FATE OF SURFACE PROTEINS OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES DURING PHAGOCYTOSIS

II. Internalization of Proteins

MARIAN WILLINGER, NICHOLAS GONATAS, and FRED R. FRANKEL

From the Departments of Microbiology and Neuropathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104. Dr. Willinger's present address is the Department of Neuropathology, Harvard Medical School, Boston, Massachusetts 02115.

ABSTRACT

The distribution of surface proteins during phagocytosis by rabbit peritoneal polymorphonuclear leukocytes was studied to determine whether the proteins of the phagocytic vesicles of these differentiated cells were representative of the entire set of plasma membrane proteins. Phagocytosis of bovine serum albumin-diisodecylphthalate emulsion by lactoperoxidase-iodinated rabbit neutrophils was linear over 15–20 min at a rate of 96 μg oil/min/mg cell protein. This rate was similar to that of unlabeled cells. Incorporation of cell-associated free iodine by endogenous myeloperoxidase during phagocytosis was inhibited by 1 mM cyanide, which had no effect on the rate of particle uptake.

The surface of intact neutrophils contained at least 13 iodinated proteins distinguishable by polyacrylamide gel electrophoresis followed by autoradiography. Isolated phagosomes were deficient in six of these proteins. The plasma membrane fraction of these cells was missing five of these same proteins which, however, were enriched in a dense surface fraction (Willinger, M., and F. R. Frankel. *J. Cell Biol.* **82:** 32–44). When experimental conditions were reversed, and the PMNs were labeled after phagocytosis, these five proteins remained on the cell surface, while at least three of the major proteins found on resting cells were depleted. Incubating the cells with colchicine, which has been shown to affect the distribution of some plasma membrane constituents during phagocytosis, had no effect on the distribution of surface proteins in our system.

These results indicate that a nonrandom interiorization of lactoperoxidase-labeled surface proteins of polymorphonuclear leukocytes occurs during phagocytosis.

KEY WORDS phagocytosis · polymorphonuclear leukocyte · surface proteins · lactoperoxidase-catalyzed iodination · plasma membrane · phagosome · colchicine

Phagocytosis is an important host defense mechanism characterized by internalization of large areas of the plasma membrane of phagocytic cells in response to interaction with bacteria and other

ingestible particles. Internalization by macrophages and neutrophils occurs at discrete sites of membrane-particle contacts. The plasma membrane is seen closely enveloping the particle via villous projections on the cell body or via lamellopodia, depending apparently on whether the particle is opsonized by IgG or by complement (18, 19). The morphological observation that phagocytosis involves localized membrane activity is confirmed by the finding that ingestion of latex does not induce the interiorization of other particles simultaneously bound to the cell (13). Also, in the case of uptake of opsonized particles by macrophages, continuous contact via plasma membrane receptors for the opsonin may be required (12, 24).

An important question concerning cell-particle interactions during phagocytosis is whether sites of membrane internalization are differentiated relative to the remainder of the cell surface. This would be expected, for example, if (*a*) specific receptors are required for recognition, (*b*) a critical density of receptors is required to induce engulfment, (*c*) the motile machinery, presumably microfilaments, utilized for engulfment becomes associated with unique components of the cell surface, or (*d*) functions or structures necessary for the integrity of the cell must be maintained at the external surface of the cell. Membrane constituents and functions that are internalized during phagocytosis by macrophages or monocytes begin to reappear at the cell surface 6 h after ingestion (21, 24, 26). Neutrophils, on the other hand, do not have the ability to resynthesize bulk membrane (11).

Tsan and Berlin (33) presented the first evidence indicating that phagocytic vesicles may not contain a random sample of the plasma membrane of the cell. The number of transport sites for lysine, adenosine, and adenine, measured by the V_{max} for transport of these molecules, remained unchanged after maximal internalization of latex by neutrophils and alveolar macrophages. Ukena and Berlin (34) further suggested that colchicine-sensitive structures, presumably microtubules, were involved in controlling the fate of these sites, because colchicine treatment led to a decrease in the number of transport sites upon phagocytosis. More recently, Oliver et al. (22) observed a decrease in the specific activity of plasma membrane-binding sites for concanavalin A with phagocytosis, implying preferential interiorization of these sites. Colchicine inhibited the decrease. They found that the specific activity of 5′-nucleotidase remained unchanged under all conditions, confirming earlier observations that the activity was distributed randomly during phagocytosis by mouse macrophages (36). In contrast to the observations of Tsan and Berlin, Dunham et al. (10), measuring potassium and lysine transport in human neutrophils, found a decrease in transport activity with phagocytosis that was unaffected by colchicine. Potassium transport was more dramatically reduced than lysine transport, however, favoring a mosaic membrane model. The above observations indicate that some components may be free to move randomly in the plane of the membrane while other components may either be restricted from, or show directed movement into, phagocytic vesicles.

The purpose of this study was to investigate this question by a more direct approach. In the companion paper, we described the lactoperoxidase-catalyzed iodination of the rabbit peritoneal neutrophil surface and isolation of a cell fraction enriched in plasma membrane. This paper will detail the fate of these surface proteins during phagocytosis. A brief report of this work has appeared elsewhere (37).

MATERIALS AND METHODS

Iodination and Analysis of Rabbit Polymorphonuclear Leukocyte (PMN) Proteins

The collection of rabbit peritoneal neutrophils, their labeling by lactoperoxidase-catalyzed iodination, and analysis of labeled peptides by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were described in the companion paper (38). Optical obsorbance scans of some of the autoradiographs were made using an SDC 300 spectrodensitometer (Schoeffel Instrument Div., Kratos, Inc., Westwood, N.J.). Band areas were measured planimetrically.

Phagocytosis

Phagocytosis was performed, with modifications, by the methods of Stossel et al. (29, 30).

PREPARATION OF PARTICLES: An emulsion of particles ranging in size from 0.5 to 5 μm was prepared by sonicating 3 vol bovine serum albumin (BSA) (crystalline, fraction V) at 20 mg/ml in Hank's balanced salt solution (HBSS) with 1 vol diisodecylphthalate (Matheson, Coleman, and Bell, from Arthur H. Thomas Co., Philadelphia, Pa.) containing 0.05 M Oil Red 0 (Fisher Scientific Co., Pittsburgh, Pa.), a lipid soluble dye which can be used to quantitate particle uptake. 1 g of Oil Red

0 was suspended in 20 ml diisodecylphthalate with a mortar and pestle. Undissolved dye was removed by centrifugation at 12,000 *g* for 30 min. The average preparation contained 0.05 M Oil Red 0 as determined by absorbance at 524 nm. The molar extinction of Oil Red 0 in dioxane at 524 nm is 2.29×10^4. For C'3 opsonization of particles, autologous serum was used fresh or was stored for short periods of time at −70°C. 1 ml of particles was incubated with 1 ml of serum at 37°C in a 5-ml polyallomer centrifuge tube. The mixture was diluted with saline and centrifuged at 100,000 *g* for 30 min. The infranatant fluid was carefully removed and the particles were washed once more in saline and suspended in 1 ml HBSS. Once the C'3 is bound to the particles, it is stable for several weeks at −20°C.

