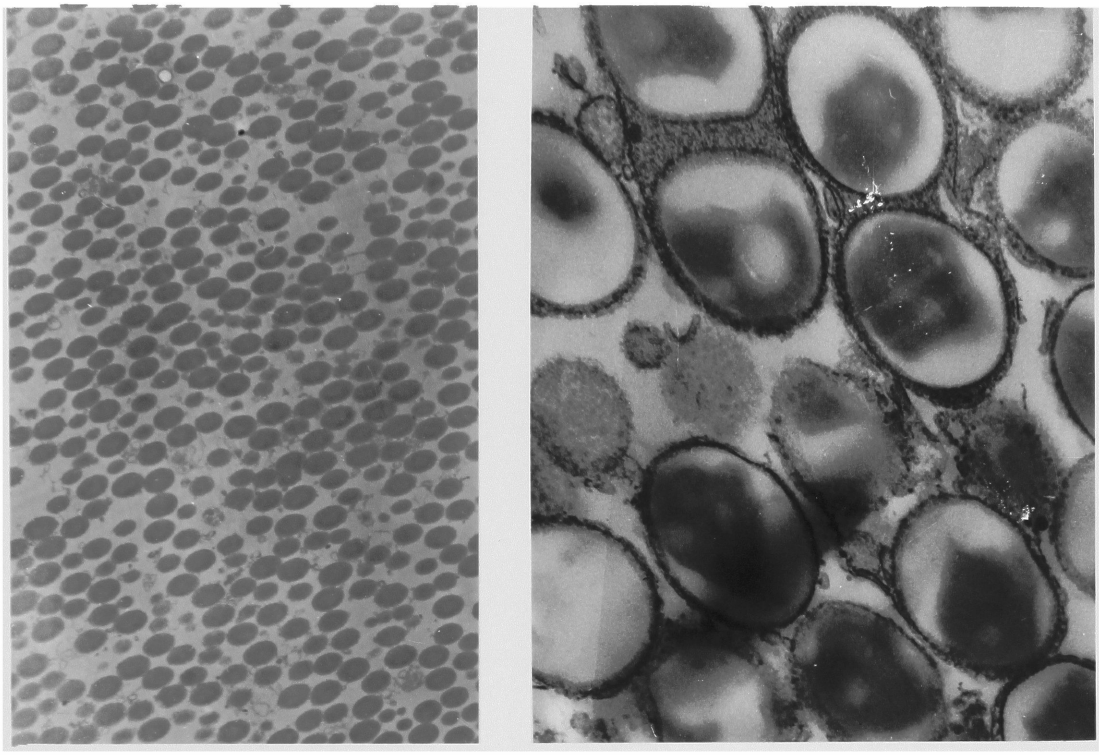
Fraction	Not Iodinated		Intracellular Iodination			Surface Iodination After The Ingestion of Unmodified Latex	
	Latex x 10^6	Total NAGase* µU	Latex x 10^6	Total NAGase* µU	TCA CPM	Latex OD_{500}	TCA CPM
10%	-+	-	-	-	-	-	-
10/25 IF	95.55 (75.8)§	37.7 (3.1)	116.03 (80.9)	33.0 (2.1)	44,948 (30.4)	0.21 (67.7)	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)	1157.8 (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 CPM) and latex, either by counting a suitable dilution in a hemacytometer 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.



a b

FIGURE 8. Electron microscopic appearance of isolated phagolysosomes. The 10/25% interface fraction was isolated from cells fed LPO-latex.
(a) Low power shows that the fraction is not contaminated by other recognizable organelles. Some fragments and vesicles of membrane, not associated with intact PL are present.
(b) High power shows membrane surrounds the latex spheres. Some rare PL profiles containing two latex beads are shown. This specimen had been stained with diaminobenzidine-H_2O_2 to visualize peroxidase activity.
a X 2,800; b X 31,200.

Table X

LATENCY OF ACID HYDROLASE ACTIVITY IN LPO-LATEX PHAGOLYSOSOMES

Enzyme	Activity (μU) Free	Total	Total/Free	% Latent* Activity
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° under isotonic conditions (Free activity) or in the presence of 0.1% Triton X-100 (Total activity).

*% latent activity $= \frac{\text{Total-Free}}{\text{Total}}$

Table XI

DISTRIBUTION OF RADIOLABELLED PROTEIN WITHIN THE PHAGOLYSOSOME

	Acid Hydrolase Activity (Relative Fluorescence Units)		
	N-Acetyl Glucosaminidase	β-Glucuronidase	TCA precipitable ^{125}I-CPM
Total sample	95.7	53	6665
Supernatant	72.4	43.2	150
% Released	75.6	81.5	2.2

Purified PL's 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 supernatant (released) activities. All data are means of duplicate samples, which agreed within 5%.

Note (Table IX) that the proportion of total radioactivity in the 25/35% interface and pellet was high relative to their latex content. Autoradiography of these fractions (Fig. 9) showed that they contained heavily labeled PL, while PL from the 10/25 interface were unlabeled when exposed for the same period.

There are several explanations for the heterogeneous distribution of radiolabeled PL in these gradients. These were tested in the following experiments:

(1) The amount of radiolabel incorporated into PL did not influence their behavior in these gradients. Cells were iodinated at concentrations of $^{125}I^-$ ranging from 1 to 1,000 μCi/ml. The specific radioactivity of each fraction rose proportionally over this thousand-fold range, but the distribution of total radioactivity within each gradient was unchanged.

(2) The PL isolated in the 10/25% interface did not contain LPO-latex whose iodinating capacity had been impaired. LPO-latex PL were first isolated from cell homogenates and subsequently exposed to the iodination conditions. These PL were then passed through the sucrose gradient a second time. All PL refloated to the 10/25 interface and all had incorporated substantial radiolabel, as judged by autoradiography (Fig. 9c).

(3) Similarly, the denser gradient fractions did not contain a subpopulation of LPO-latex possessing greater enzymatic activity. PL were isolated from homogenates of cells fed LPO-latex. The 10/25 interface sample was diluted to contain the same latex concentration as the 25/35 interface. These PL fractions were passed through three cycles of freezing and thawing, then used to iodinate an excess of soluble protein. The same amount of radiolabel was incorporated into protein in both samples (Table XII).

We believe that the denser fractions contain the subpopulation of heavily labeled PL seen by EM-ARG (Fig. 7a). The heterogeneity appears to be due to restrictions on the iodination reaction within the cell rather than any permanent change in LPO activity. We do not know why the PL that are heavily labeled within the cell also possess a greater buoyant

density. However, SDS-PAGE analysis revealed that the same polypeptides were labeled in all gradient fractions. (See Section VIII, Figure 10b)

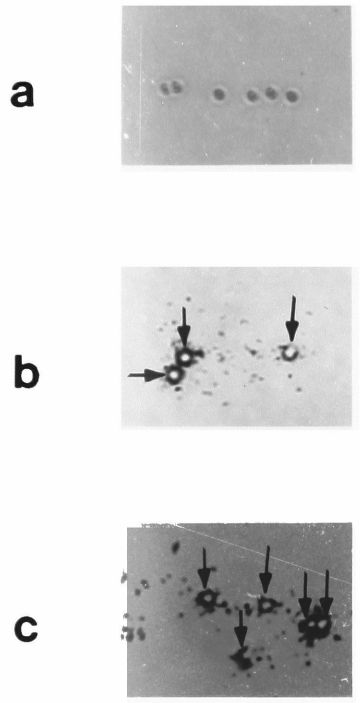


FIGURE 9. Light microscope autoradiography of gradient fractions. Homogenates of macrophages iodinated intracellularly were fractionated and samples of the 10/25% (a) and 25/35% (b) interface fractions were fixed on glass coverslips and processed for LM-ARG. After 1 day of exposure, PL from the 25/35% interface were surrounded by exposed silver grains (arrows) while the PL of the 10/25% interface bore few grains. These PL had not lost their capacity to iodinate, however. PL were harvested from the 10/25% interface fraction of a preparation of unlabeled cells, then exposed to $^{125}I^-$, GO and glucose in PBS. The iodinated PL were then refloated. The PL recovered from the 10/25% interface of the second gradient were prepared for LM-ARG. All PL were heavily labeled (arrows) within 1 day of exposure (c). a, b, and c X 1,600.

TABLE XII

THE 25/35% INTERFACE DOES NOT CONTAIN A SUBPOPULATION OF LPO-LATEX WITH INCREASED IODINATING CAPACITY

Fraction	Total Latex ($\times 10^6$)	TCA-Precipitable Radioactivity (CPM; Mean $\pm$ S.D.)	CPM/10^6 latex beads
10/25	9.50	47,520 $\pm$ 1981	5,002
10/25 Diluted	0.57	2,201 $\pm$ 267	3,861
25/35	0.85	1,723 + 177	2,207

Macrophages in culture were fed LPO-latex and homogenized as usual. Following fractionation on the discontinuous sucrose gradient, latex particles in the 10/25% and 25/35% interface fractions were counted in a hemocytometer chamber. A portion of the 10/25% fraction was diluted in 30% sucrose to approximately the latex concentration of the 25/35% fraction. 0.1 ml FCS was added to 0.3 ml of each sample, then the samples were frozen and thawed three times. ^{125}I and GO in PBS + 20 mM glucose were added to final concentrations of 40 µCi/ml and 0.24 mU/ml, respectively. Iodination proceeded for 10 min at 25° and was terminated by the addition of NaN_3 to 2%. Triplicate aliquots of 50 µl were taken for each sample for determination of acid-insoluble radioactivity.

VIII. THE POLYPEPTIDES OF INTERNALLY LABELED PHAGOLYSOSOME MEMBRANE

The spectrum of PL proteins iodinated intracellularly was analyzed by SDS-PAGE of cell lysates. Twenty-four bands ranging in apparent molecular weight from 250,000 to 12,000 were visible in autoradiograms of the gels (Fig.10 and 12). The same pattern was observed whether macrophages were derived from the NCS or SW variety of Swiss white mice. The identical iodination pattern was obtained whether LPO-latex beads were ingested in PBS or medium containing 10% FCS (Compare Figs.10 and 11). The same iodination pattern was also obtained whether LPO-latex was centrifuged onto the cell monolayer prior to phagocytosis or allowed to settle on the cells in culture at 37° (Fig. 11). When GO was omitted from the reaction mixture and iodination catalyzed by endogenous H_2O_2, the specific activity of incorporated radiolabel was reduced, but the spectrum of iodinated polypeptides was identical to that of cells which received GO.

A band migrating slightly behind the LPO standard was routinely visible. However, the following control experiments demonstrated that none of the bands in the autoradiogram corresponded to LPO or fragments thereof: The LPO on LPO-latex was iodinated to high specific activity. These [^{125}I]LPO-latex beads were washed and either processed for SDS-PAGE or fed to macrophage monolayers. Ingestion of [^{125}I]LPO-latex was allowed to proceed as usual. Then cells were washed entensively, lysed and processed for SDS-PAGE. Samples containing 15,000 TCA-precipitable CPM were run on a gel, which was subsequently processed for autoradiography. Figure 11 shows the autoradiogram. No radioactivity from either sample of the [^{125}I]LPO beads is seen (lanes B and L). This exposure is long enough to readily visualize 15,000 CPM even when distributed over an entire iodination profile (lanes S and C).

The iodination pattern is quite different from the protein staining pattern of the whole cell lysate; none of the major cytoplasmic proteins are labeled. No radiolabel comigrates with actin or myosin standards (Fig.10a) nor with the major iodinatable serum proteins from the culture

medium, BSA and IgG (Fig. 12a). 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 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. 12 a), except for minor and variable differences in the intensity of two high molecular weight bands (asterisks, Fig. 12 a). The pattern of polypeptides radioiodinated intracellularly was unchanged if the cells were maintained at 37° for 1 hr after latex uptake and then iodinated (Fig. 12 a, lane 1). This suggests that the reduced intensity of these two bands is not due to rapid digestion of the corresponding polypeptides within the PL.

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. 12b). Therefore, it is unlikely that the differences in labeling intensity seen in Fig. 12a 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.

To further investigate the striking similarity of PL and PM, we pursued two lines of investigation: (1) to more efficiently iodinate LPO-latex PLs and (2) to improve resolution of membrane components by two dimensional gel electrophoresis.

Fusion of lysosomes with LPO-latex PL was allowed to continue for an extra hour between ingestion and iodination, but again no differences in the composition of the lysosome and plasma membranes were detected (Compare lanes 1 and 2, Fig. 12a).

Fusing lysosomes may bring novel membrane polypeptides which are not detected because, as the pH of the phagolysosome drops, LPO iodinates the

membrane of these PLs less efficiently. To test this possibility, we next performed the intralysosomal iodination in the presence of chloroquine. Following ingestion of LPO-latex, macrophage cultures were incubated for 10 min in PBS containing 100 μM chloroquine prior to iodination. Ohkuma and Poole (40) have shown that this concentration of chloroquine will cause the pH of mouse macrophage lysosomes to increase from 4.7 to 6.3 within 2 min.

The chloroquine-treated cells incorporated 3-6 times more radiolabel than controls. This is consistent with the expectation that raising the lysosomal pH would improve the efficiency of iodination, and hence our ability to detect differences between PM and PL. However, when lysates of cells iodinated in the presence of chloroquine were analyzed by SDS-PAGE, the spectrum of iodinated proteins was identical to that of the unaltered phagolysosomes and to that of PM.

The same pattern was seen when chloroquine was present during ingestion of LPO-latex as well as iodination, and when chloroquine was present during ingestion, but washed out of the cells before iodination (Fig. 13).

We have begun experiments to compare PM and PL using the greater resolving power of two dimensional gel electrophoresis. In these initial attempts, we have encountered some difficulty getting all of the radiolabel to move into the isoelectric focusing gel. However, comparison of the radiolabel which is resolved in this system reveals the same striking similarity of iodination patterns (Fig. 14).

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 which can be detected by these techniques.

Recall (Table XI) that we could not detect labeling of soluble content proteins in isolated PL. Neither can we detect any proteins other than membrane proteins by SDS-PAGE. Since $\geq$ 98% of the iodinated protein in isolated PL is membrane protein, detection of any iodinated

content proteins would require very sensitive techniques. The following experiment demonstrated that soluble proteins, at least from exogenous sources, could be iodinated in LPO-latex PL. Macrophages were cultured overnight in 10% normal mouse serum. Serum components enter the cell by pinocytosis, and macromolecules accumulate in secondary lysosomes. These cells were washed extensively and then allowed to ingest LPO-latex. Following intracellular iodination, cell lysates were subjected to immune precipitation with an antibody which recognized mouse IgG. Radiolabeled IgG was detected in the precipitates from these cells (Fig. 15, Ppt. lane 1) but not in precipitates of cells not exposed to mouse serum (Fig. 15, Ppt lane 3). If macrophages were exposed to mouse serum only during ingestion of LPO-latex, even more radiolabeled IgG was precipitated (Fig. 15, Ppt lane 2). Mouse IgG sticks to LPO-latex by hydrophobic interactions (data not shown). This binding may have allowed more of it to enter LPO-latex PL and/or to be in a sterically more favorable position with respect to the LPO. Iodination of IgG(and presumably other serum components not precipitated by the antibody) within the PL did not alter the iodination pattern (Fig. 15). Note that the samples subjected to immune precipitation contained eight times the radioactivity as the samples run in the "Pre" and "Post" lanes.

