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Changes in membrane microviscosity associated with phagocytosis: Effects of colchicine

(fluorescence polarization/microtubules/lateral phase separation)

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ABSTRACT **The effects of phagocytosis on plasma membrane microviscosity were studied by fluorescence depolarization techniques. It was shown that lipophilic probes are accumulated in intracellular vesicles to a significant degree in fibroblasts and neutrophils. Microviscosity was thus determined from the behavior of probes in isolated membranes. Phagocytosis of oil emulsions or polystyrene beads by rabbit polymorphonuclear leukocytes induces a marked decrease in plasma membrane microviscosity that parallels the extent of phagocytosis. Liposomes made from extracts of membrane lipid show qualitatively the same changes, indicating that the alteration of microviscosity results at least in part from changes in lipid composition. The decrease in microviscosity is abolished when colchicine is present during phagocytosis. Addition of colchicine to membranes previously isolated from control or phagocytic cells has no effect on their microviscosity. The results suggest that phagocytosis is accompanied by a microtubule-dependent reorganization of membrane lipids.**

During phagocytosis, particles are enveloped by plasma membrane and subsequently internalized. In polymorphonuclear leukocytes (PMN), this process is accompanied by striking changes in the composition of the residual plasma membrane. For example, certain lectin receptors are selectively removed from the surface (1) while transport proteins are selectively spared (2). Treatment of cells with low doses of colchicine, an inhibitor of microtubule assembly, prevents the induced topographical changes without affecting the rate or amount of ingestion (1, 3). The existence of these colchicine-sensitive phenomena indicates a role for microtubules in the control of membrane topography. Further, the large and rapid membrane reorganization accompanying phagocytosis provides a unique system for analyzing the consequences of microtubule-membrane interaction at the molecular level.

We report here studies of the microviscosity of the plasma membrane of phagocytic cells. Microviscosity was inferred from the fluorescence depolarization of lipophilic probes embedded in isolated plasma membranes and in liposomes made from extracts of the membranes. Large changes in microviscosity are observed which parallel the degree of phagocytosis. Colchicine has no direct effect on membrane microviscosity, but blocks changes induced by phagocytosis.

METHODS

The fluorescence polarization method used for the study of membrane microviscosity has been described extensively (4) and has been used with artificial liposomes (4, 5,), membranes (6), and whole cells (7, 8). The polarization of fluorescence is determined by the rotation of the probe during the lifetime of the excited state. The rotation of the probe in turn is dependent on the viscosity of its environment ("microviscosity")—rapid rotation leading to depolarization. By determining the polarization of the probe in media of known viscosity a correlation between polarization values and microviscosity can be made. Unknown microviscosities of membranes can thus be inferred provided the fluorescent state lifetimes are known. The rotational depolarization of fluorophore is described by the Perrin equation:

$$(r_0/r) = 1 + C(r)(T\tau/\eta)$$

where r and r_0 are the measured and limiting anisotropy, respectively; T, the absolute temperature; τ the excited state lifetime; η the viscosity of the medium, and $C(r)$ a parameter relating to the molecular shape and orientation of the absorption and emission dipoles which is essentially determined empirically.

For our studies, polarization-viscosity curves were obtained for both perylene and 1,6-diphenyl-1,3,5-hexatriene (DPH) in S60 standard viscosity oil (Cannon Instrument Co.). Fluorescence lifetime measurements were made by a monophoton counting technique (9). We found these to be 6.5 nsec for perylene and 10.0 nsec for DPH in oil, membranes, and liposomes at 22°. When changes in intensity with temperature were observed, we assumed that the lifetime varied as the intensity (10). However, in most cases a significant change in intensity of fluorescence with temperature was not observed and we assumed constancy of the excited state lifetime. The constancy of intensity was not the result of alterations in the amount of membrane-bound probe because it was observed whether or not the membranes were washed free of excess probe. (The quantum yields of the probes in aqueous solution are negligible.) However, it proved important to label initially at high temperature (42°). A period of about 30 min was required to develop maximum intensity. Further incubation of membranes at 42° for up to 2 hr did not alter polarization values.