ASSAY OF PHAGOCYTOSIS: All glassware in contact with cells was siliconized to prevent cell adhesion. The cells were incubated at a concentration of 2–2.5×10^7 cells/ml in HBSS buffered with 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, Mo.) at 36.5°C and maintained in suspension by shaking on a Burrell wrist action shaker (Arthur H. Thomas Co.) set at 1.0 (~120 excursions/min). At zero time, 0.2 vol of warmed particle suspension was added. To determine the initial rate of particle uptake, 0.1 ml of the reaction mixture was removed at zero time and at various intervals thereafter into 3 ml of ice-cold saline containing 1 mM *N*-ethylmaleimide (NEM) (Sigma Chemical Co.). The cells were separated from uningested particles by centrifugation at 1,000 rpm for 20 min in an International PRJ centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) at 4°C. The cell pellets were suspended by gentle mixing and washed twice with cold PBS. To solubilize the Oil Red 0, the cell pellets were extracted in 1 ml dioxane (Spectroquality; Matheson, Coleman, and Bell from Arthur H. Thomas Co.) for 1 h at room temperature. The particulate material was removed by centrifugation at 2,000 rpm for 20 min, and the absorbance of the supernate at 524 nm was measured. In general, rates were expressed as Δ OD_{524}/min.

PHAGOCYTOSIS BY PRE-LABELED CELLS: Cells washed three times after surface iodination were suspended at 2.5×10^7 cells/ml in HBSS/HEPES containing 1 mM KCN to prevent incorporation of residual iodine into trichloroacetic acid-precipitable material. The cells were incubated and processed as described above.

Subcellular Fractionation of Phagocytizing Cells

The method of Stossel et al. (31) for the isolation of phagosomes was modified to permit concomitant isolation of phagocytic vesicles and plasma membrane. After 5–15 min of phagocytosis of emulsion, 6×10^7 cells were diluted into 6–10 vol of ice-cold 1 mM NEM in saline and washed twice with PBS. The cells were homogenized, centrifuged at low speed to remove intact cells and nuclei, and then sedimented through a step sucrose gradient as described for resting cells (38). Because the emulsion has a low density, emulsion-bound membrane (phagocytic vesicles) was removed as a floating red layer. The material at the 35%–45% sucrose interface (plasma membrane) was collected as described previously for resting cells (38).

RESULTS

Phagocytosis by Rabbit Peritoneal PMNs of BSA-Diisodecylphthalate Emulsion

KINETICS OF PARTICLE UPTAKE: To analyze the fate of membrane proteins during phagocytosis, it was desirable to use a relatively pure cell population that was homogeneous in phagocytic activity and obtainable in large quantities. Rabbit peritoneal PMNs were chosen because they are highly phagocytic and of uniform maturity. 4-h glycogen-induced peritoneal exudates contained 4–12×10^8 cells, >98% of which were PMNs and 100% of which were viable. Electron microscopy of these cells showed the typical morphology of PMNs with lobed condensed nuclei, heterogeneous granules, localized stores of glycogen, as well as a paucity of mitochondria and ribosomes.

Rabbit PMNs were fed on emulsion of BSA and an oil, diisodecylphthalate, which was phagocytized linearly for 15–20 min at a rate of 70–100 μg oil/min/mg cell protein. Guinea pig peritoneal granulocytes phagocytize at a similar rate but uptake is linear for only 4 min (30). Temperatures below 18°C totally inhibited particle uptake, whether measured spectrophotometrically or by phase-contrast microscopy before washing. Examination of cells exposed to particles for 5, 10, or 15 min by phase microscopy, or by light microscopy of thick sections, revealed that >95% of the cells had particles associated with them. Transmission electron microscopy showed intracellular particles bounded by plasma membrane (result not shown).

The initial rate of phagocytosis was independent of particle concentration above an emulsion volume of 10%. Therefore, a 20% emulsion volume was chosen for all subsequent work. At this substrate concentration, particle uptake was directly proportional to cell concentration between 0.5 and 2.5×10^7 cells/ml. The initial rate of phagocytosis displayed the following characteristics: (*a*) irreversible inhibition by concentrations of NEM between 5×10^{-5} and 10^{-3} M, (*b*) inhibition by 10^{-2}

and 5×10^{-2} M NaF, and (*c*) lack of inhibition by 10^{-3} M KCN or 10^{-2} M NaN_3 (3, 25).

PHAGOCYTOSIS OF OPSONIZED AND UNOPSONIZED PARTICLES BY TRYPSINIZED CELLS: Trypsinization of rabbit peritoneal PMNs resulted in the release of cell surface polypeptides (38). To determine whether trypsin-sensitive proteins were necessary for phagocytosis, trypsinized or control cells were incubated with C'3-opsonized or unopsonized particles and the initial rates of particle uptake were measured. The rate of phagocytosis of BSA-diisodecylphthalate emulsion was increased 60% when the particles were opsonized with fresh serum (Table I). Trypsinized PMNs phagocytized either particle at about the same rate as untreated cells. Therefore, a trypsin-sensitive C'3 receptor was not observed in this system.

PHAGOCYTOSIS BY IODINATED PMNS: When lactoperoxidase-labeled cells which had phagocytized BSA-oil emulsion were analyzed by SDS-PAGE, ^{125}I was detected in the same 13 proteins previously seen on resting PMNs (38). In addition, label was detected in three proteins of 67,000, 40,000, and 27,000 daltons. It was possible that phagocytized BSA (67,000 daltons) had been iodinated by endogenous myeloperoxidase and accumulated soluble ^{125}I and was then degraded by lysozomal protease to 40,000- and 27,000-dalton cleavage products. To test this hypothesis, PMNs were incubated with ^{125}I-labeled BSA either in solution or as an emulsion. Fig. 1 shows that the labeled BSA was cleaved to the two smaller peptides (CP1 and CP2) and that this cleavage of BSA was dependent on phagocytosis of the emulsion.