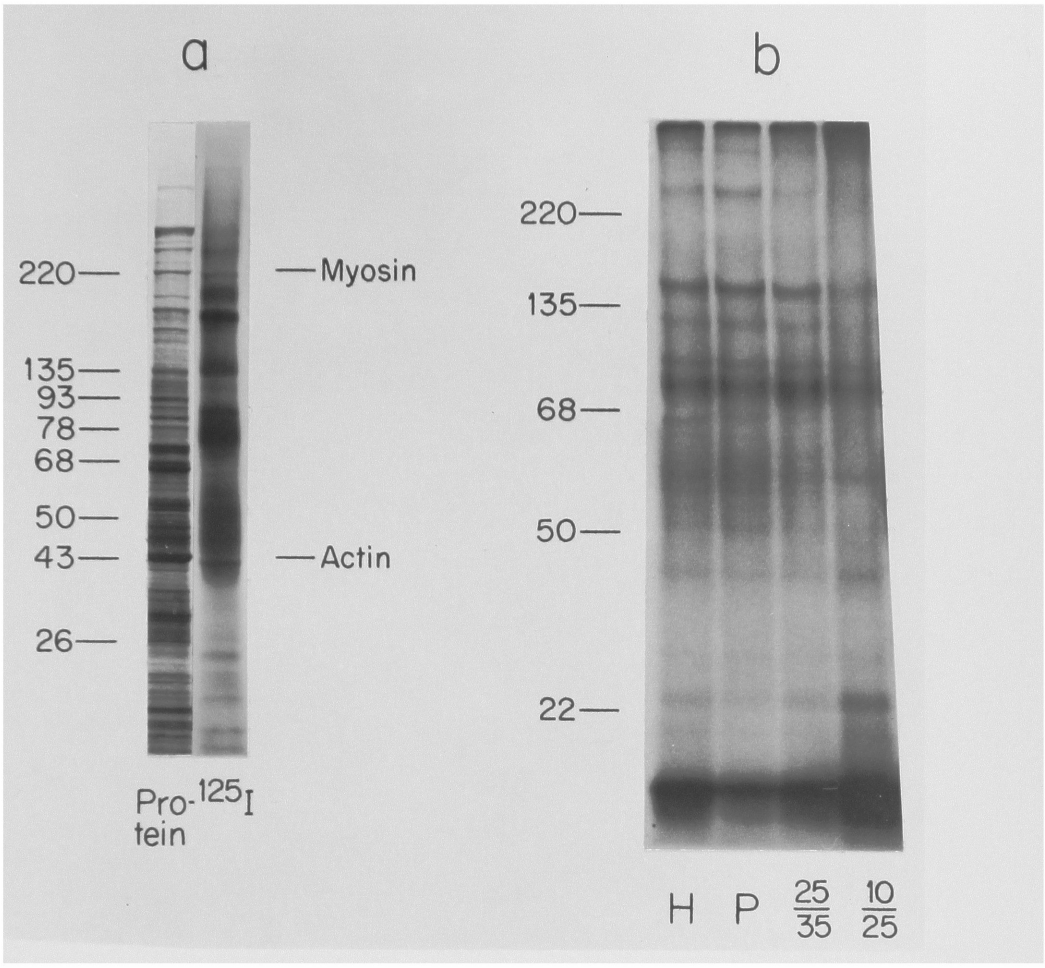


FIGURE 10. (a) LPO-latex mediates the iodination of a select group of cell proteins. Macrophages were iodinated from within the PL and cell lysates separated by SDS-PAGE with a 4-11% gradient gel. The gel was stained with Coomassie blue (Protein), dried, and exposed for autoradiography (^{125}I) on DuPont Cronex film for 4d 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).

FIGURE 11. Controls for LPO-latex catalyzed intracellular iodination. (See Section III. F.2 for experimental details)

1. No gel bands are due to radioactive LPO. [^{125}I]LPO-latex spheres were fed to macrophages by the usual protocol. Following ingestion, cells were lysed and processed for SDS-PAGE as usual. 15,000 TCA precipitable CPM were loaded onto lane L. Lane B received 15,000 CPM of the original [^{125}I]LPO-latex. Upon prolonged exposure a trace of radiolabel at $\sim$ 78,000 daltons appeared in lane B, but not in lane L. Arrow indicates position of authentic ^{125}I-LPO run as a standard.

2. The iodination pattern of phagolysosomes is not a consequence of the method of delivery of LPO-latex. LPO-latex was delivered to macrophage monolayers by centrifugation (lane C) or by allowing them to settle onto the cells (lane S). Following ingestion in complete medium cells were iodinated, washed and lysed as usual. Approximately 15,000 TCA precipitable CPM were run on this 4-11% gradient gel. Compare these iodination patterns to Figure 10a where ingestion of LPO-latex took place in PBS. One day exposure on Kodak XR-1. *E. coli* RNA polymerase was run as a gel standard. Subunit molecular weights = 140,000, 90,000, and 40,000.

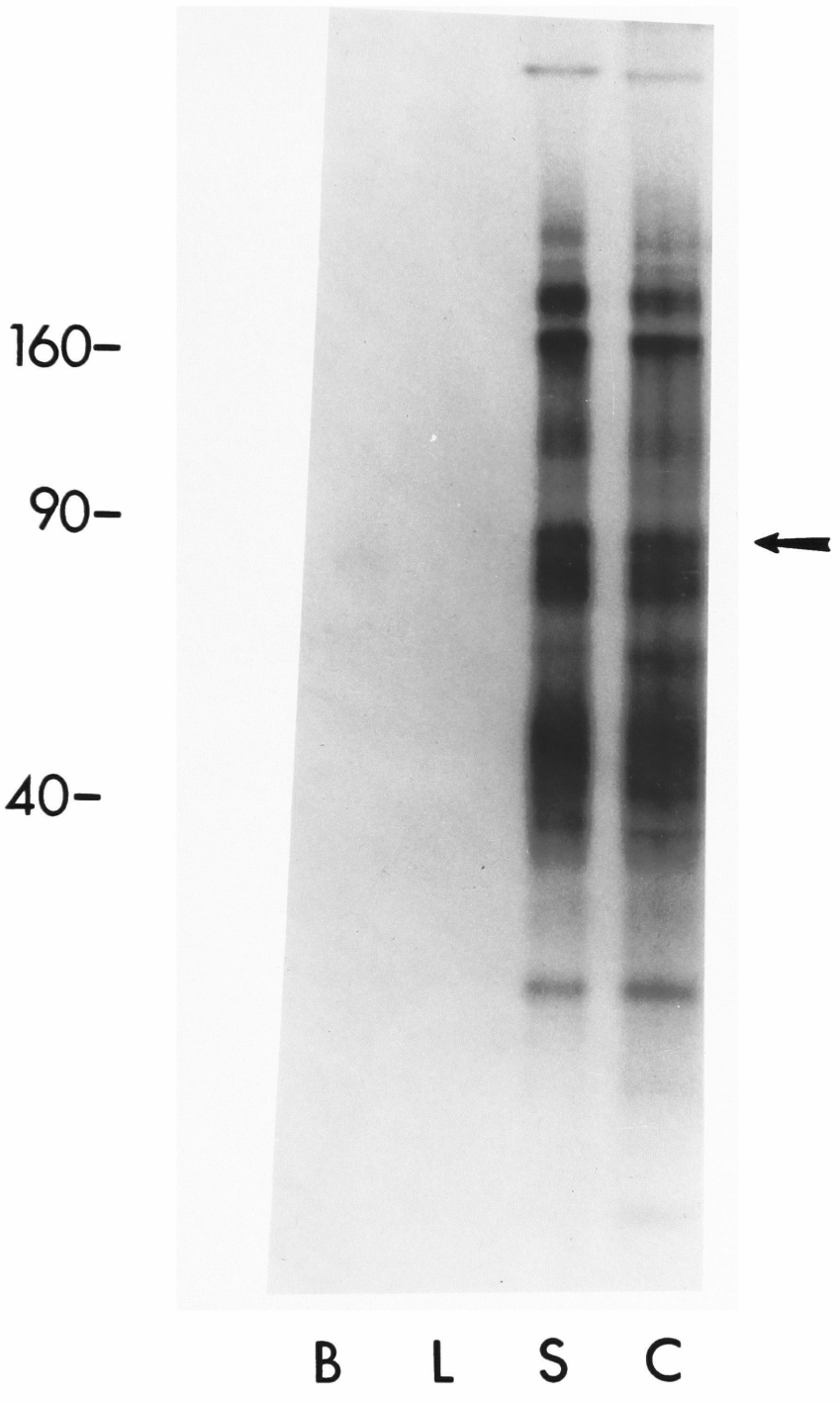

160–
90–
40–
B
L
S
C

FIGURE 12.

(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% of 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 (X 10^3) are indicated at the left.

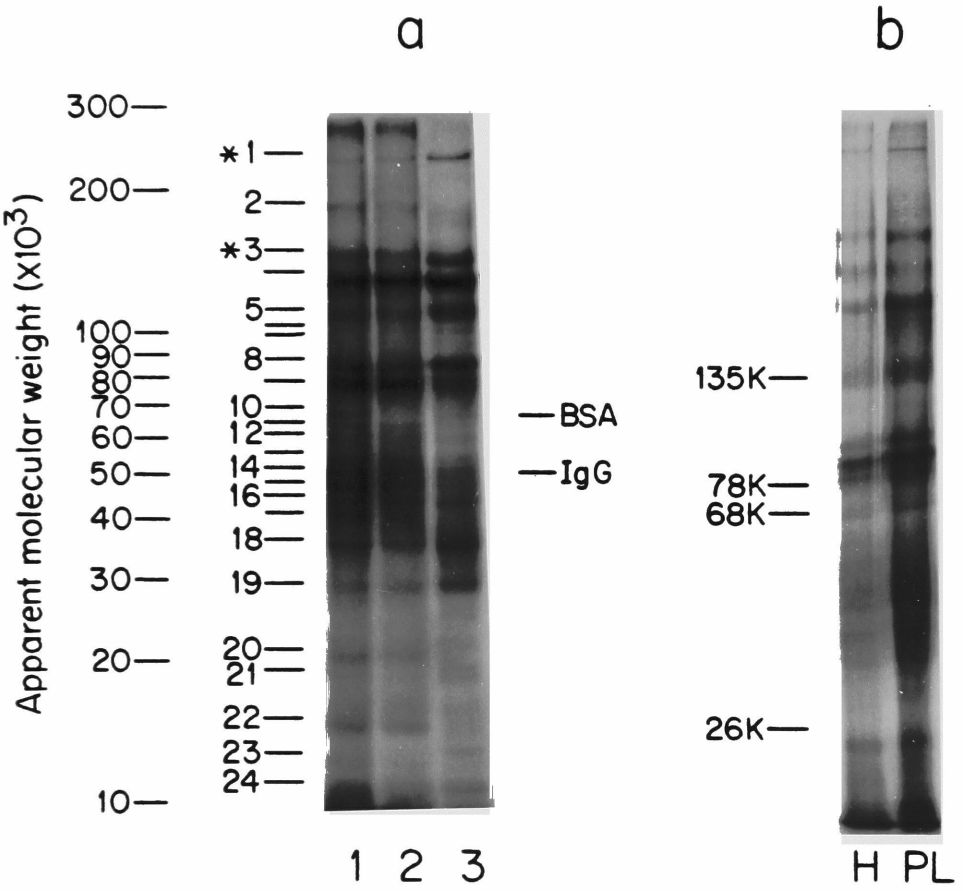

a
b
Apparent molecular weight (X10^3)
300
200
100
90
80
70
60
50
40
30
20
10
*1
2
*3
5
8
10
12
14
16
18
19
20
21
22
23
24
BSA
IgG
1 2 3
135K
78K
68K
26K
H PL

FIGURE 13. Effect of chloroquine on the iodination pattern of PL. Macrophages were allowed to ingest LPO-latex in the presence or absence of 100 μM chloroquine, then iodinated on ice in the presence or absence of 100 μM chloroquine. Approximately 40,000 TCA-precipitable CPM from each sample were run on a 4-11% SDS gel. 3 day exposure on Cronex film. E = plasma membrane iodinated extracellularly by LPO-latex. I, Q, Q', Q" and S = PL iodinated intracellularly by LPO-latex. I = normal procedure for intracellular iodination. Q = chloroquine present during ingestion and iodination. Q' = chloroquine present during ingestion only. (Cells were washed extensively on ice before iodination.) Q" = chloroquine present during iodination only. S = control to test the effect of lysosome swelling on iodination pattern, since chloroquine also causes lysosomes to swell. Cells were incubated in 100 mM sucrose for 2 hr prior to ingestion of LPO-latex. This procedure causes swelling of lysosomes including those containing LPO-latex, but does not change the efficiency of iodination.