Fluorescence Measurements. Steady-state polarization measurements were made with a Perkin-Elmer Hitachi MPF-4 spectrofluorimeter with corrected emission spectra. Fluorescence polarization and intensity were obtained by measurements of fluorescence parallel and perpendicular to the direction of polarization of the excitation beam. Corrections for the intrinsic polarization of the exciting beam were applied (4).

Temperature was carefully controlled and determined by immersing a thermocouple directly into the sample.

For perylene, excitation was at wavelengths of 436 nm; emission, 474 nm. For DPH, excitation was at 357 nm; emission, 430 nm. Readings were taken within 10 sec of light exposure to reduce photoisomerization (10).

Samples of isolated membranes, or liposomes derived from them, were prepared so that the contribution by scatter was less than 2% of the signal. To facilitate scatter corrections, we de-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PMN, polymorphonuclear leukocytes; BSA, bovine serum albumin.

termined scatter on membrane suspensions prior to labeling with fluorescent probes added in negligible volumes. Scatter corrections were applied to all measurements.

Membranes from the sucrose gradient (see below) were diluted with phagocytosis medium to the desired concentration. The final concentration of sucrose was usually about 0.2 M, but we found that varying sucrose concentrations from 0.1 to 1.0 M and the electrolyte concentrationsoover a similar 10-fold range did not affect polarization values.

Cells. Rabbit PMN were obtained from sterile peritoneal exudates (11).

Phagocytosis. Two kinds of particles were used: a finely divided emulsion of paraffin oil and bovine serum albumin (BSA) (12), and polystyrene beads of 0.81 μm diameter. Phagocytosis of the former was quantified by measurement of oil Red O dissolved in the paraffin oil (12). Cell-associated polystyrene beads were quantified by extraction into dioxane and measurement of specific absorption at 253 nm (13).

Cells were suspended at 10^7/ml in medium at pH 7.5 containing 140 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 10 mM KCl, 5 mM glucose, 0.01 mM $CaCl_2$, and 0.2 mM $MgSO_4$ (phagocytosis medium). They were incubated for various lengths of time at 37°C with 5% (vol/vol) of emulsion or beads at a ratio of roughly 300 particles to one PMN, then collected by centrifugation and washed to remove excess emulsion or beads. Ingestion of emulsion was rapid for approximately 15 min, after which the process appeared to be saturated. In control experiments, cells were incubated with 1% BSA alone (the same concentration present in the emulsion). Under our conditions maximal phagocytosis of polystyrene required 45–60 min.

Subcellular Fractionation. For isolation of membranes, homogenates were centrifuged through a discontinuous sucrose gradient as described (1). In these experiments, homogenates were prepared using a nitrogen bomb (Artisan) by decompressing the cell suspension from 600 lbs./inch^2 of nitrogen pressure. Using this gentle procedure, more than 90% of β-glucuronidase was recovered in the pellet of the first centrifugation, indicating that little lysosomal breakage occurred. A second centrifugation through the sucrose gradient was used to further purify the membranes, which were enriched 10-fold with respect to protein for the plasma-membrane-specific enzyme 5'-nucleotidase (14).

The final contamination of the membrane fraction by lysosomal membranes was estimated from the content of the lysosomal enzyme β-glucuronidase. A crude lysosomal pellet was obtained by centrifugation of homogenates at 1500 rpm for 10 min (to remove nuclei and whole cells), followed by recentrifugation of the supernatant fraction at 13,000 rpm for 30 min to pellet lysosomes but not membranes. Phospholipid phosphorus per unit of β-glucuronidase activity was determined in the pellet. Then, given β-glucuronidase with respect to phospholipid phosphorus in the membrane fraction, the percent of phospholipid in isolated membranes resulting from lysosomal contamination was calculated. In three experiments this averaged 7% for control membranes and 11% for phagocytic membranes.