TABLE I
Phagocytosis by Trypsinized PMNs

	Rate	% control
	OD_{524}/min	
1. Untreated cells + emulsion	0.010	100.0
2. Trypsinized* cells + emulsion	0.011	110.0
3. Untreated cells + opsonized‡ emulsion	0.016	160.0
4. Trypsinized cells + opsonized emulsion	0.017	170.0

* 2×10^7 PMNs/ml were incubated with 50 μg/ml trypsin in PBS for 20 min at 4°C. The reaction was stopped with 50 μg soybean trypsin inhibitor. The cells were centrifuged, washed once with PBS, and resuspended in HBSS/HEPES for the phagocytic assay.

‡ Washed opsonized or unopsonized emulsion was prepared as described in Materials and Methods.

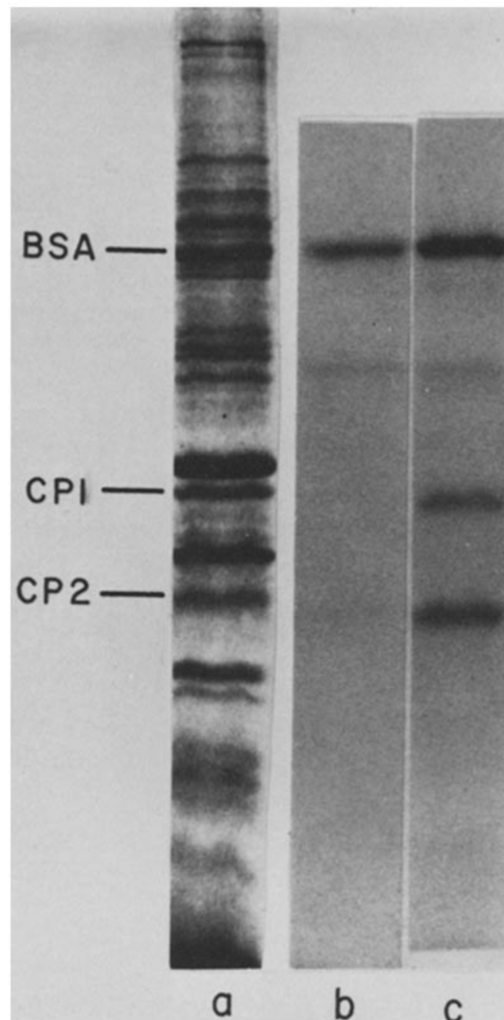

FIGURE 1 Cleavage of ingested BSA. 2×10^7 cells were incubated either with 0.2 ml ^{125}I-BSA (15 mg/ml, 4×10^6 cpm/ml) or with 0.2 ml ^{125}I-BSA/oil emulsion (4×10^6 cpm/ml) in 1 ml HBSS/HEPES at 36.5°C. Aliquots of 4×10^6 cells were removed into ice-cold 10^{-3} M NEM in saline, washed twice with PBS, and processed for SDS-PAGE. (*a*) Coomassie blue stain of whole cells 10 min after phagocytosis. (*b*) Cells incubated with soluble ^{125}I-BSA for 20 min. (*c*) Cells incubated with ^{125}I-BSA/oil emulsion for 20 min. *CP1* and *CP2* mark the position of migration of BSA cleavage products.

BSA ingested during a 5-min incubation was 80% degraded during the next 10 min.

We wished to inhibit this iodination so that only the radioactivity incorporated before phagocytosis would be monitored in subsequent experiments. The data in Fig. 2 show that phagocytizing gran-

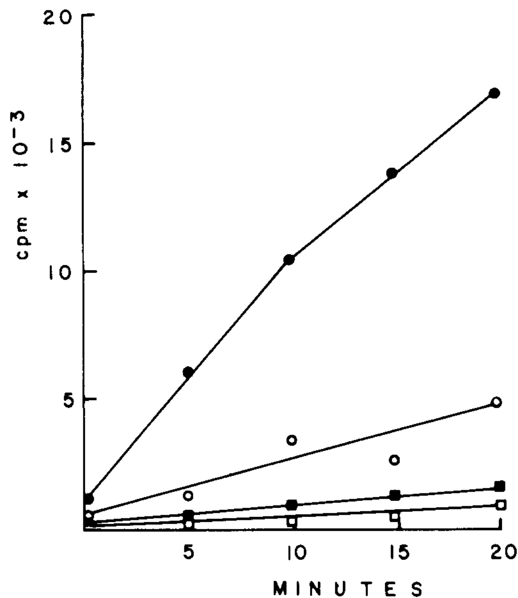

FIGURE 2 The effect of cyanide on the incorporation of ^{125}I into acid-precipitable material by phagocytizing PMNs. 2×10^7 cells in 1 ml HBSS/HEPES were incubated at 36.5°C with either 3 mg/ml BSA (○), 0.2 ml emulsion (●), 3 mg/ml BSA plus 10^{-3} M KCN (□), or 0.2 ml emulsion plus 10^{-3} M KCN (■). 23 μCi ^{125}I were added and 0.1-ml aliquots were removed at 0, 5, 10, 15, and 20 min into 10^{-3} M NEM/saline. The cells were washed three times with 10^{-3} M NaI in PBS, and the cell pellets were TCA-precipitated and counted as described in Materials and Methods.

ulocytes in the absence of added enzymes incorporate iodine into acid-insoluble material (27); however, 1 mM KCN inhibited this activity. Under these conditions, ^{125}I was not incorporated into BSA and the total acid-insoluble radioactivity of whole cells did not increase upon phagocytosis. Labeled cells in the presence of cyanide phagocytized emulsion at the same rate as unlabeled cells (Fig. 3).

Fate of Plasma Membrane During Phagocytosis

Fig. 4 shows the experimental scheme that we used to examine the distribution of pre-existing plasma membrane proteins upon phagocytosis. Labeled cells were incubated with particles under various conditions, phagocytosis was terminated with NEM, and the cells were homogenized and fractionated. The labeled proteins of the various isolated fractions were then analyzed by SDS-PAGE.

ISOLATION OF PHAGOCYTIC VESICLES AND PLASMA MEMBRANE FROM PHAGOCYTIZING CELLS: Because extensive aggregation of nuclei with other organelles and particles occurred after homogenization of phagocytizing cells, experiments were performed to determine to what extent the phagosome fraction might also be contaminated with nonphagosome labeled plasma membrane. In one test, labeled resting cells were mixed at 4°C with unlabeled phagocytizing cells and homogenized. Table II shows that very little radioactive plasma membrane of resting cells was found in the phagosome fraction derived from unlabeled, phagocytizing cells. In another test, labeled cells were mixed at 4°C with emulsion and homogenized in the absence of phagocytosis. Table II shows that the floating fraction did not bind any radioactivity. Thus, almost all the label recovered in the floating fraction was due to phagocytic vesicle formation. When the floating fraction was examined by electron microscopy, only vesicles or fragments of unit membrane thickness as well as the collapsed remains of free BSA-oil emulsion were seen.