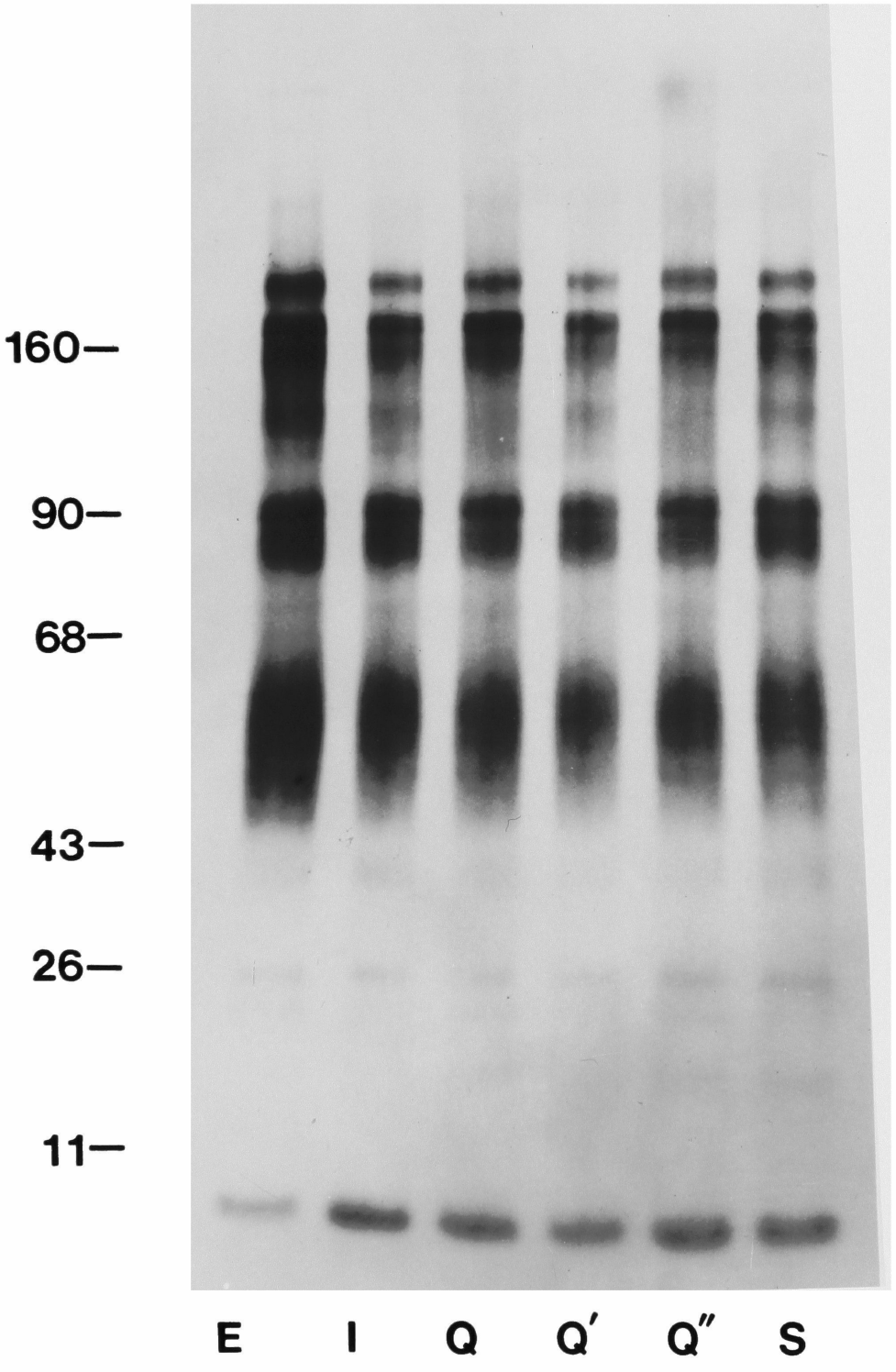
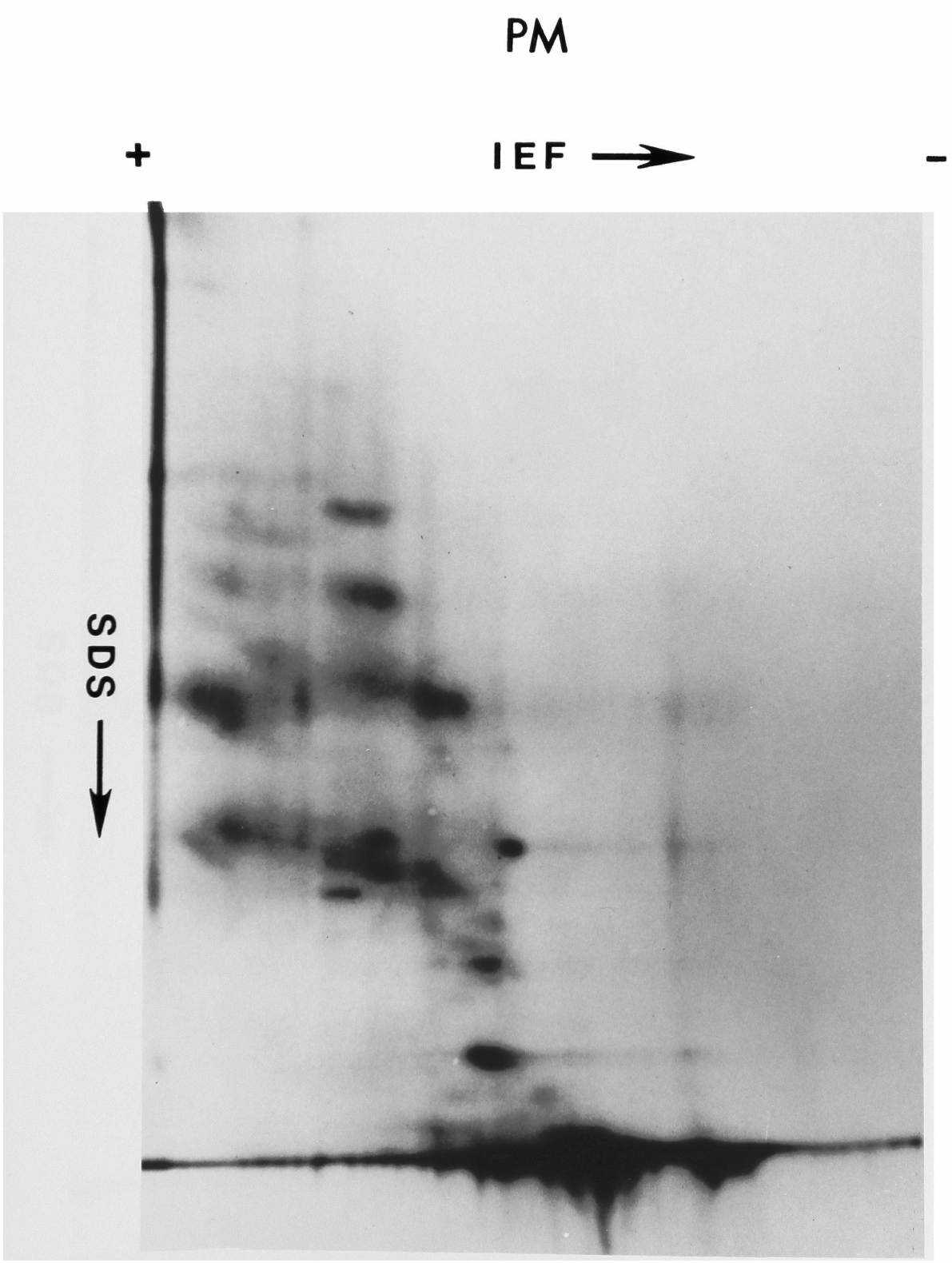

160—
90—
68—
43—
26—
11—
E
I
Q
Q′
Q″
S

FIGURE 14. Comparison of iodinated plasma membrane (PM) with phagolysosome membrane (PL) by two dimensional gel electrophoresis. IEF = isoelectric focusing (first dimension); + and - indicate anodal and cathodal ends, respectively. The pH gradient spanned the range from 4.2 to 8.0. 4.5 day exposures on Kodak XR-1 film.


PM
+
IEF
-
SDS

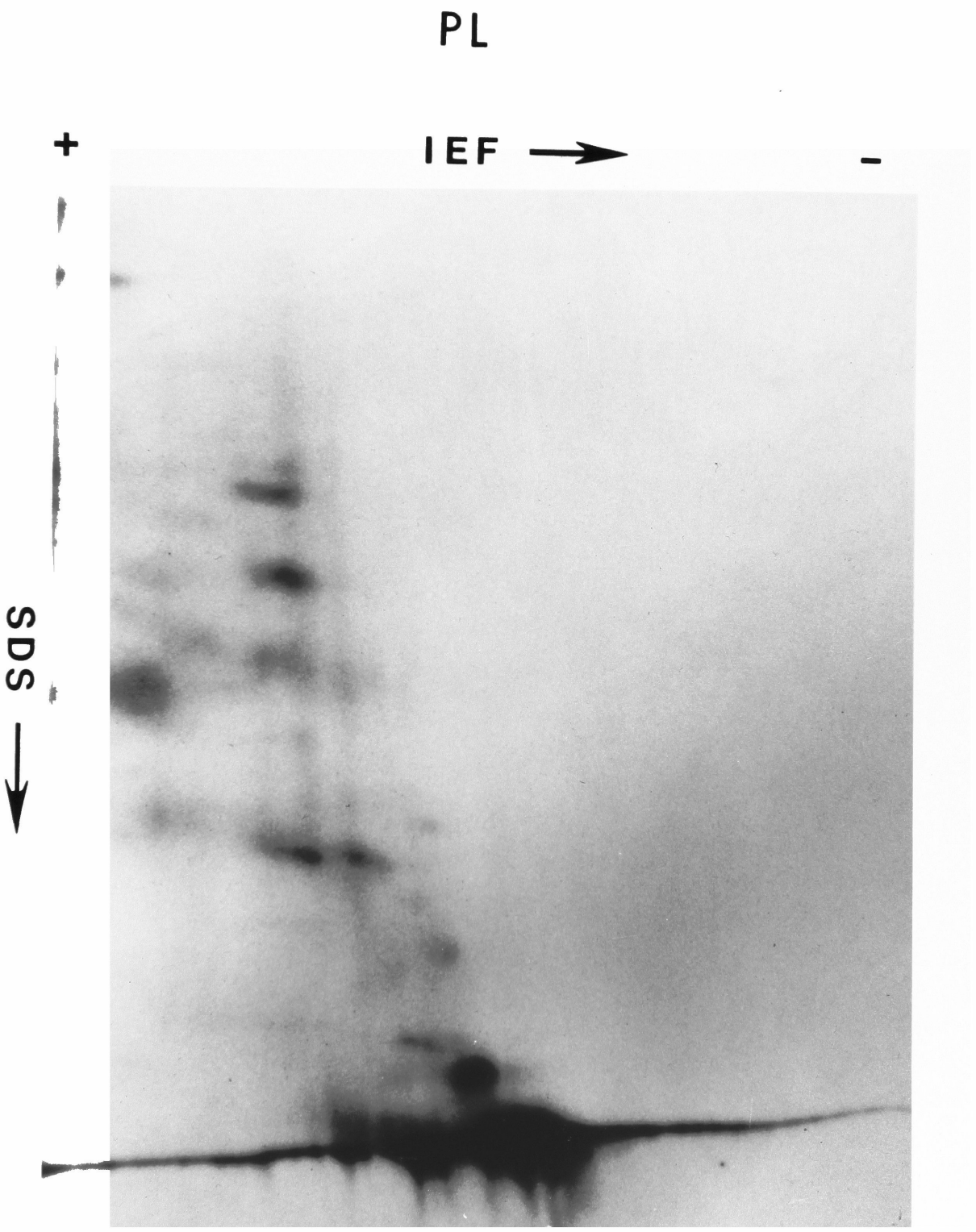
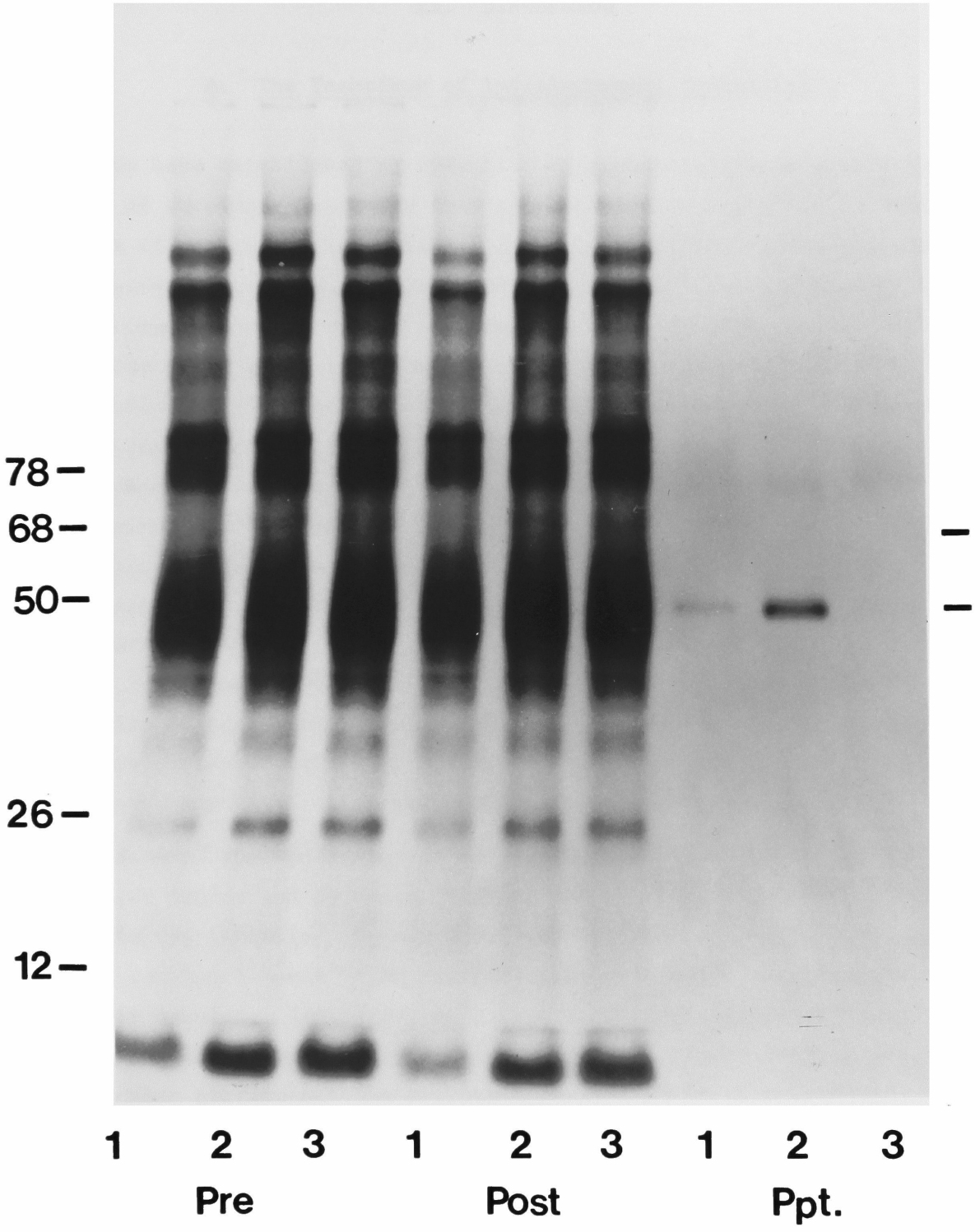

PL
+
IEF
-
SDS

FIGURE 15. Iodination of mouse IgG in LPO-latex PL. (See section III. F.3 for experimental details.) Macrophages were exposed to normal mouse serum prior to ingestion of LPO-latex (lanes 1), during ingestion of LPO-latex (lanes 2) or not at all (lanes 3). Following intracellular iodination, cell lysates were subjected to immune precipitation with an antibody against mouse IgG. The precipitate from 200 µl of lysate was analyzed by SDS-PAGE (Ppt.). 25 µl of whole cell lysate from each sample before (Pre) and after (Post) immune precipitation were also run. Cronex film, 1 day exposure. Gel standards include BSA (68,000) and IgG (50,000).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IX. DISCUSSION

A. The Technique of Intralysosomal Iodination

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 below neutrality 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 (34), 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 (45) and Root (46) 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 concentrations 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.10b).

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. It may

PART TWO

BIDIRECTIONAL FLOW OF MEMBRANE BETWEEN LYSOSOMES AND THE CELL SURFACE

X. INTRODUCTION

In Part One we described a technique for selectively iodinating the luminal surface of the phagolysosomal (PL) membrane by interiorizing lactoperoxidase conjugated to latex spheres. Radiolabel was recovered as monoiodotyrosine and was present in at least 24 separate polypeptides (12,000-250,000 daltons) when displayed on one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. These labeled proteins were essentially identical to those labeled in the plasma membrane by surface iodination.

In this report we use these methods to gain further insight into the fate of these proteins when cells are returned to culture.