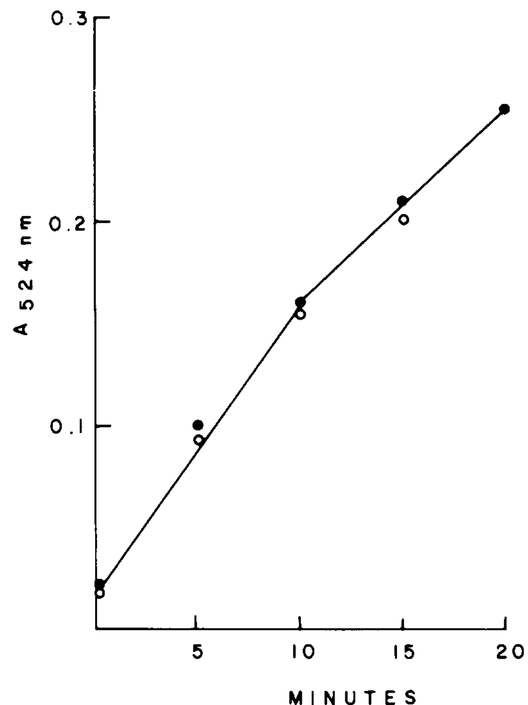

FIGURE 3 Initial rate of phagocytosis by iodinated PMNs. Initial rates were measured as described in Materials and Methods. Unlabeled cells phagocytizing in HBSS/HEPES (●). Lactoperoxidase-labeled cells phagocytizing in 10^{-3} M KCN in HBSS/HEPES (○).

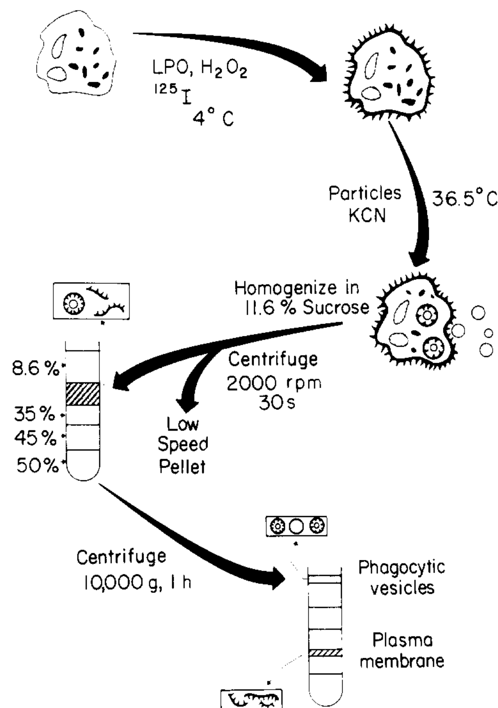

FIGURE 4 Experimental scheme employed to determine the distribution of preexisting cell surface proteins after phagocytosis.

TABLE II
Acid-Insoluble Radioactivity Co-isolating with Emulsion

	% homogenate cpm* in floating fraction
1. Iodinated cells phagocytizing 12 min	23.0, 24.6 (h)‡
2. Iodinated resting cells + unlabeled cells phagocytizing 12 min	1.0, 1.8 (h)
3. Iodinated resting cells + emulsion	0.0

Resting cells were mixed with phagocytizing cells or emulsion at 4°C and then homogenized and fractionated as described in Materials and Methods.

$$* \frac{\text{cpm}_{\text{floating fraction}}}{\text{cpm}_{\text{homogenate}}} \times \frac{\text{OD}_{\text{homogenate}}}{\text{OD}_{\text{floating fraction}}} \times 100$$

‡ Fractionation in the presence of heparin: (h)

Our plasma membrane fraction, the 35%–45% interface, was free of contaminating phagocytic vesicles as judged by the lack of Oil Red 0 in this fraction. Its purity was further evidenced by the absence of BSA cleavage produces, CP1 and CP2, which are major polypeptide constituents of phagosomes (Fig. 5). The protein profile of the 35%–45% interface from homogenates of phagocytizing cells was qualitatively similar to the same fraction from resting cells.

Yields of phagosomes (OD_{524} of floating fraction/OD_{524} of homogenate) were between 10 and 15%. Inclusion of heparin (100–200 U/ml) in the homogenizing medium increased the yield to 30–40%. Heparin increased yields by promoting cell lysis and inhibiting aggregation, but it also caused lysis of nuclei (4). We were unable to obtain a plasma membrane fraction from heparin homogenates of either resting or phagocytizing cells.

DISTRIBUTION OF EXTERNAL MEMBRANE PROTEINS AFTER PHAGOCYTOSIS: Although extensive degradation of BSA took place within 15 min of its phagocytosis (see Fig. 1), the acid-precipitable radioactivity of lactoperoxidase-labeled cells decreased by only 10–15% and was identical to that of control cells incubated only with soluble BSA. Autoradiography of SDS-polyacrylamide gels in which equal numbers of labeled cells before or after phagocytosis were analyzed revealed little change in the labeled protein profile. In a few cases, diffuse label accumulated between bands 6 and 7 (Fig. 6 *a–d*); however, most experiments did not show this material (Fig. 6 *e* and *f*). The origin of this material is not known. It cannot represent membrane protein degradation, because

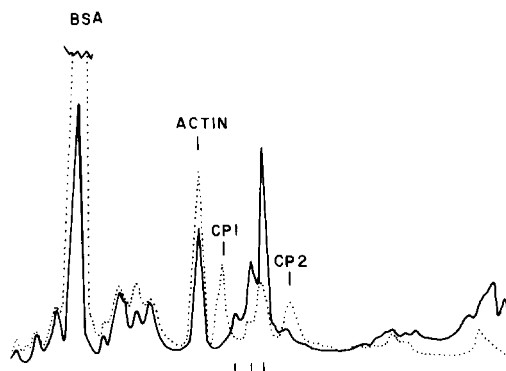

FIGURE 5 Electrophoretic analysis of subcellular fractions of phagocytizing polymorphonuclear neutrophils. Optical absorbance scans of Coomassie blue-stained gels. 7.5 × 10^7 iodinated cells were incubated with particles for 15 min and fractionated as described in Materials and Methods. Each sample applied to the gel contained equal amounts of acid insoluble radioactivity. (—): plasma membrane; (·····): phagocytic vesicles. *CP1* and *CP2*, cleavage products of BSA.