Previous studies of the fate of content (15 , 54) and membrane (24 ,59) tracers brought into the lysosome by endocytosis were unidirectional in design. They demonstrated that the membrane and content of these vacuoles were digested in these secondary lysosomes.

We, too, observe rapid digestion of part of the iodinated PL membrane proteins. However, because the radiolabel is incorporated selectively into membrane proteins of the PL, we are able to observe in addition, that most of the radiolabel which is initially in the PL membrane moves rapidly to the cell surface and is randomly associated with the plasma membrane. Isolation of plasma membrane after the redistribution of label indicates that all the polypeptides initially iodinated within the PL take part in this flow. Simultaneously, plasma membrane with its full complement of polypeptides is being interiorized as pinocytic vesicles and is entering the PL compartment. These studies demonstrate in a direct fashion the continuous and bidirectional flow of membrane polypeptides between the vacuolar apparatus and the cell surface.

XI. FATE OF RADIOLABELED PHAGOLYSOSOME MEMBRANE POLYPEPTIDES

Initial studies were focused on the turnover of the iodinated polypeptides of the PL membrane. Phagocytosed LPO-latex was used to iodinate the PL membranes of mouse macrophages and the cells were returned to culture. The macrophages remained viable for at least 1 wk, and their morphologic features were indistinguishable from those of cells receiving a phagocytic load of unmodified latex. Quantitation of cell-associated latex by direct microscopic examination at high power, and by light scattering of cell lysates, showed that all of the LPO-latex originally ingested remained within the cells. LPO activity of the ingested latex remained stable for $\sim$ 5 h (triiodide enzymatic assay) but fell to one-tenth the original activity by 24 h.

The TCA-precipitable radiolabel that had been incorporated into PL was lost from the monolayer with biphasic kinetics (Fig. 16). Identical kinetics were obtained whether or not trypsinization was employed to remove extracellular beads. There was first a rapid loss ($t_{\frac{1}{2}} \cong$ 1 hr) of 20-50% of cell-associated radioactivity. The size and rate of this rapid component was unaffected by the amount of radiolabel incorporated within the range we have tested (Fig. 16). This early rapid loss of TCA-precipitable counts was the result of extensive digestion of radiolabeled proteins to the amino acid level. Lost TCA-precipitable counts were recovered quantitatively as radiolabeled monoiodotyrosine ($M^{125}IT$) when the culture medium was chromatographed on Sephadex G-25 to separate MIT from the free iodide that also eluted (Table XIII).

When macrophages were maintained on ice for 3 h following intralysosomal iodination and washing, there was no decrease in TCA-precipitable radiolabel associated with these cells. When these cells were warmed to 37°, digestion of radiolabel began immediately and exhibited the biphasic kinetics demonstrated by cells returned to culture directly following iodination (Fig. 17).

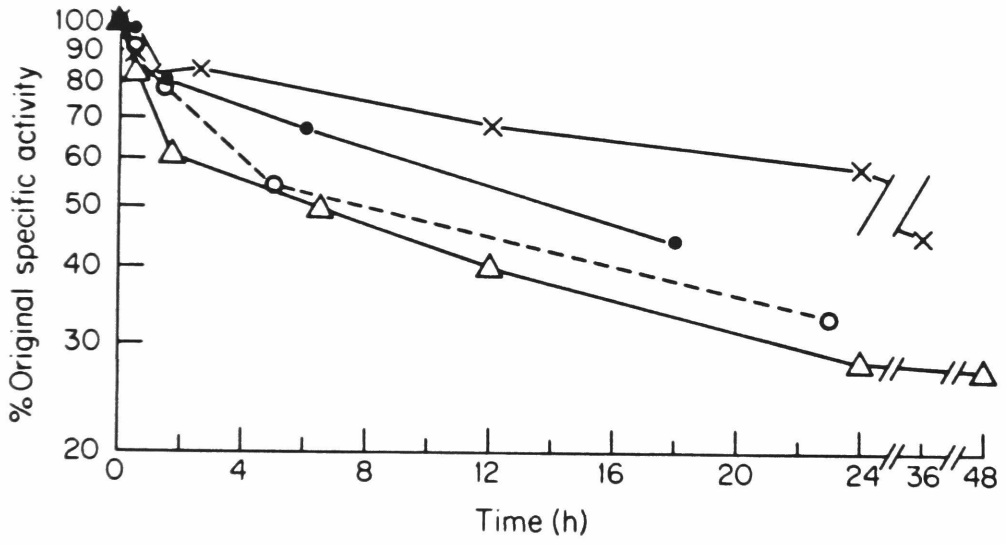


FIGURE 16. Kinetics of radiolabel loss from internally iodinated macrophages. After intracellular iodination, cells were returned to culture for the times indicated, then washed, lysed, and assayed for TCA-precipitable radioactivity and protein. The curves are from four separate experiments performed over the course of 1 yr. Each point represents the mean of triplicate measurements. The standard deviation was always within 10%. (The original specific activities [cpm/μg of cell protein] were: X=1,662, O=37,352, ●=758, and △=584.

Table XIII

Identification of Radiolabelled Species in Cells and Culture Medium

Sample	Time in Culture (hr)	RADIOACTIVITY	
		Void Volume	MIT
Cells	0	15,363	0[§]
Cells	2	5,882	238
Medium	2	0	10,042

Following intracellular iodination or 2 hr after return of cells to culture, the medium was aspirated and cells scraped from the dish with a plastic policeman. The medium and cells were lyophilized immediately and the concentrated material was chromatographed on Sephadex G-25.

[§]Not detectable above background.

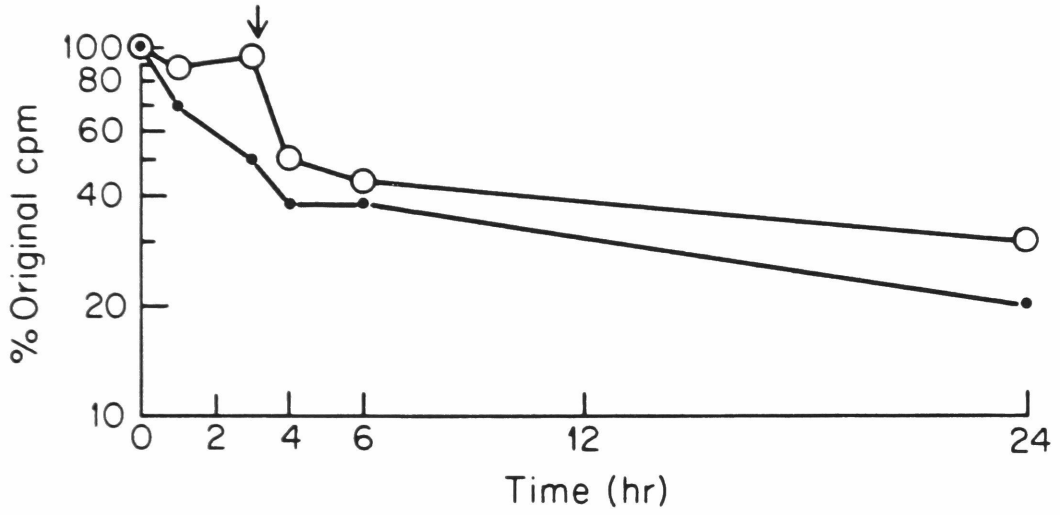
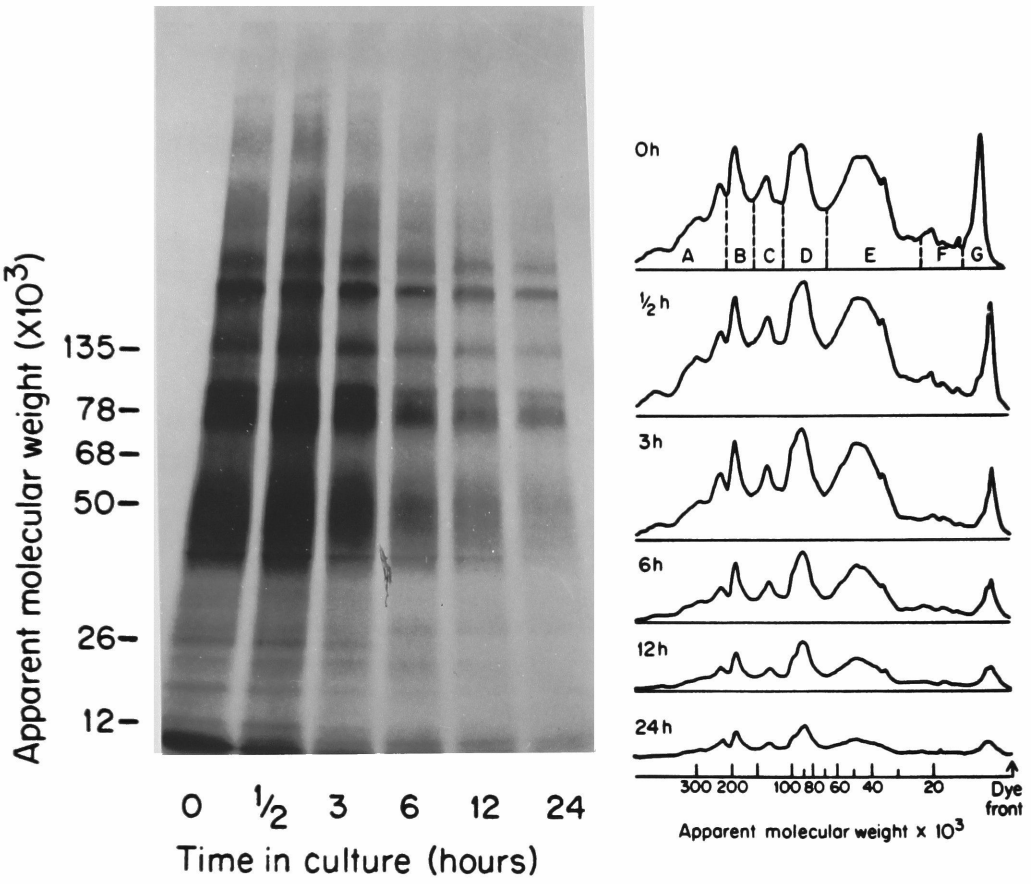


FIGURE 17. Digestion of radiolabel is inhibited at low temperatures. Macrophages cultured 2 days in Costar wells were iodinated using intralysosomal LPO-latex. Cells were then washed and returned to culture at 37° (●) or incubated on ice in ice-cold culture medium (0). At the times indicated triplicate cultures were lysed and assayed for TCA-precipitable radioactivity. After the 3 hours time point, all remaining cultures were given fresh warm culture medium and returned to the incubator at 37° (arrow). The data shown are the means of the triplicate samples. Standard deviations were always less than 13%. Original TCA precipitable radioactivity was 32,200 cpm/culture for (●) and 21,300 cpm/culture for (0).

The bulk of the label (50-80%) was degraded slowly, with a $t_{1/2}$ of 24-30 h or longer (Fig. 16). SDS-PAGE was then employed to characterize cell-associated radiolabel at various times after return to culture (Fig. 18). Although the total radioactivity decreased with time, the same iodinated species were observed at all times. In addition, quantitative densitometric analysis of the autoradiogram showed that the relative intensity of isotope among the bands was preserved (Fig. 18). This indicated that all of the labeled polypeptides were subject to both rapid and slow phases of degradation and turned over at a similar rate.

The presence of a large pool of free ^{125}I immediately after iodination raised the possibility that radiolabel might continue to be incorporated and generate an artificial slow phase. We therefore exposed LPO-latex-containing cells to the standard iodination conditions in the presence or absence of 6 mM (0.02%) sodium azide, which reversibly inhibits LPO activity. Although azide completely inhibited intracellular iodination (Table XIV and see Table V), a pool of free ^{125}I accumulated nevertheless. When these cells were washed and returned to culture, they failed to incorporate any radiolabel into TCA-precipitable material over a 2-h period (Table XIV). As a control, we incubated azide-treated cells with fresh ^{125}I and GO. The ^{125}I was incorporated, proving that the LPO-latex was still enzymatically active. We conclude that after the initial intracellular iodination at 4°C in our system, macrophages did not incorporate additional ^{125}I after return to culture.

FIGURE 18. Turnover of labeled phagolysosomal polypeptides. After intralysosomal iodination, macrophages were returned to culture. At the times indicated, cultures were lysed and samples containing equal amounts of protein were subjected to SDS-PAGE (4-11% gradient gel). Top left, autoradiogram of dried gel (4-d exposures on Kodak XR-1 film with enhancing screen). Molecular weights of gel standards are indicated at the left. Top right, densitometer tracings of the autoradiogram. The curve has been broken up into peak regions (indicated by the vertical lines) by an integrating circuit attached to the densitometer that also calculated the percent of total area under each peak. These values are tabulated.



Hours After Return to Culture	REGION A	B	C	D	E	F	G
	% of Total Area						
0	16.5	9.9	9.7	17.5	27.9	6.5	7.7
0.5	16.4	8.3	10.2	17.9	29.1	5.9	7.1
3	16.9	9.6	9.1	18.3	31.1	5.0	5.9
6	16.1	9.5	8.8	18.1	29.5	7.6	7.2
12	16.2	9.6	6.8	19.8	34.6		8.9
24	16.5	9.6	6.5	18.6	25.7	5.7	8.3

Table XIV

Residual $^{125}I^-$ Is Not Incorporated Upon Return To Culture

Azide During Iodination	Return To Culture in	Time in Culture (hr)	Specific Radioactivity (CPM/μg cell protein)
-		0	1569 ± 238
+		0	52 ± 16
+	Culture medium	0.5	39 ± 10
+		2.0	50 ± 23
+	$^{125}I^-$, glucose, GO	0.5	1687 ± 444
+		2.0	1753 ± 235

2×10^6 resident peritoneal cells were plated in 16 mm Costar wells and cultured 2 days in medium 199 supplemented with 10% FCS. Following ingestion of 1:800 LPO-latex, they were exposed to iodinating conditions (40 μCi/ml $^{125}I^-$, 0.24 mU/ml GO) on ice in the presence or absence of 6 mM (0.02%) NaN_3. The latter treatment blocks iodination but allows the cells to accumulate $^{125}I^-$.

After 30 min some of the azide-treated cultures were washed returned to the 37° incubation for 1/2 or 2 hr in either culture medium or fresh iodinating reagents.