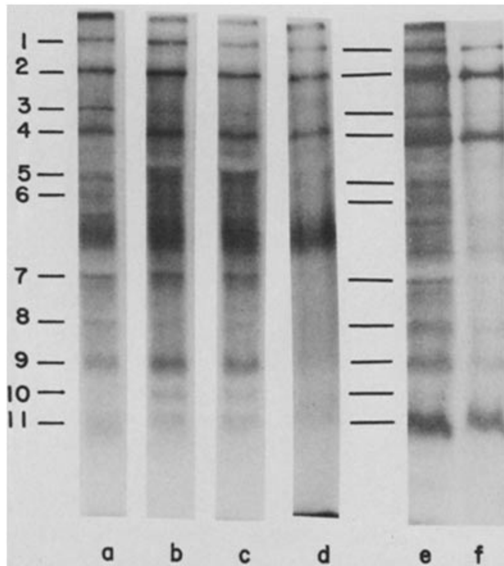

FIGURE 6 Electrophoretic analysis of subcellular fractions of phagocytizing polymorphonuclear neutrophils. Autoradiography. Cells were iodinated and incubated with particles for 15 min. The cells were fractionated as described in Materials and Methods. An equal number of acid-insoluble counts (20,000 cpm) from whole cells (*a*), plasma membrane (*c*), and phagosomes (*d*) were applied to the gel. (*b*) is plasma membrane from cells before phagocytosis. (*e*) and (*f*) show the whole cells and the phagosome fraction, respectively, from another phagocytosis experiment.

the major pre-existing labeled polypeptides were unaltered.

Although the total protein composition of phagosomes and plasma membrane of PMNs was similar, some differences were detected. Fig. 5 shows optical absorbance scans of the Coomassie blue-stained proteins of these two fractions. The membrane fraction contains three proteins, indicated by three short vertical lines, that are depleted in phagosomes. Proteins which co-migrated with actin and myosin were associated with both phagosomes and plasma membrane. The 45,000-dalton protein co-migrating with actin represented ~30% of the total protein in phagosomes (exclusive of the ingested BSA) and 7–10% of whole cell proteins. Preliminary attempts to release this protein with MgATP in low ionic strength failed.

The composition of labeled proteins in phagosomes, plasma membrane, and whole cells after 15 min of phagocytosis is shown in Fig. 6. Samples containing equal amounts of radioactivity were analyzed. Bands 3, 5, and 6 were consistently absent from both phagocytic vesicles (channels *d* and *f*) and plasma membrane (channel *c*). These bands were found in the pellet after centrifugation of the total homogenate at low speed (see Materials and Methods, and reference 38). Band 10, a minor component in the whole cell pattern, was enriched in the isolated plasma membrane fraction of phagocytizing cells (channel *c*) as well as resting cells (channel *b*). However, it was not present in phagosomes in 7 out of 10 independent experiments (channels *d* and *f*). Also depleted in the phagosome fraction were bands 7 and 9, two proteins which showed only partial sensitivity to trypsinization of whole cells. The labeled protein content of phagosomes, plasma membrane, and whole cells calculated from scanned autoradiographs from another experiment is presented in Table III. The areas under each peak were normalized to make band 2 equal in all the samples. The data show that bands 3, 5, and 6 were absent from membranes and phagosomes; band 10 was enriched in membranes but absent from phagosomes; and bands 7 and 9 were present in membranes but reduced in phagosomes. In this experiment there was an unusual amount of material in band 11 of phagosomes, which was dependent upon phagocytosis by the cells (see also Fig. 6, channel *f*).

The earliest time after phagocytosis that we examined was 5 min. Although intraphagosomal degradation cannot be ruled out entirely, loss of band 10 was not evident in whole cells. The lost proteins did not appear when the gels were exposed to film for two or three times longer than necessary to achieve the result shown in Fig. 6. Because the yield of phagosomes was low, it could be argued that we have monitored the peptide composition of a unique population of phagosomes. However, analysis of phagosomes from heparin homogenates (yield 30–40%) also revealed the depletion of bands 3, 5, 6, and 10.

An alternative method to examine the effect of phagocytosis on the distribution of cell surface proteins is to label cells after phagocytosis has occurred. Only those proteins that fail to be interiorized should become labeled. Unfortunately, this experiment is very difficult to perform because phagocytosis markedly activates the endogenous enzymes that contribute to high labeling backgrounds. Nevertheless, Fig. 7 shows the results of a successful experiment of this type. BSA and its cleavage products were significantly labeled. However, it can be seen that bands 1, 2, and 4 were markedly reduced on phagocytizing whole cells

TABLE III

Labeled Protein Composition of Membrane and Phagosome Fractions of Resting or Phagocytosing Cells in the Presence and Absence of Colchicine

Band	Whole cells			Membranes			Phagosomes	
	Resting	Phagocytosis − colchicine	Phagocytosis + colchicine	Resting	Phagocytosis − colchicine	Phagocytosis + colchicine	Phagocytosis − colchicine	Phagocytosis + colchicine
1	0.52	0.36	0.48	0.64	0.65	0.44	0.27	0.26
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3	0.14	0.19	0.29	0.04	0.02	0.01	0.01	0.0
4	0.83	0.86	0.81	0.96	1.47	1.44	0.87	0.91
5	0.33	0.36	0.39	0.01	0.0	0.0	0.0	0.0
6	0.06	0.03	0.07	0.02	0.0	0.0	0.0	0.0
7	0.56	0.75	0.77	0.22	0.50	0.58	0.11	0.11
8	0.20	0.19	0.16	0.07	0.02	0.01	0.07	0.09
9	0.86	1.00	1.13	0.96	1.24	0.97	0.53	0.71
10	0.22	0.22	0.29	0.36	0.35	0.28	0.0	0.06
11	0.89	1.61	1.65	0.60	0.50	0.50	1.67	1.97

Iodinated PMNs, after phagocytosis for 10 min, were fractionated as described in Materials and Methods. An equal number of acid-insoluble counts were analyzed by SDS-polyacrylamide gel electrophoresis. Autoradiographs of these gels were scanned and the area under each band was measured. These areas were normalized for band 2. Some of the cells were pre-incubated with colchicine as described in the text.

(channel *b*) as compared with resting cells (channel *a*). Presumably, these proteins have been interiorized. In contrast, bands 3, 5, and 6 have remained accessible to labeling at the cell surface.

EFFECT OF COLCHICINE ON THE DISTRIBUTION OF LACTOPEROXIDASE-LABELED PROTEINS AFTER PHAGOCYTOSIS: It has been reported that colchicine modulates the distribution of plasma membrane proteins during phagocytosis (22, 34). Therefore, it was of interest to examine the effect of colchicine on the localization of bands 3, 5–7, 9, and 10. Cells were pre-incubated for 5 min in the presence of 5×10^{-5} M colchicine and then fed emulsion for 10 min in the presence of the drug. This treatment was found to be sufficient to inhibit PMN aggregation, a phenomenon which requires intact microtubules (20). However, the rate of particle uptake by colchicine-treated cells, 0.043 $OD_{524}/min/5 \times 10^6$ cells, was not significantly different from that of control cells, 0.039 $OD_{524}/min/5 \times 10^6$ cells. Furthermore, ingested BSA was cleaved to CP1 and CP2 as occurred during normal conditions of phagocytosis. As shown in Table III, the distribution of most of the labeled polypeptides in the plasma membrane and phagocytic vesicle fractions of colchicine-treated cells was unaltered. Bands 9 and 11 of phagocytic vesicles show slight differences from normal, but these results were not seen in two other experiments.

DISCUSSION

Phagocytosis

The purpose of this study was to directly examine the distribution of external membrane proteins in phagocytizing and resting rabbit peritoneal PMNs. By labeling surface proteins before particle uptake, and comparing the labeled protein composition of whole cells with the phagocytic vesicle and the remaining cell surface, we hoped to determine whether a segregation of specific membrane components to or from sites of particle internalization occurred. This approach was different from those previously described because the fate of identifiable membrane proteins, rather than surface-related functions, was analyzed during phagocytosis. Lactoperoxidase-catalyzed iodination was employed to label the membrane proteins, and slab gel electrophoresis followed by autoradiography permitted their identification.

Rabbit peritoneal PMNs ingested BSA-diisodecylphthalate emulsion avidly. Uptake of the emulsion exhibited first-order and saturation kinetics as was observed with guinea pig neutrophils (30), human peripheral neutrophils (28), alveolar macrophages (28), and *Acanthamoeba* (35). Unlike latex-phagocyte interactions, nonspecific absorption of these particles was not observed. Measurements of particle uptake were reproducible and showed little variation from rabbit to rabbit. Par-

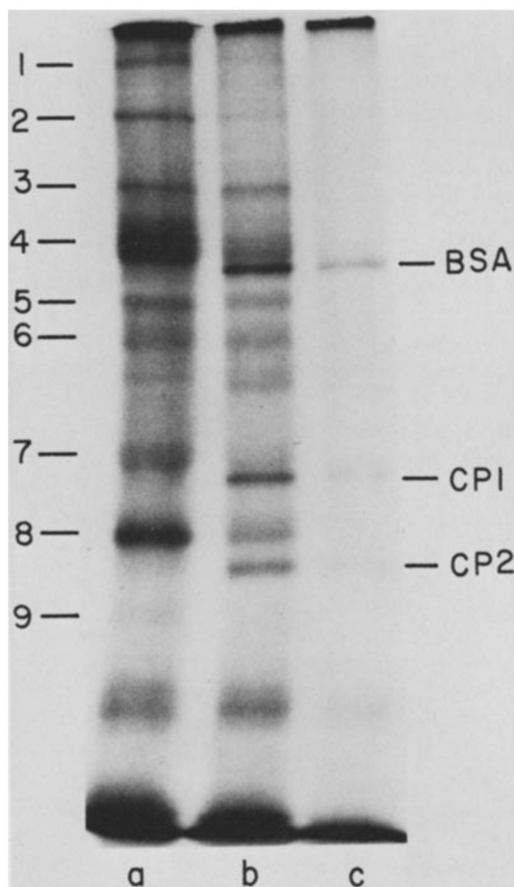

FIGURE 7 Enzymatic iodination of neutrophils after phagocytosis. Neutrophils were incubated with particles for 8 min at 36.5°C, or on ice. The cells were then washed twice with PBS, resuspended in PBS at 5×10^7 ml, and iodinated as described previously. (*a*) Cells incubated at 0°C where phagocytosis fails to occur, and then labeled with complete reaction mixture. (*b*) Cells incubated at 36.5°C and then labeled with complete reaction mixture. (*c*) Cells incubated at 36.5°C but labeled without lactoperoxidase.

ticle uptake displayed similar sensitivity to metabolic inhibitors as phagocytosis of BSA-paraffin oil emulsion by guinea pig peritoneal granulocytes (30).

Opsonization by fresh serum increased the rate of particle uptake between 50 and 60%. Stossel (28) found that opsonization had no affect on uptake of emulsion by guinea pig neutrophils but doubled the initial rate of phagocytosis by rabbit alveolar macrophages and almost tripled uptake by human leukocytes. He demonstrated that opsonization by fresh serum was a result of divalent cation-dependent binding of the third component of complement to particles. The fact that uptake of opsonized particles in our system was not trypsin sensitive indicates that an exposed surface protein may not be involved in C'3 action.

Distribution of Cell Surface Proteins During Phagocytosis

The surface of rabbit neutrophils was labeled by the lactoperoxidase-catalyzed iodination procedure described before (38). During 10 min of phagocytosis, 20–30% of the cell surface was internalized as measured by the amount of acid-insoluble surface radioactivity in phagocytic vesicles. Direct examination of the subcellular distribution of the various iodinated membrane proteins after phagocytosis provided the following information (see Table IV): (*a*) Three of the most prominent iodinated proteins, bands 1, 2, and 4, were found in phagocytic vesicles isolated at 5 and 15 min of phagocytosis. (*b*) As early as 5 min, bands 3, 5–7, and 10 were either not detected at all or detected in reduced amounts in phagocytic vesicles. Bands 3, 5, and 6 were also absent from the plasma membrane fraction of resting and phagocytizing cells. We have shown (38) that those polypeptides are concentrated in a low speed pellet fraction of PMN homogenates. (*c*) Colchicine had little affect on the distribution of labeled proteins in phagosomes. A reservation that must be considered when examining this distribution of labeled surface proteins into various cell fractions is the low yield of phagocytic vesicles that we obtained. Although we

TABLE IV
Distribution of Surface Labeled Proteins of Phagocytizing Neutrophils

Band	Whole cells	Plasma membrane	Phagocytic vesicles
1	+	+	+
2	+	+	+
3	+	−	−
4	+	+	+
5	+	−	−
6	+	−	−
7	+	+	±
8	+	+	+
9	+	+	±
10	+	++	−
11	+	+	+

showed that this fraction was uncontaminated by plasma membrane, it was also clear that phagosomes, with their included red dye, were present not only at the top of the sucrose gradient but at the 8.6%–11.6% and 11.6%–35% interfaces. Homogenization in the presence of heparin reduced the losses to these interfaces and increased the yield of phagocytic vesicles to ~40%. Analysis of heparin vesicles revealed the same distribution of labeled peptides as in normal phagocytic vesicles.