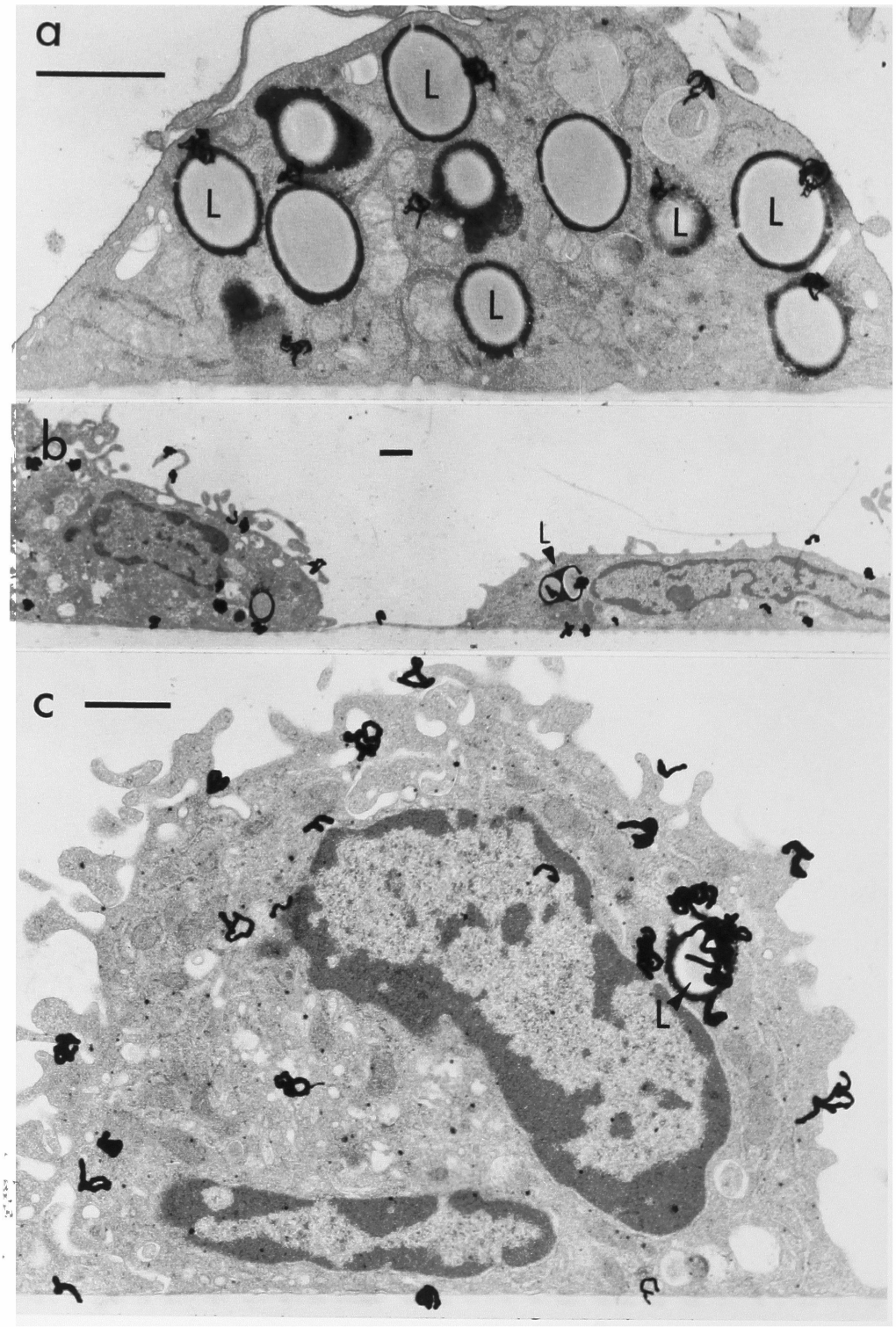
XII. REDISTRIBUTION OF RADIOLABEL FROM THE PHAGOLYSOSOME MEMBRANE

A. Electron Microscope Autoradiography

The preceding studies indicated that a large fraction of the polypeptides initially iodinated in the PL membrane remained intact and cell associated. We next employed EM-ARG to examine their distribution in cells incubated at 37°C. We had previously found that, immediately after intracellular iodination, exposed silver grains were located over LPO-latex PL (Fig.19a). When the cells were returned to culture for 30 min, a striking redistribution of grains was observed (Fig.19b-e). Now, large numbers of grains were present on the cell surface and found over the PM. Some grains also lay over the cytoplasm, particularly in vesicle-rich areas, and some labeling of LPO-latex PL was still evident (Fig. 19). The redistribution of label to the PM was readily detected within 15 min of culture (the earliest time-point studied). In addition, the distribution of grains in EM-ARG performed 0.5, 1, and 24 h after return to culture was similar and suggested that a steady state had been reached at the earliest time-point. For quantitative analysis, autoradiograms were prepared by the flat substrate method and analyzed by the probability circle method as described in the previous paper. Analysis of 530 grains in 48 randomly selected cell profiles showed that 78% of the grains were located over PM at 30 min (Table XV). The density of grains on the dish and upper surfaces of the PM were equal. Relatively few grains were associated with LPO-latex at this time, although the grain density of this compartment approached that of the PM, when counts were corrected for the relative areas of PM and PL membrane (Table XV). Compartments other than PM and PL contained little label.

We conclude that by 30 min after return to culture the radiolabel had redistributed from the PL to the PM.

Attempts were made to quantitate the rate of radiolabel appearance on the cell surface by means of short periods of proteolysis (100 μg/ml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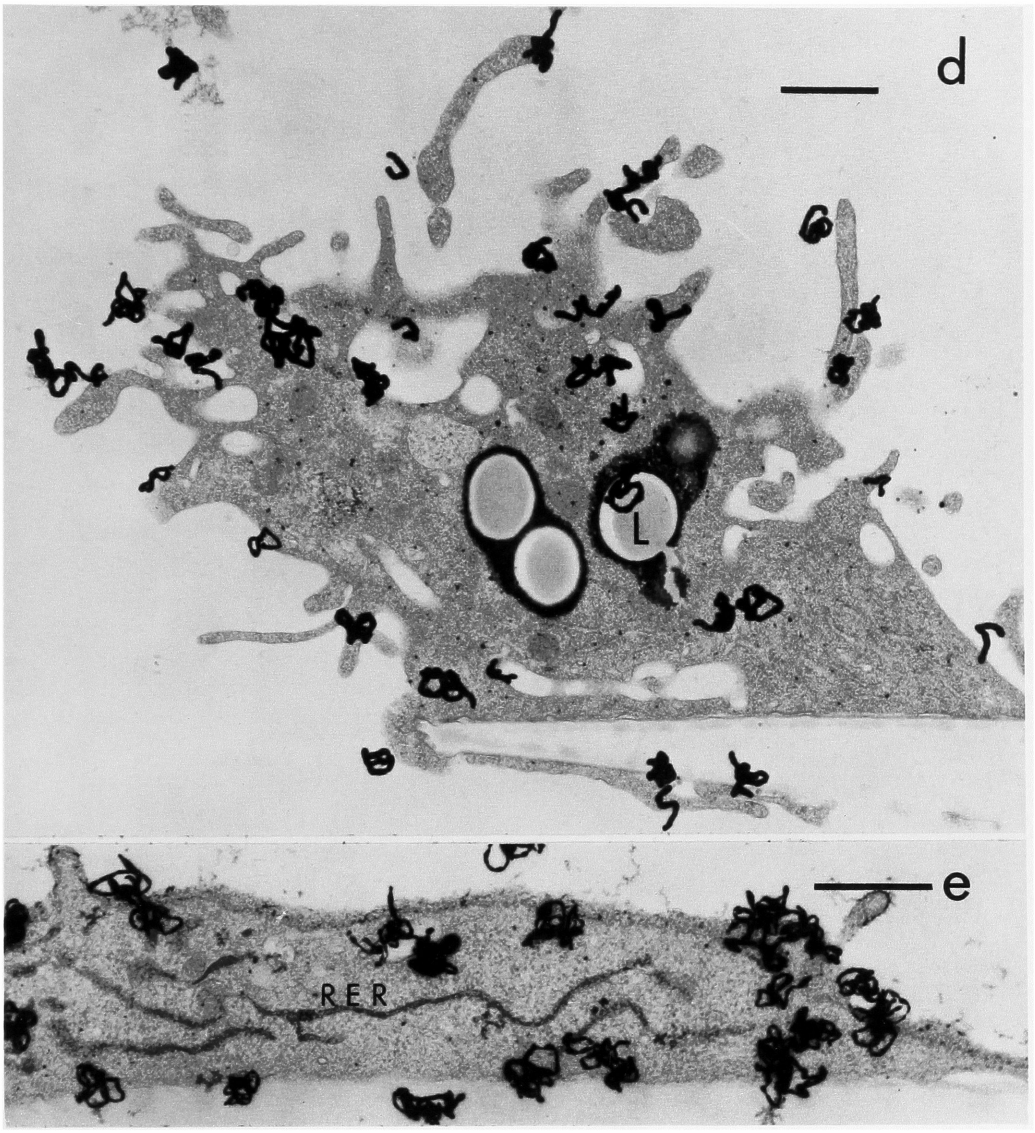

FIGURE 19. Redistribution of radiolabel from PL to plasma membrane. (a) Cell fixed immediately after iodination shows grains associated with LPO-latex PL (L). (b-e) Macrophages returned to culture for 30 min before fixation. Most of the grains are distributed over entire PM including the flat surface adherent to the culture dish. Some grains are still seen in association with LPO-latex. The cells shown in b, c, and d were stained with DAB-H_2O_2 for 10 min to mark LPO-latex. Ilford L4 emulsion, Microdol X developer, 2-wk exposure. The cells in a and e were from a different experiment. They were exposed to DAB-H_2O_2 for 30 min, a treatment that visualized peroxidase activity in rough endoplasmic reticulum (RER) and the perinuclear cisterna as well as LPO-latex PL. Ilford L4 emulsion; Kodak D-19 developer. 3.5-d exposure. Bars, 1 μm. (a) X 19,500, (b) X 5,000, (c) X 13,000, (d) X 13,000, (e) X 15,600.

Table XV

DISTRIBUTION OF GRAINS THIRTY MINUTES AFTER

RETURN TO CULTURE

		Plasma Membrane	LPO-Latex	Cytoplasm*	Nucleus	Mitochondria
Total Grains (Uncorrected)		383	25	89	33	0
Grains Corrected for Radiation Spread from Plasma Membrane‡		383 (78.0%)	25 (5.1%)	61 (12.4%)	22 (4.5%)	0
Relative Grain Density§	A	1.91	0.86	0.42	0.21	
	B	1.92	1.11	0.40	0.21	

*Cytoplasm includes ground cytosol, RER, Golgi, Vacuoles and all other structures not in separate categories. A grain was assigned to the cytoplasm compartment only when no other organelle fell within one half-radius from its center.

‡ Corrected for spillover of grains from the heavily labelled compartment as in Ref. 17

§ Percent of total grains assigned to the compartment divided by the percent of total cell area which that compartment occupies. The data in row A were calculated based on the relative area of the entire LPO-latex bead profile. The data in row B were calculated using the relative area of the rim of the LPO-latex phagolysosome as the denominator.

chymotrypsin for 5 min at 37°C) at various times after return to culture. Such a short period was necessary because redistribution of label was so rapid and because chymotrypsin could enter the cell by pinocytosis. The material released into the extracellular medium was concentrated and analyzed by Sephadex G-25 chromatography. Immediately after iodination, cell-associated radiolabel was completely resistant to extracellular protease (Table XVI). Within 30 min of additional culture, however, maximal quantities of protease-sensitive radioactivity were observed. The percent of total cell radiolabel susceptible to proteolysis was small in absolute terms (∿ 2.5% of the total), but corresponded to half the level that could be removed by a similar treatment of macrophages iodinated externally (Table XVI). We conclude that some of the radiolabel redistributing form PL to PM is protease sensitive; however, the low levels of radioactivity released under these conditions prevented further use of this approach.

B. Isolation and Identification of the Phagolysosome Polypeptides Appearing on the Plasmalemma

Next, it was necessary to characterize the iodinated PL polypeptides that returned to the cell surface. We therefore isolated samples of PM from cells that had been iodinated intracellularly, either immediately after iodination or 30 min after return to culture. To accomplish this, we performed the experiment outlined in Fig. 20 . Two separate cultures of macrophages ingested LPO-latex and were iodinated intracellularly on ice as usual. Then, immediately following iodination, low density styrene-butadiene latex spheres (S-B latex) were centrifuged down on top of one of the monolayers at 4°C. The other cells were returned to the incubator for 30 min to allow redistribution of radiolabeled PL membrane. Then this culture was chilled and given the same S-B latex dose. Once the cells had received S-B latex, the culture was rapidly warmed to 37° for 5 min to allow ingestion of this second latex load. The S-B latex became surrounded by membrane which was at the cell surface when the beads were ingested as well as any which may have fused with the forming phagosomes during the 5 min ingestion period.

Table XVI

Protease Sensitivity of Cell Radiolabel

	Time In Culture After Iodination (hr)	Radiolabelled Polypeptide Released (CPM removed per 10^5cpm of cell radiolabel)
Surface Iodination LPO-latex attached to surface of cell monolayers in the cold	0	4,709
Intracellular Iodination LPO-latex phagocytosed by macrophages	0	0
	0.5	2,366
	5	2,694
	19	1,302

Following iodination cells were returned to culture for the times indicated, washed and exposed to chymotrypsin (100 µg/ml) for 5 min at 37°. The supernatants were chromatographed on Sephadex G-25. See Materials and Methods for experimental details. Protein counts were not released at any time in the absence of chymotrypsin.

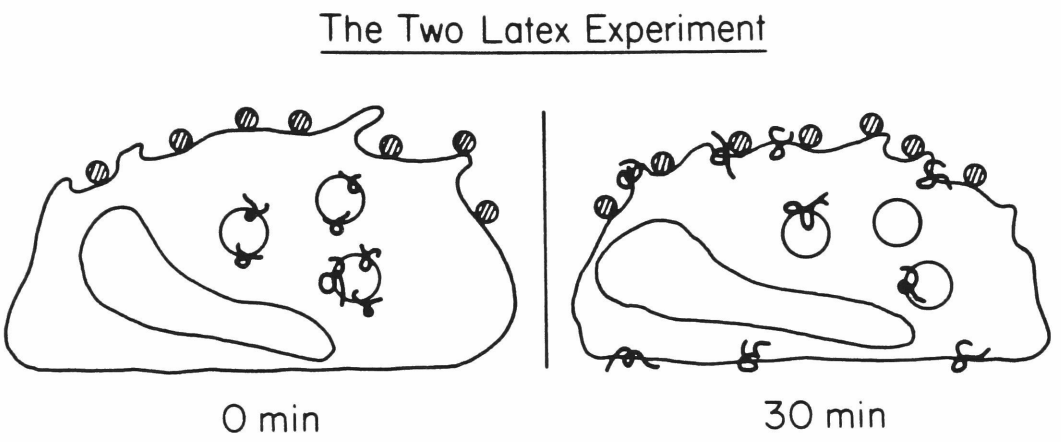

FIGURE 20. Outline of protocol used to retrieve PM from macrophages following intracellular iodination. See text for details.

The cultures were then homogenized, and S-B latex phagosomes were separated from LPO-latex PLs by virtue of their lower density. Discontinuous sucrose density gradients could be used to retrieve some two-thirds of the S-B latex phagosomes from cell homogenates (5/15% interface) free from LPO-latex PL, which banded at the 15/35% interface (see Section III. N.). SDS-PAGE gels were then obtained, using equal portions of the 5/15% interface from cells homogenized 5 and 35 min after intracellular iodination. It should be noted that 5 min represents the minimum time required for the ingestion of S-B latex and precludes recovery of PM immediately after intracellular iodination. At 5 min, relatively little radioactivity could be retrieved in S-B latex phagosomes (Fig. 21). In contrast, after 30 min in culture, larger amounts of all the radioactive polypeptides were present on the cell surface and were captured in S-B latex phagosomes. Quantitative densitometric analysis of this autoradiogram showed that the relative intensities of the major bands (see Fig. 21) of the 5/15% fraction were the same at 5 and 35 min, although four times as much radioactivity was recovered at 35 min. This movement of label to the cell surface during 30 min of culture is even more striking, considering that the total radioactivity in the cells decreased in that interval (compare the 15/35% fraction at 5 and 35 min). The spectrum of radiolabeled polypeptides in S-B latex PL was identical to that of the whole-cell homogenate (Fig. 21). We conclude that a representative sample of PL membrane proteins, iodinated intracellularly by LPO-latex, can return to the PM intact. Because PL membrane polypeptides are migrating in concert, the data suggest that intact segments of membrane are flowing from PL to PM.

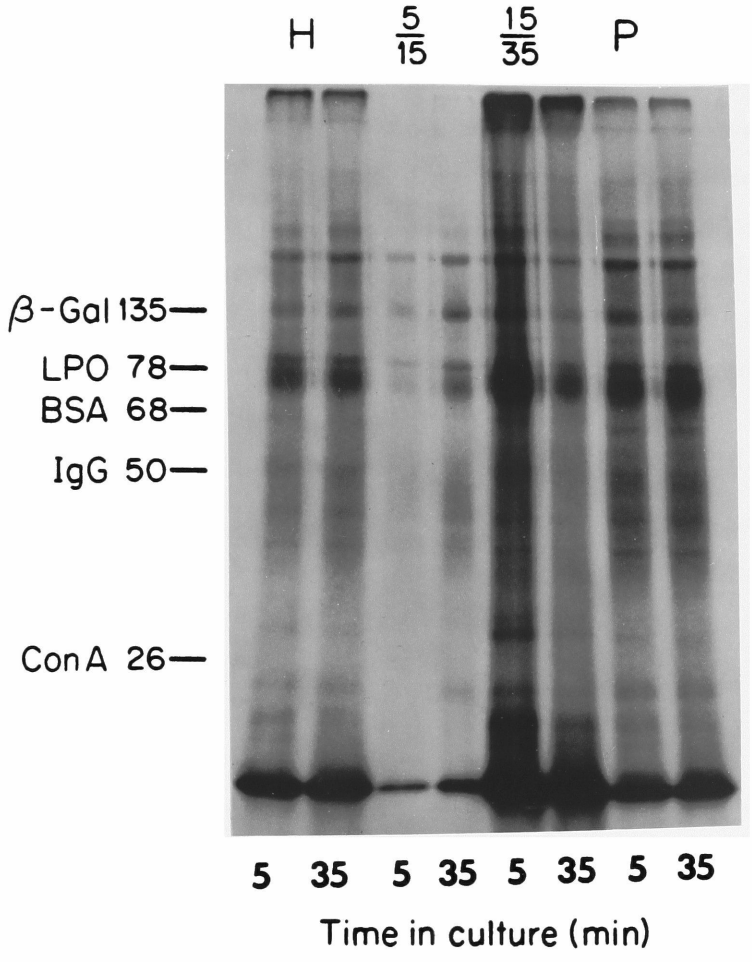

FIGURE 21. **Identification of radiolabeled polypeptides in PM.** Immediately after intralysosomal iodination (5) or after a 30-min return to culture (35), macrophages were given a 5-min phagocytic pulse of S-B latex, homogenized, and fractionated on a 5/15/35% discontinuous sucrose gradient. The fractions collected were analyzed by SDS-PAGE (4-11% slab gel). Samples of the total homogenate (H) and pellet (P) fractions contained equal TCA-precipitable radioactivity, whereas samples of the two interface fractions contained equal latex. The dried gel was exposed for autoradiography on Kodak XR-1 film with an enhancing screen. Molecular weights of gel standards (X 10^3) are at the left.

XIII. THE INFLUX OF PM INTO THE LATEX PL

The movement of membrane from PL to PM without shrinkage of the phagolysosome implies that PL membrane must be replaced from other sources. Pinocytic vesicles (PV) fusing with PL would provide a logical new source of membrane (55). Evidence for this pathway was first obtained from the accumulation of extracellular pinocytic markers into PL. Transmission EM indicated that Thorotrast was delivered to all pre-existing LPO-latex PL (Fig. 22). Quantitative enzymatic assays showed that HRP, a marker of fluid-phase pinocytosis, accumulated linearly in latex PL for at least 2 h (Fig. 23), paralleling its continuous uptake into cells (Fig. 23). About 1% of the total ingested HRP was recovered in the PL fraction, a value similar to those found for endogenous lysosomal hydrolases.

Evidence was next obtained that PV membrane (*i.e.*, PM) itself became part of the PL. For this purpose we allowed macrophages to ingest unmodified latex and then iodinated the cell surface with soluble LPO at 4°C. The cells were homogenized immediately or 30 min after return to culture, and the PL fractions (10/25% interface) were analysed by SDS-PAGE (Fig. 24). At time 0, little radiolabel was noted in the latex PL, but by 30 min over four times as much radiolabel (quantitated densitometrically) was recovered in the PL fraction (Fig. 24 , PL). This PL radiolabel represented interiorized PM, because the iodination pattern was identical to that seen in the whole-cell lysate (Fig. 24 , H). We conclude that PV can deliver representative samples of iodinated PM to latex PL and that PM flows rapidly into and out of the vacuolar system.

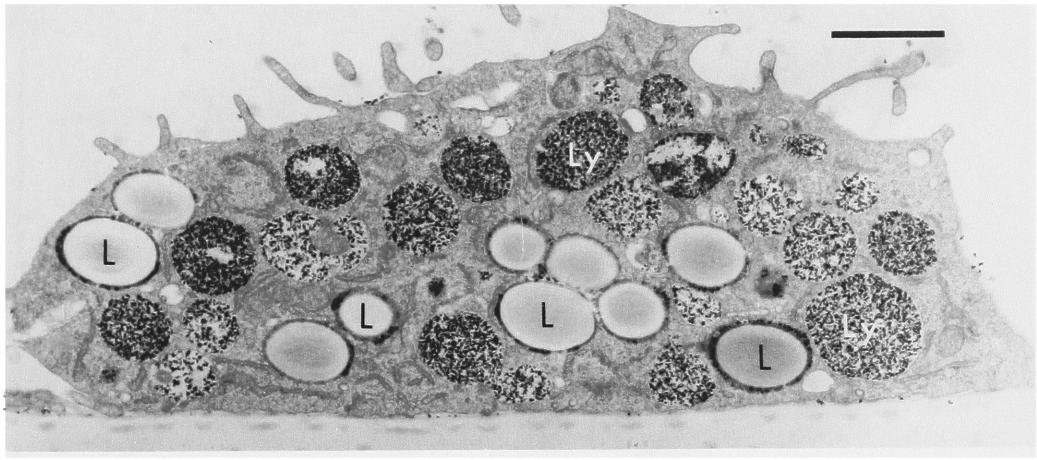

FIGURE 22. Accumulation of Thorotrast in cells previously fed LPO-latex. After ingestion of LPO-latex, cells were washed and returned to culture for 9 h in medium supplemented with Thorotrast. Note colloid particles within the latex phagolysosomes (L). Some enlarged lysosomes (Ly) are filled with colloid. The cells had been stained with DAB to mark LPO-latex with an electron-dense rim. Bar, 1 μm. X 16,000.

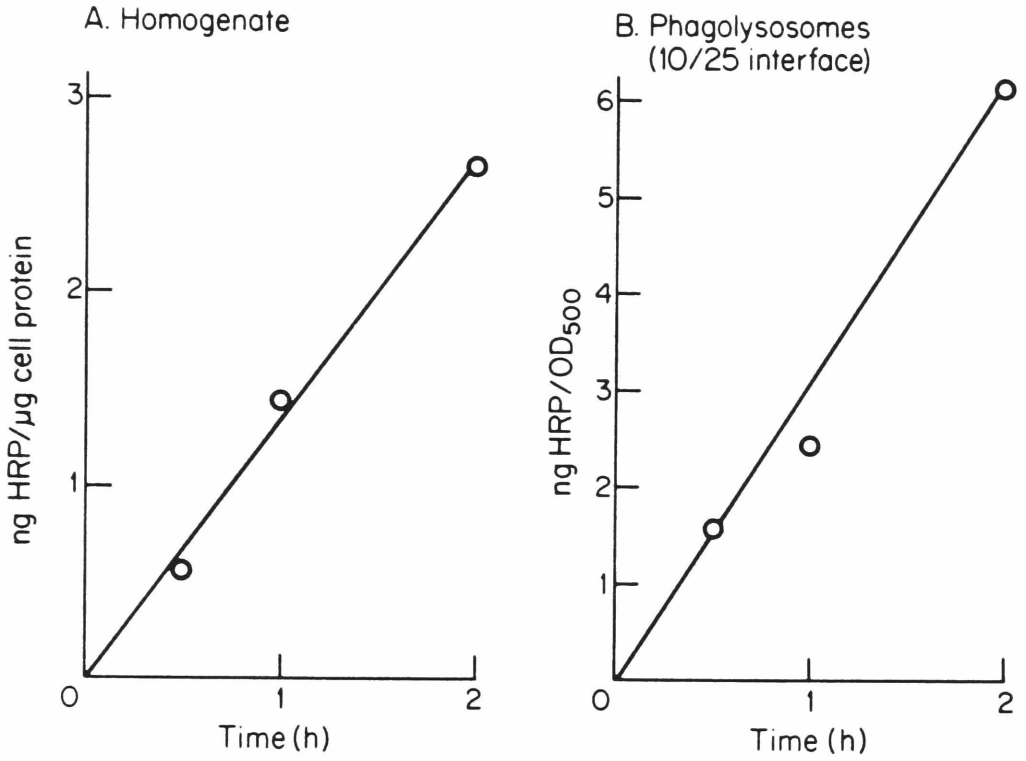

FIGURE 23. Uptake of HRP into latex PL. After ingestion of polystyrene latex particles, cells were exposed to HRP in culture medium for the times indicated, homogenized, and fractionated. (A) HRP activity in whole cell homogenate. (B) HRP activity recovered from purified PL, using OD_{500} to quantitate latex. Each point 0 represents the average of duplicate cultures fractionated in separate gradients. Placement of the lines was calculated by the method of least squares.

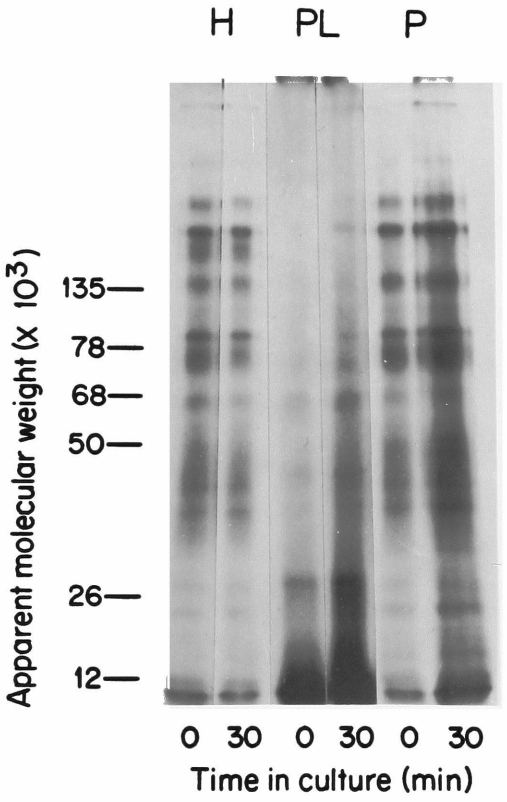

FIGURE 24. Movement of radioiodinated PM proteins into latex PL. After ingestion of PS-latex, macrophage surfaces were iodinated at 4°C, using soluble LPO. Cells were homogenized and fractionated immediately (0) or after return to culture for 30 min (30). Samples of the 10/25% interface fraction (PL) at both time-points, containing an equal amount of latex, were compared by SDS-PAGE. The homogenate (H) and pellet (P) lanes received approximately equal amounts of radiolabel (predominantly PM). The dried gel was exposed on Kodak XR-1 film with an enhancing screen.

XIV. DISCUSSION

The data presented in this paper demonstrate directly that PM interiorized into PL can return intact to the cell surface. The experimental approach was to radioiodinate membrane proteins of the phagolysosome within living cells and then follow the fate of radiolabel when cells were returned to culture. Part of the radiolabeled protein was digested within the lysosome, as expected from previous studies (24,59), but most of it returned to the plasmalemma.

A. Turnover of Radiolabeled Phagolysosome Proteins

When macrophages were returned to culture after intracellular iodination of PL membrane proteins, there was a rapid loss of 20-50% of the TCA-precipitable radioactivity (Fig. 16). No TCA precipitable radiolabel was lost from cells maintained on ice for 3 hr, but the rapid loss began immediately upon warming the cells to 37° (Fig. 16). Radioactivity lost from cells was recovered as $M^{125}IT$ in the culture medium (Table XIII), indicating that extensive digestion of labeled proteins took place.

Lysosomal digestion of PM proteins interiorized by phagocytosis is a well-established phenomenon. Werb and Cohn (59) found that the PM enzyme 5'-nucleotidase was inactivated by macrophages after a latex meal, while Hubbard and Cohn (24) demonstrated that radioiodinated PM proteins of L cells were digested in lysosomes after latex ingestion. In these studies the percent of total PM marker digested could be increased by driving more membrane into the PL compartment with a larger phagocytic load.

We, too, observe rapid digestion of 20-50% of the PL membrane which we iodinate. However, $\leq$ 5% of the PM is internalized during phagocytosis of our dose of LPO-latex. Thus, we cannot detect significant loss of cellular 5'-nucleotidase activity. Werb and Cohn (59) drove much more PM into the PL compartment with their higher latex doses. The point to be made here is not that PM marker enzymes are immune to degradation

in LPO-latex PLs, but merely that the low dose of latex used in these experiments minimally perturbed the cells, as assessed by the turnover of this intrinsic PM enzyme.

We show that the radiolabeled PL membrane proteins of macrophages maintain the same intensities relative to one another on SDS-PAGE autoradiograms at all times (Fig. 18). Hubbard and Cohn (24) obtained similar results with externally iodinated L cell PM proteins interiorized by phagocytosis. The fact that all PL polypeptides are digested in concert suggests that membrane degradation is a quantal process. For example, digestion may involve exposing whole segments of membrane to the lysosomal milieu, as in multivesicular bodies.

That the rapid and slow components of membrane turnover involve the same polypeptides suggests either that the cell recognizes portions of the membrane and marks these for rapid digestion or that a constant aliquot of membrane is randomly processed. If we consider a selective process to be more feasible, then what determines the nature of the signal? One possiblility that has been invoked for other autophagic processes is that altered or denatured membrane components are responsible. These may either occur through natural pathways or, in this specific case, be the result of iodination. In either case, lysosomal acid hydrolases would find these denatured proteins more readily digestible substrates (11). Perhaps membrane function, not structure alone, must be impaired to generate this signal. Trypsin rapidly cleaves two major macrophage surface polypeptides (bands 4 and 8 in Fig. 12a) into fragments which remain in the membrane. However, the turnover rate of these fragments is indistinguishable from that of the native molecules (data not shown). Another possibility is that the lifetime of a PL membrane protein is related to its length of exposure to the acidic and hydrolase-rich environment of the lysosome. Rapidly digested membrane may be that immobilized in the lysosome, whereas membrane that exhibits a longer lifespan does so by virtue of leaving the PL for the cell surface, rather than any intrinsic insusceptibility.