To conclude that bands 3, 5–7, and 10 were segregated from sites of membrane interiorization, degradation must be ruled out. The extent of intraphagosomal membrane protein degradation at early times after phagocytosis was difficult to assess. 80% of the ingested BSA was degraded during this time, yet the profile of labeled surface proteins did not change. Of interest was that colchicine-treated cells cleaved BSA to the same extent as controls (see also references 23 and 30). Hydrolysis of a limited number of surface proteins might not be detected when total acid-insoluble radioactivity was measured before and after phagocytosis. To make such a measurement meaningful, it would be necessary to quantitate the amount of radioactivity in the individual proteins. To do so, labeling to a higher specific activity must be achieved. ^{131}I has been used successfully in place of ^{125}I for this purpose (32). Another approach, however, would be to label whole cells after phagocytosis to determine whether the cells bear more accessible 3, 5, 6, and 10 on their surface than 1, 2, 4, 9, and 11. Because of the activation of oxidases and hydrolases upon phagocytosis, this experiment has been difficult. Nevertheless two experiments supported the prediction: post-phagocytic cells showed a depletion of bands 1, 2, and 4 and a retention of bands 3, 5, and 6 on their surface.

Evidence in the literature is consistent with the concept that some plasma membrane components of differentiated cells may be internalized at random while others may not be. 5′-nucleotidase is internalized during phagocytosis by mouse macrophages (36), rabbit peritoneal neutrophils (22), and KB cells (6), in proportion to the estimated amount of plasma membrane internalized. Oliver et al. (22) found that the specific activity of 5′-nucleotidase in isolated membrane remained constant throughout 30 min of phagocytosis. However, 5′-nucleotidase may not be a plasma membrane marker in rabbit neutrophils (1, 2), but rather a component of the membrane of azurophil granules. The specific activities of 5′-nucleotidase, Na^+- and K^+-ATPase, and NADPH-cytochrome *c* reductase of KB cells membrane ghosts and phagosomes were identical (6). However, DePierre and Karnovsky (9) detected ATPase, AMPase, and *p*-nitrophenylphosphatase activity on the surface of intact guinea pig neutrophils whose activities remained unchanged after internalization of an estimated 70% of the cell surface during phagocytosis.

Tsan and Berlin (33) were the first to report the segregation of membrane functions from sites of particle interiorization. There was no reduction in the V_{max} of transport of lysine, adenosine, and adenine after phagocytosis by rabbit macrophages and neutrophils. In contrast, Dunham et al. (10), measuring lysine and K^+ fluxes in human neutrophils, did observe a decrease in the number of transport sites. Each transport system was affected to a different extent. The same decreases in transport were also observed when zymosan was attached to the surface in the presence of cytochalasin B. They concluded that membrane heterogeneity may occur before internalization, during particle binding. However, conclusions concerning membrane-localized activities in intact cells should be made with caution because the physiology of the cell changes dramatically upon phagocytic activation. By cytochemical techniques, Briggs et al. (5) showed that NADH oxidase is a latent plasma membrane enzyme. It is detected on the surface and in the phagosomes of phagocytic cells, but not on resting cells.

There have been two studies, in addition to the one reported in this paper, in which the fate during phagocytosis of external membrane proteins labeled by lactoperoxidase has been analyzed (14–16). L cells interiorize 15–30% of their surface during 1 h of phagocytosis of latex beads. By slab gel electrophoresis, Hubbard and Cohn detected 19–20 iodinated proteins of >50,000 daltons on the surface of L cells grown in suspension culture. All of these lactoperoxidase-labeled proteins were found in isolated phagosomes. Hunt and Brown (16) also observed that all the proteins labeled on L cells grown in suspension were present in the phagocytic vesicle. L cells grown in monolayer, however, possessed only two major iodinated protein species. The phagocytic vesicle contained one, a high molecular weight glycoprotein similar to the LETS protein of Hynes (17).

Our results, in conjunction with those described above, suggest that in differentiated phagocytic cells, such as neutrophils, four classes of membrane macromolecules may exist. Members of class I have an inherent random distribution, are free to move in the plane of the membrane, and are internalized randomly during phagocytosis. Bands 1, 2, 4, 8, and 11 and 5′-nucleotidase would belong to this class. Members of class II are free to diffuse in the bilayer of resting cells, but their distribution is not homogeneous during phagocytosis, i.e., they are either segregated from sites of vesicle formation or preferentially internalized. Submembranous structures such as microtubules or microfilaments may anchor them or actively direct their movement (8, 22, 39). Alternatively, particle binding itself could induce receptor cross-linking and patch formation. Lectin-binding sites, transport sites, and bands 7, 10, and perhaps 9 may be class II macromolecules. Class III components are distributed nonrandomly on resting cells but are internalized with bulk membrane. The large glycoprotein of adherent L cells may belong to this class. The corresponding protein of NIL8 fibroblasts is located in a unique fraction of the plasma membrane and is observed as fibrils on the cell surface (17). Class IV components are proteins which have an inherent heterogeneous distribution, being concentrated at or away from sites of phagocytosis. Bands 3, 5, and 6, which are not found in the predominant fraction of neutrophil plasma membrane, may be examples of such proteins. Additional biochemical information concerning the structure and function of the iodinated protein species of neutrophils as well as morphological analysis of their location and mobility will help determine whether the various classes of membrane proteins listed above actually exist and what their role in cell interactions may be. This study has shown qualitative differences in the external membrane protein composition of membrane fractions isolated from phagocytizing cells. Future experiments quantitating the distribution of these proteins in all of the membrane compartments of the cell would be useful and could be achieved, perhaps, by the development of monospecific antibodies directed against them.

We thank T. Stossel for suggesting the use of diisodecylphthalate.

Received for publication 29 July 1977, and in revised form 28 February 1979.