B. The Bidirectional Flow of Vacuolar Membrane

The unique finding of this study is that membrane proteins introduced into the PL can escape degradation and return to the cell surface. Previous studies on the fate of PL membrane (24,59) were not designed to detect this. We performed three types of experiments to demonstrate the extensive efflux of membrane components within 15-30 min after internal iodination of the cells. (a) EM-ARG indicated that the majority of the radiolabel originally on the PL membrane was now distributed on the PM. (b) The radiolabeled polypeptides became susceptible to extracellular proteolysis. (c) The PM could be isolated and shown to contain a spectrum of labeled proteins identical to those iodinated on the PL.

Our observations that return of all PL polypeptides to the PM is rapid and concerted suggests that the process occurs by a bulk movement of assembled membranes. We favor the idea that small membrane vesicles bud from the PL and subsequently fuse with the PM. These vesicles and their route of return have not been identified directly.

Several alternative explanations for the presence of radiolabel on the PM were considered. (a) Radiolabeled phagolysosome membrane was not being brought back to the surface because of exocytosis of LPO-latex. Sensitive light-scattering measurements of cell lysates and direct counts of particles in cells by phase microscopy showed that all of the latex originally ingested remained within the cell. (b) Monoiodotyrosine is not reused by the cell in protein synthesis (47). Were this occurring, the spectrum of radiolabeled proteins in the cell would shift with time to resemble the spectrum of the major cell proteins, but this did not occur (Fig. 18). (c) No new iodination occurred when cells were returned to culture (Table XIV). Therefore the radiolabeled polypeptides found on the PM moved there from the site of iodination in the PL.

Although membrane returns from PL to the cell surface, the contents of the lysosome matrix, including acid hydrolases and exogenous molecules, do not seem to follow such a pathway. Neither serum albumin (15) nor

HRP (54) once internalized by the cell is found in the extracellular milieu. It is possible, however, that molecules that are tightly associated with the luminal surface of the PL maintain this contact during membrane flow. The mechanism by which the constituents of the lysosomal matrix are excluded is speculative. The size of the channel or pore into the forming vesicle may provide a barrier to macromolecules, much as does the pore-mediated barrier that presumably restricts transport of proteins in endothelial endocytic vacuoles and fenestrae (53). Alternatively, if the lysosome contents are in a viscous state, rapid vesiculation might remove membrane before large macromolecules (but not water and small ions) could equilibrate within the buds. Finally, the hydrostatic pressure within the forming vesicle may be larger than that in the remaining PL, preventing contents from entering.

Is membrane returned to all areas of the cell surface, or are there specific sites for insertion? By 15 min, autoradiographic grains appear randomly distributed on the plasma membrane. Measurements of the diffusion constants for membrane proteins in the plane of the bilayer are in the range of 5×10^{-9} to 10^{-11} cm^2/s (14,28,44). Only at the fastest rate would lateral diffusion of membrane proteins from a small number of specialized insertion sites produce the rapid randomization of radiolabel we observe; but, even then, these sites would have to be distributed evenly around the plasmalemma. We think it likely that membrane returns to multiple sites on both the apical and basal surfaces of cultured macrophages, after which diffusion could produce further randomization of radiolabeled proteins. Returning membrane may pass through the Golgi complex (16) or GERL (19). Perhaps by conducting EM-ARG studies at very early time-points we shall be able to determine whether membrane passes preferentially (or obligatorily)through a certain region of the cell.

The existence of rapid membrane movement from PL to PM implies that there must be a new source of membrane to maintain the size of the PL compartment. Our previous stereological analysis suggested that the major source of this membrane could be newly derived PV (55). Additional results derived by the different techniques employed in the present study

also support this idea. (a) Thorotrast is delivered to all LPO-latex PL (Fig. 22), indicating that all are undergoing fusion with PM-derived PV. (b) HRP accumulates in PL at a constant rate for at least 2 hr (Fig. 23). (c) After iodination of the cell surface, cultured cells internalize a representative sample of PM into the PL (Fig. 24). The studies of Mellman *et al.* using LPO-catalyzed iodination within PV indicate directly that PV membrane is equivalent in composition to PM. Thus, there appears to be a continuous movement of PM into PL via PV that we think largely balances the flow of membrane from PL to PM. We would assume that movement of PM into and out of the latex PL is a constitutive process, even though we only followed some aspects of it, *e.g.*, the movement of radiolabel from PL to PM for 30 min. For example, PV deliver HRP to latex PL at a constant rate for at least 2 hr; yet the latex PL, like other secondary lysosomes (55), never swell with excess membrane, so the PM that joins PL via PV most likely returns to the surface as well.

C. Various Approaches to the Study of Membrane Recycling

Several groups of investigators have presented data consistent with recycling of the membrane involved in endocytic or exocytic processes. Although the techniques differ, the results of these studies support the conclusion that whole segments of membrane, or at least various components thereof, pass into the cell and return to the cell surface maintaining their physical and functional integrity. The approaches taken to study this phenomenon can be grouped into three major categories: (1) Ultrastructural studies, often employing a soluble tracer of pinocytosis, (2) studies following the fate of tracers bound non-covalently to the plasma membrane and (3) studies following the fate of radiolabel covalently incorporated into membrane. The advantages and disadvantages of each approach will be reviewed briefly with representative examples from the literature.

In several different cell types, evidence for membrane recycling during endocytosis and exocytosis has come from electron micrographic studies. In most of these, an exogenous tracer was used to mark endocytic vacuoles. The evidence from these studies is, at best, indirect.

Static electron micrographs can only suggest the nature of membrane interactions occurring in the living cell. No information concerning the composition of vesicle membrane or the direction of vesicle movement can be obtained. These restrictions notwithstanding, such studies provided the first clues that a coupled bidirectional flow of membrane might occur during these processes.

Many investigators have demonstrated an increased pinocytic uptake of HRP in gland cells and neurons following stimulation of secretion [reviewed in Holtzman *et al.* (22)]. The role of pinocytosis is presumably to retrieve membrane brought to the cell surface in secretory events. There is conflicting evidence as to whether the secretory granule membrane is maintained as a discrete and separate component or whether randomization and mixing of this membrane with PM occurs.

The endocytosed HRP in adrenal medulla cells is found predominantly in multivesicular bodies (1) although using another tracer in a different cell type Farquhar (17 , and see below) demonstrated some movement of the tracer to Golgi and secretory granules. Endocytosed HRP in a variety of neurons is found in multivesicular bodies and membrane limited axonal channels as well as in vesicles resembling synaptic vesicles which also line up along synaptic ribbons (51). Heuser and Reese (21) demonstrated that when neuromuscular junction preparations were preloaded with HRP, stimulation of the neuron resulted in disappearance of HRP from the vesicles. This suggested that membrane retrieved from the synaptic cleft could be reused in future secretory events. The amount of membrane which participates in recycling cannot be determined from these studies, since HRP is delivered to organelles other than synaptic vesicles and less than half of the synaptic vesicle profiles are ever seen to contain HRP (22). For similar reasons, the route(s) retrieved membrane follows can not be ascertained.

Protozoa such as *Paramecium* and *Tetrahymena* ingest their prey in food vacuoles which form from the plasma membrane in their oral region (cytopharynx). These vacuoles shrink as the food is digested following fusion with lysosomes, then re-expand as they move to the anal region (cytoproct) where they fuse with the plasma membrane and discharge

ingested contents. During active feeding, the surface area of food vacuoles formed each minute may be several times the surface area of the cytopharyngeal membrane from which they are derived (31). Feeding may continue for some time at this rate with no decrease in cell size.

Allen and coworkers (2,3 ,4 ,5) have proposed that recycling of membrane from the cytoproct to the cytopharynx allows feeding to continue in the absence of new membrane synthesis. They have demonstrated that following fusion at the cytoproct, food vacuole membrane rapidly vesiculates (3 , 4). Within 30 seconds of fusion, only flattened tubular vesicles were seen in the cytoproct area. If the cells were bathed in HRP at the moment of defecation, these vesicles took up some of the tracer (5). It was thus demonstrated that some of these vesicles lined up along microtubules which run from the anal region back to the oral region. It was proposed that these vesicles would be guided back to the cytopharynx to participate in the formation of new food vacuoles, but this has yet to be demonstrated directly.

Many of the problems inherent in the interpretation of static electron micrographs were overcome in the stereologic analysis of pinocytosis by Steinman *et al.* (55). The authors used HRP as a marker for fluid phase pinocytosis. Cytochemistry was used to determine where the tracer was, and could distinguish pinosomes from secondary lysosomes. Stereological analysis of electron micrographs allowed determination of the sizes of those membrane compartments. Biochemical assays of enzyme activity were used to determine the absolute amount of HRP taken up. The authors found that HRP accumulated in cells at a large and constant rate for at least 3 hr. During this steady state pinocytosis, macrophages and L cells interiorized 186% and 48% of their surface area, respectively, each hour as pinocytic vesicles. The surface area and volume of incoming pinosomes were 10 times the dimensions of the secondary lysosome compartment. Yet, the surface area and volume both of the cells and of the vacuolar apparatus did not change appreciably.

In the steady state, plasma membrane turnover in these cells is slow (24 , 26 , 59) so rapid degradation of pinosome membrane matched by *de novo* synthesis of plasma membrane seemed an unlikely explanation for this paradox. The authors postulated that PM internalized by pinocytosis fused with lysosomes then bud off and returned to the cell surface intact. HRP is not a membrane marker and the centrifugal flow of membrane could not be detected directly. However, the kinetics of membrane flow in this system made recycling seem the only reasonable explanation.

Many investigators have studied the fate of tracers bound non-covalently to plasma membrane. These tracers include ligands bound to specific receptors on the plasma membrane, lectins and cationized ferritin bound to multiple determinants on the cell surface and antibodies generated against isolated plasma membrane. These systems offer the advantage of studying the fate of the particular PM molecules to which the tracers bind. In all cases it is assumed that the tracer, which contains the radiolabel, fluorescent probe or electron-dense particle, remains associated with the site to which it originally bound as the membrane moves into the cell. In most cases, however, these tracers are found to fall off the membrane to a greater or lesser degree once the internalized membrane fuses with lysosomes. The subsequent fate of the internalized membrane must be inferred from the pathway taken by what little labeled tracer, if any, remains associated with it. The small amount and loose association of residual membrane-bound tracer raises the question of whether the pathway followed by this tracer represents a major physiological route in the cell and whether it is still bound to the same membrane on which it entered the cell.

Studies on the fate of receptor-bound ligands are quite numerous. Two examples will be described which are illustrative of this field in general. Brown and Goldstein and co-workers **(18)** have studied the low density lipoprotein (LDL) receptor in fibroblasts. Kaplan (27) has studied the macrophage receptor for α-macroglobulin-protease complexes. In both cases, ^{125}I-labeled ligand was shown to bind specifically to defined receptor sites on the cell surface. At 37°

the ligand was rapidly internalized and delivered to lysosomes where it was digested. In the presence of excess ligand, far more was internalized than could be bound at any one time to the receptors on the cell surface. Assuming that the receptor entered the cell with the ligand, the investigators in both cases proposed that internalized receptor returned to the cell surface where it was reutilized. The uptake of α-macroglobulin-trypsin complexes by macrophages was unaffected by cycloheximide for 3 hr suggesting that *de novo* synthesis of receptors was not responsible for the accumulation of ligand (27). Studies such as these cannot directly demonstrate return of receptors to the cell surface. However, assuming that receptors are internalized along with the ligand, the data they provide on the kinetics of ligand uptake argue strongly in favor of receptor reutilization. Kaplan calculated that the receptors he studied were utilized 5-10 times per hour (27). Goldstein *et al.* (18) calculated the half time for internalization of fibroblast LDL receptors was around 3 minutes. These rates of membrane movement are even higher than those calculated by Steinman *et al.* (55).

While the ligand-receptor studies suggest a rate for membrane recycling, studies with other membrane markers suggest a route. Gonatas *et al.* (19) incubated sympathetic neurons from cultured ganglion cells with ricin-HRP. The lectin bound to the plasma membrane at 4°, presumably to galactose containing glycoproteins and glycolipids. When cells were warmed to 36° for 3 hr, HRP reaction product was found in the lumens of elongated Golgi cisterna as well as in clusters of vesicles near elongated Golgi stacks, while reaction product on the cell surface decreased to trace levels. The authors concluded that the internal labeling represented translocated PM in dynamic relationship with the Golgi apparatus, perhaps reflecting recycling of the ricin "receptors" to their site of synthesis. Some of the electron micrographs presented show labeled profiles suggestive of GERL and of lysosomes. The authors do not comment on the former and state that lysosomes are not labeled.

Farquhar and coworkers have tagged the surfaces of rat anterior pituitary cells (16) and of plasma cells and myeloma cells (41) with cationized ferritin. They demonstrated that the electron-dense marker was pinocytosed and subsequently found in lysosomes, Golgi and condensing granules. In myeloma and plasma cells it could be demonstrated in the same Golgi cisternae which stained for the IgG secretory product (41). Assuming that the movement of cationized ferritin was tracing the natural movement of PM into secretory granules, endocytosed PM could then be recycled for use in subsequent secretory events. It could not be determined whether the cationized ferritin passed through the lysosomes *en route* to Golgi, or whether PM followed distinct pathways to lysosomes and Golgi However, much more cationized ferritin was found in lysosomes than in Golgi or secretory granules. Moreover, the intracellular label seen by Farquhar *et al.* and Gonatas *et al.* was in the lumen of these vesicles, not bound to the membrane.

Schneider *et al.* (52) reported that rat fibroblasts that had internalized fluoresceinated goat anti-rabbit IgG released some of this label when rabbit anti rat PM IgG was added to cultures. They postulated that the rabbit IgG bound to the PM, was interiorized by pinocytosis, complexed with the goat anti-rabbit IgG within the lysosomes, and shuttled it back to the cell surface where it was released into the culture medium. This provided further evidence that plasma membrane could enter lysosomes and return intact to the cell surface. However, the time required for this process to occur and the amount of membrane involved could not be precisely established by these studies.

The approach employed in my studies was to introduce a nonreutilizable covalent label selectively into membrane proteins of the phagolysosome. The advantages of this approach are that the radiolabel originates in the lysosome and remains on the original labeled membrane proteins wherever they may move. By this technique we demonstrate that the same spectrum of some 2 dozen membrane polypeptides participate in both latex phagocytosis and fluid phase pinocytosis, intermix in the 2° lysosome and return to the cell surface, all within minutes. We have not yet "caught"

the recycling process before it reached equilibrium. One disadvantage of this method is that the resolution of EM autoradiography may not be fine enough to allow us to detect the route of recycling.

The only other published work using a selectively incorporated covalent label to study membrane recycling comes from Thilo and Vogel (57). They used galactosyl transferase to incorporate [^{3}H] galactose into terminal *N*-acetyl glucosamine moieties on the cell surface of *Dictyostelium discoideum* at 0°C. Upon warming, some of this label became resistant to hydrolysis by β-galactosidase presumably because it had entered the cells in pinocytic vesicles. If labeled cells were chilled and treated with β-galactosidase to remove residual surface label, the authors could demonstrate a return of β-galactosidase sensitivity upon subsequent warming. The actual differences they obtained were small.

It was never demonstrated that the ^{3}H galactose was incorporated totally into plasma membrane (as opposed to the extracellular slime) or that the radiolabel itself was not transferred from one molecule to another. Nevertheless, the data are consistent with a return of endocytosed labeled plasma membrane to the cell surface.

D. Possible Roles of Membrane Recycling

The vacuolar apparatus has traditionally been viewed as a system of structurally unique and well-defined compartments. The route to the lysosome was considered a unidirectional path taken by exogenous molecules and membrane destined for digestion. The concept of the vacuolar system that emerges from this study is one of a dynamic system of membranes that interact continuously and extensively with the plasmalemma. The lysosomal milieu, with its low pH, acid hydrolases and other contents is then available to interact with plasma membrane. It is possible that such interactions in the lysosome may be important to plasma membrane physiology just as are events occurring on the cell surface. Several possible roles which membrane recycling might play in the physiology of the cell are considered below. These all derive from

the dynamic nature of vacuolar system membrane.

Membrane recycling allows for the efficient delivery of receptor-bound ligands to the lysosomes without obligatory loss or degradation of the receptor itself. Anderson *et al.* (7) have proposed that recycling of fibroblast low-density lipoprotein (LDL) receptors accounts for the extensive and continued uptake of LDL in the absence of receptor synthesis. In contrast, interaction of other ligands, such as epidermal growth factor, with their receptors leads to selective removal of the receptors from the cell surface (10), or so-called down regulation. It is possible that a consequence of ligand binding is to block recycling of membrane, trapping the complex in the lysosome. We favor the idea that some feature of the receptor or complex rather than the ligand may determine whether the complex is trapped in the lysosome (down regulation) or whether the ligand is discharged in the lysosome, followed by recycling of the free receptor. Such selective membrane removal could be involved in membrane turnover. Our data show that interiorized PM may fuse with lysosomes but escape degradation. Perhaps membrane destined for digestion is specifically recognized and rapidly degraded in lysosomes. Degradation of normal membrane may be a random event so that very little is digested on any one trip through the lysosome. The extensive flow of membrane into and out of the vacuolar system would provide the cell with a mechanism to rapidly and continuously survey the integrity of the PM. This flow combined with lateral diffusion in the plane of the membrane might also allow any novel components added by lysosome fusion and *de novo* synthesis to mix in with preexisting PM.

In addition to selective membrane alteration, vesicular membrane flow might be used by the cell to deliver bulk membrane from one area to another. As discussed in section XIV C, in various secretory cells and neurons it has been proposed that following fusion of secretory granules with the plasmalemma, the secretory granule membrane is endocytosed and reused in future secretory events. Although recycling appeared to be random under our culture conditions, it is possible that this flow could be directed to bring large areas of membranes to specific regions where they are needed when the cell moves, changes shape, or extends pseudopods during phagocytosis.

Finally, there is the suggestion that the vacuolar system could serve as an intermediate in the renewal and synthesis of PM. This is based on the information that the polypeptide compositions of the PM and PL are virtually indistinguishable (Fig.12). The input of new membrane into the vacuolar apparatus presumably comes from the membrane of the primary lysosome, which in turn originates in the Golgi or GERL area of the cell. If typical PM surrounds the primary lysosome, this would imply that new segments of PM are first inserted into the secondary lysosome and then reach the cell surface during the recycling process.

The role that membrane recycling plays in these functions still remains to be determined directly. However, these are all examples of how the lysosome, previously demonstrated to be a digestive organelle may, in addition, by the vehicle of membrane recycling, influence several aspects of cell physiology.

XV. REFERENCES

1. Abrahams, S. and E. Holtzman. 1973. Secretion and endocytosis in insulin-stimulated rat adrenal medulla cells. J. Cell Biol. 56:540-558.

2. Allen, R. D. 1974. Food vacuole membrane growth with microtubule-associated membrane transport in *Paramecium*. J. Cell Biol. 63: 904-922.

3. Allen, R. D. and R. W. Wolf. 1974. The cytoproct of *Paramecium caudatum:* Structure and function, microtubules, and fate of food vacuole membranes. J. Cell Sci. 14:611-631.

4. Allen, R. D. and R. W. Wolf. 1979. Membrane recycling at the cytoproct of *Tetrahymena*. J. Cell Sci. 35:217-227.

5. Allen, R. D. and A. K. Fok. 1980. Membrane recycling and endocytosis in *Paramecium* confirmed by horseradish peroxidase pulse-chase studies. J. Cell Sci. 45:131-145.

6. Ames, G. F-L., and K. Nikaido. 1976. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry 15:616-623.

7. Anderson R. G. W., J. L. Goldstein, and M. S. Brown. 1977. A mutation that impairs the ability of lipoprotein receptors to localize in coated pits on the cell surface of human fibroblasts. Nature (Lond.) 270:695-699.

8. Avruch, J. and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. Biochim. Biophys. Acta 233:334-347.

9. Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

10. Carpenter, G. and S. Cohen. 1976. ^{125}I-labeled human epidermal growth factor. Binding, internalization and degradation in human fibroblasts. J. Cell Biol. 71:159-171.

11. Coffey, J. W. and C. de Duve. 1968. Digestive activity of lysosomes. I. The digestion of proteins by extracts of rat liver lysosomes. J. Biol. Chem. 243:3255-3263.

12. Constantino-Ceccarini, E., P. M. Novikoff, P. H. Atkinson, and A. B. Novikoff. 1978. Further characterization of HeLa S_3 plasma membrane ghosts. J. Cell Biol. 77:448-463.

13. Dobyns, B. M. and S. R. Barry. 1953. The isolation of iodinated amino acids from throid tissue by means of starch column chromatography. J. Biol. Chem. 204:517-531.

14. Edidin, M. and D. Fambrough. 1973. Fluidity of the surface of cultured muscle fibers. Rapid lateral diffusion of marked surface antigens. J. Cell Biol. 57:27-53.

15. Ehrenreich, B. A. and Z. A. Cohn. 1967. The uptake and digestion of iodinated human serum albumin by macrophages *in vitro*. J. Exp. Med. 126:941-958.

16. Farquhar, M. G. 1978. Recovery of surface membrane in anterior pituitary cells. Variations in traffic detected with anionic and cationic ferritin. J. Cell Biol. 77:R35-R42.

17. Farquhar, M. G., J. J. Reid and L. W. Daniell. 1978. Intracellular transport and packaging of prolactin: A quantitative electron microscope autoradiographic study of mammotrophs dissociated from rat pituitaries. Endocrinology 102:296-311.

18. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. Cell 7:85-95.

19. Gonatas, N. K., A. Steiber, S. U. Kim, D. I. Graham, and S. Avrameas. 1975. Internalization of neuronal plasma membrane ricin receptors into the Golgi apparatus. Exp. Cell Res. 94:426-431.

20. Graham, R. C., Jr. and M. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291-302.

21. Heuser, J. E. and T. S. Reese. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. 57:315-344.

22. Holtzman, E., S. Schacher, J. Evans, and S. Teichberg. 1977. Origin and fate of the membranes of secretion granules and synaptic vesicles: membrane circulation in gland cells and retinal photoreceptors in Cell Surface Reviews Vol. 4. The Synthesis, Assembly and Turnover of Cell Surface Components. G. Poste and G. L. Nicholson (eds.). Elsevier/North-Holland Biomedical Press. The Netherlands. 165-246.

23. Hubbard, A. L. and Z. A. Cohn. 1975. Externally disposed plasma membrane proteins. I. Enzymatic iodination of mouse L cells. J. Cell Biol. 64:438-460.

24. Hubbard, A. L. and Z. A. Cohn. 1975. Externally disposed plasma membrane proteins. II. Metabolic fate of iodinated polypeptides of mouse L cells. J. Cell Biol. 64:461-479.

25. Izzo, J. L., A. Roncone, M. J. Izzo, R. Foley, and J. W. Bartlett. 1972. Degradation of ^{131}I-insulins by rat liver. J. Biol. Chem. 247:1219-1226.

26. Kaplan, G., J. C. Unkeless, and Z. A. Cohn. 1979. Insertion and turnover of macrophage plasma membrane proteins. Proc. Natl. Acad. Sci. USA 76:3824-3828.

27. Kaplan, J. 1980. Evidence for reutilization of surface receptors for α-macroglobulin·protease complexes in rabbit alveolar macrophages. Cell 19:197-205.

28. Koppel D. E., D. Axelrod, J. Schlessinger, E. L. Eison and W. W. Webb. 1976. Dynamics of fluorescent marker concentration as a probe of mobility. Biophys. J. 16:1315-1329.

29. Laskey, R. A. and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335-341.

30. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

31. McKanna, J.A. 1973. Cyclic membrane flow in the ingestive-digestive system of peritrick protozoans. I. Vesicular fusions at the cytopharynx. J. Cell Sci. 13:663-675.

32. Mellman, I. S., R. M. Steinman, J. C. Unkeless, and Z. A. Cohn. 1980. Selective iodination and polypeptide composition of pinocytic vesicles. J. Cell Biol. 86:712-722.

33. Murray, H. W., C. W. Juangbhanich, C. F. Nathan, and Z. A. Cohn. 1979. Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. J. Exp. Med. 150:950-964.

34. Nathan, C. F., and R. K. Root. 1977. Hydrogen peroxide release from mouse peritoneal macrophages. Dependence on sequential activation and triggering. J. Exp. Med. 146:1648-1662.

35. Nathan, C. F., L. H. Brukner, S. C. Silverstein, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. I. Pharmacologic triggering of effector cells and the release of hydrogen peroxide. J. Exp. Med. 149:84-99.

36. Nathan, C. F., S. C. Silverstein, L. H. Brukner, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. J. Exp. Med. 149:100-113.

37. Nathan, C. F., N. Nogueira, C. Juangbhanich, J. Ellis, and Z. A. Cohn. 1979. Activation of macrophages *in vivo* and *in vitro*. Correlation between hydrogen peroxide release and killing of *Trypanosoma cruzi*. J. Exp. Med. 149:1056-1068.

38. Neville, D. M., Jr. 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. 246:6328-6334.

39. Novikoff, P.M., A. B. Novikoff, N. Quintana, and J. J. Hauw. 1971. Golgi apparatus,GERL, and lysosomes of neurons in dorsal root ganglia, studied by thick section and thin section cytochemistry. J. Cell Biol. 50:859-886.

40. Ohkuma, S. and B. Poole. 1978. Fluorescence probe measurement of the lysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA 75:3327-3331.

41. Ottosen, P. D., P. J. Courtoy, and M. G. Farquhar. 1980. Pathways followed by membrane recovered from the surface of plasma cells and myeloma cells. J. Exp. Med. 152:1-19.

42. Peters, T. J., M. Müller and C. de Duve. 1972. Lysosomes of the arterial wall. I. Isolation and subcellular fractionation of cells from normal rabbit aorta. J. Exp. Med. 136:1117-1139.

43. Piperno, G., B. Huang and D. J. L. Luck. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA 74:1600-1604.

44. Poo, M., and R. A. Cone. 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. Nature (Lond.). 247:438-441.

45. Reed, P. W. 1969. Glutathione and the hexose monophosphate shunt in phagocytizing and hydrogen peroxide-treated rat leukocytes. J. Biol. Chem. 244:2459-2464.

46. Root, R. K. 1974. Correction of the fuction of chronic granulomatous disease (CGD) granulocytes (PMN) with extracellular H_2O_2. Clin. Res. 22:452A (Abstr.)

47. Ryser, H. J.-P. 1963. Comparison of the incorporation of tyrosine and its iodinated analogs into the proteinsof Ehrlich ascites tumor cells and rat liver slices. Biochim. Biophys. Acta. 78:759-762.

48. Salpeter, M. M. and L. Bachman. 1964. Autoradiography with the electron microscope. A procedure for improving resolution, sensitivity, and contrast. J. Cell Biol. 22:469-477.

49. Salpeter, M. M. and F. A. McHenry. 1973. Electron microscope autoradiography. Analyses of autoradiograms. In Advanced Techniques in Biological Electron Microscopy. Vol. I. J. K. Koehler, editor. Springer-Verlag, New York.113-152.

50. Salpeter, M. M., H. C. Fertuck, and E.E.Salpeter. 1977. Resolution in electron microscope autoradiography. III. Iodine-125, the effect of heavy metal staining, and a reassessment of critical parameters. J. Cell Biol. 72:161-173.

51. Schacher, S., E. Holtzman and D. C. Hood. 1976. Synaptic activity of frog retinal photoreceptors: A peroxidase uptake study. J. Cell Biol. 70:178-192.

52. Schneider, Y.-J., P. Tulkens, C. de Duve, and A. Trouet. 1979. Fate of plasma membrane during endocytosis. II. Evidence for recycling (shuttle) of plasma membrane constituents. J. Cell Biol. 82:466-474.

53. Simionescu, N., M. Simionescu, and G. E. Palade. 1975. Permeability of muscle capillaries to small heme-peptides. Evidence for the existence of patent transendothelial channels. J. Cell Biol. 64:586-607.

54. Steinman, R. M. and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages *in vitro*. J. Cell Biol. 55:186-204.

55. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A stereologic analysis. J. Cell Biol. 68:665-687.

56. Steinman, R. M., G. Kaplan, M. D. Witmer, and Z. A. Cohn. 1979. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers and maintenance *in vitro*. J. Exp. Med. 149:1-16.

57. Thilo, L. and G. Vogel. 1980. Kinetics of membrane internalization and recycling during pinocytosis in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA 77:1015-1019.

58. Tsan, M.-F., and R. D. Berlin. 1971. Effect of phagocytosis on membrane transport of nonelectrolytes. J. Exp. Med. 134:1016-1035.

59. Werb, Z., and Z. A. Cohn. 1972. Plasma membrane synthesis in the macrophage following phagocytosis of polystyrene latex particles. J. Biol. Chem. 247:2439-2446.

60. Wetzel, M. G., and E. D. Korn. 1969. Phagocytosis of latex beads by *Acanthamoeba castellanii* (Neff). III. Isolation of the phagocytic vesicles and their membranes. J. Cell Biol. 43:90-104.

61. Willinger, M. and F. R. Frankel. 1979. Fate of surface proteins of rabbit polymorphonuclear leukocytes during phagocytosis. I. Identification of surface proteins. J. Cell Biol. 82:32-44.

62. Willinger, M., N. Gonatas, and F. R. Frankel. 1979. Fate of surface proteins of rabbit polymorphonuclear leukocytes during phagocytosis. II. Internalization of proteins. J. Cell Biol. 82:45-56.

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