REFERENCES

1. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. I. Histochemical staining of bone marrow smears. *J. Cell Biol.* **39**:286–298.
2. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophils and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. *J. Cell Biol.* **39**:299–317.
3. Beck, W. S. 1968. The control of leukocyte glycolysis. *J. Biol. Chem.* **232**:251–270.
4. Bornens, M. 1973. Action of heparin on nuclei: solubilization of chromatin enabling the isolation of nuclear membranes. *Nature (Lond.).* **244**:28–30.
5. Briggs, R. T., D. B. Drath, M. L. Karnovsky, and M. J. Karnovsky. 1975. Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. *J. Cell Biol.* **67**:566–586.
6. Charalampous, F. C., N. K. Gonatas, and A. D. Melbourne. 1973. Isolation and properties of the plasma membrane of KB cells. *J. Cell Biol.* **59**:421–435.
7. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leukocytes. I. Observations on the requirements and consequences of particle ingestion. *J. Exp. Med.* **111**:667–687.
8. dePetris, S. 1975. Con A receptors, IgG and the antigen of the lymphocyte surface. Interactions with Con A and with cytoplasmic structures. *J. Cell Biol.* **65**:123–146.
9. dePierre, J., and M. L. Karnovsky. 1972. Ectoenzymes, sialic acid and the internalization of cell membrane during phagocytosis. *In* Inflammation, Mechanisms and Control, I. H. Lepow and P. A. Ward, editors. Academic Press, Inc., New York. 55–70.
10. Dunham, P. B., I. M. Goldstein, and G. Weissmann. 1974. Potassium and amino acid transport on human leukocytes exposed to phagocytic stimuli. *J. Cell Biol.* **63**:215–226.
11. Elsbach, P. 1972. Lipid metabolism by phagocytes. *Semin. Hem.* **9**: 227–239.
12. Griffin, F. M., J. A. Griffin, J. E. Leider, and S. C. Silverstein. 1975. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle bound ligands to specific receptors on the macrophage plasma membrane. *J. Exp. Med.* **142**:1263–1282.
13. Griffin, F. M. and S. C. Silverstein. 1974. Segmental response of the macrophage plasma membrane to a phagocytic stimulus. *J. Exp. Med.* **139**:323–336.
14. 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.
15. 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**:461–471.
16. Hunt, R. C., and J. C. Brown. 1975. Identification of a high molecular weight transmembrane protein in mouse L cells. *J. Mol. Biol.* **97**:413–422.
17. Hynes, R. O. 1974. Role of surface alterations in cell transformation: the importance of surface proteases and surface proteins. *Cell.* **1**:147–156.
18. Kaplan, G., G. Gaudernack, and R. Seljelid. 1975. Localization of receptors and early events of phagocytosis in the macrophage. *Exp. Cell res.* **95**:365–375.
19. Kaplan, G., and R. Seljelid. 1976. Difference in the mode of internalization with F_c and C3 in macrophages. *J. Cell Biol.* **70**(2, Pt. 2):28*a*. (Abstr.).
20. Lackie, J. M. 1974. The aggregation of rabbit PMN: effects of antimitotic agents, cyclic nucleotides and methylxanthines. *J. Cell Sci.* **16**: 167–180.
21. Lutton, J. D. 1973. The effect of phagocytosis and spreading on macrophage surface receptors for Con A. *J. Cell Biol.* **56**:611–617.
22. Oliver, J. M., T. E. Ukena, and R. D. Berlin. 1974. Effects of phagocytosis and colchicine on the distribution of lectin binding sites on cell surfaces. *Proc. Natl. Acad. Sci. U. S. A.* **71**:394–398.
23. Pesanti, E. L., and S. G. Axline. 1975. Colchicine effects on lysosomal enzyme induction and intracellular degradation in the cultivated macrophage. *J. Exp. Med.* **141**:1030–1045.
24. Rabinovitch, M., M. Manejias, and V. Nussenzweig. 1975. Selective phagocytic paralysis induced by immobilized immune complexes. *J. Exp. Med.* **142**:827–838.
25. Sbarra, A. J., and M. Karnovsky. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.* **234**:1355–1362.

26. SCHMIDT, M. E., and S. D. DOUGLAS. 1972. Disappearance and recovery of human monocyte IgG receptor activity after phagocytosis. *J. Immunol.* **109**:914–917.
27. SIMMONS, S. R., and M. L. KARNOVSKY. 1973. Iodinating ability of various leukocytes and their bacteriocidal activity. *J. Exp. Med.* **138**: 44–63.
28. STOSSEL, T. P. 1973. Quantitative studies of phagocytosis. Kinetic effects of cations and heat labile opsonins. *J. Cell Biol.* **58**:346–356.
29. STOSSEL, T. P., C. A. ALPER, and F. S. ROSEN. 1973. Serum-dependent phagocytosis of paraffin oil emulsified with bacterial lipopolysaccharide. *J. Exp. Med.* **137**:690–705.
30. STOSSEL, T. P., R. J. MASON, J. HARTWIG, and M. VAUGHN. 1972. Quantitative studies of phagocytosis by polymorphonuclear neutrophils: use of emulsions to measure the initial rate of phagocytosis. *J. Clin. Invest.* **51**:615–624.
31. STOSSEL, T. P., T. D. POLLARD, R. J. MASON, and M. VAUGHN. 1971. Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. *J. Clin. Invest.* **50**:1745–1757.
32. TENG, N. N. H., and L. B. CHEN. 1976. Thrombin sensitive surface protein of cultured chick embryo fibroblasts. *Nature (Lond.).* **259**:278–280.
33. TSAN, M. T., and R. D. BERLIN. 1971. Effect of phagocytosis on membrane transport of non-electrolytes. *J. Exp. Med.* **134**:1016–1035.
34. UKENA, T., and R. D. BERLIN. 1972. Effect of colchicine and vinblastine on the topographical separation of membrane functions. *J. Exp. Med.* **136**:1–7.
35. WEISMAN, R. A., and E. D. KORN. 1967. Phagocytosis of latex beads by Acanthamoeba. I. Biochemical properties. *Biochemistry.* **6**:485–497.
36. WERB, Z., and Z. A. COHN. 1972. Plasma membrane synthesis in macrophages following phagocytosis of polystyrene latex. *J. Biol. Chem.* **247**:2439–2446.
37. WILLINGER, M. and F. R. FRANKEL. 1974. Studies on the fate of surface proteins by polymorphonuclear leukocytes. *J. Cell Biol.* **63**(2, Pt. 2): 373*a*. (Abstr.).
38. 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.
39. YAHARA, I., and G. M. EDELMAN. 1975. Modulation of lymphocyte receptor mobility by locally bound Con A. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1579–1583.