Liposomes from Extracted Lipids. Membrane suspensions that had been used for polarization measurements were extracted overnight with 20 volumes of chloroform/methanol (2:1) under nitrogen at 4° and washed with 0.05 M KCl and Folch upper phase (15). The samples were then evaporated to dryness under nitrogen at room temperature. A small volume of phagocytosis medium was added to the dried samples with several 1-μm glass beads. This mixture was first mixed on a Vortex mixer and then sonicated to produce a mixture of liposomes with low scatter (signal-to-scatter intensities of >50:1).

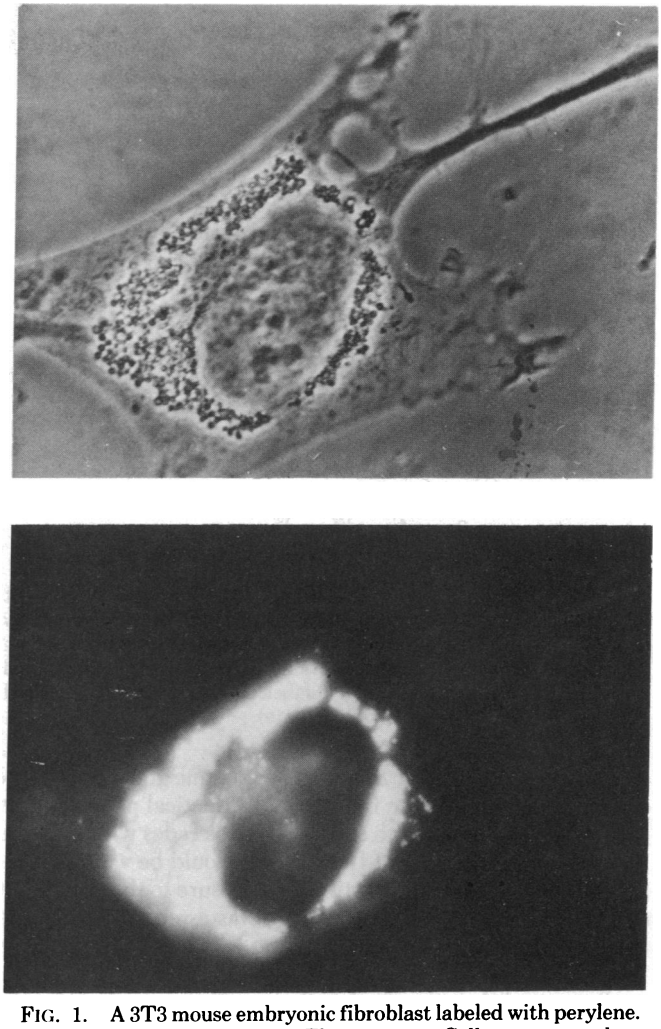

FIG. 1. A 3T3 mouse embryonic fibroblast labeled with perylene. *Upper.* Phase contrast. *Lower.* Fluorescence. Cells grown on glass coverslips were incubated with 0.1 μM perylene in phosphate-buffered saline containing Mg and Ca for 30 min at 37° and rinsed extensively in the same buffer. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and checked periodically for mycoplasmic contamination. Fluorescence was observed through a Zeiss Photomicroscope equipped for epiillumination. A no. 400 dichroic mirror with a Corning glass filter (Pomfret Optical Co.) that transmits well in the 300- to 400-nm region was used for excitation of perylene or DPH. A Zeiss (Schott) no. 44 barrier filter was used for observation of perylene, and a no. 41 filter for DPH. Original magnification ×1250. Stock solutions of the probes were made in dioxane and were diluted rapidly 10^4 × in phosphate-buffered saline immediately before use.

RESULTS

Rationalization for Study of Isolated Membranes. The fluorescence depolarization of DPH or perylene used to label whole cells has been used recently to infer the microviscosity of their plasma membranes (7, 8). However, direct examination of such labeled cells by fluorescence microscopy revealed that the regions of greatest fluorescence intensity were intracellular vesicles or granules and not cytoplasmic membrane. Fig. 1 shows phase contrast and fluorescence micrographs of a 3T3 mouse embryonic fibroblast. The usual perinuclear refractile granules are observed by phase contrast (Fig. 1 *Upper*). An intense fluorescence is observed in the corresponding areas after

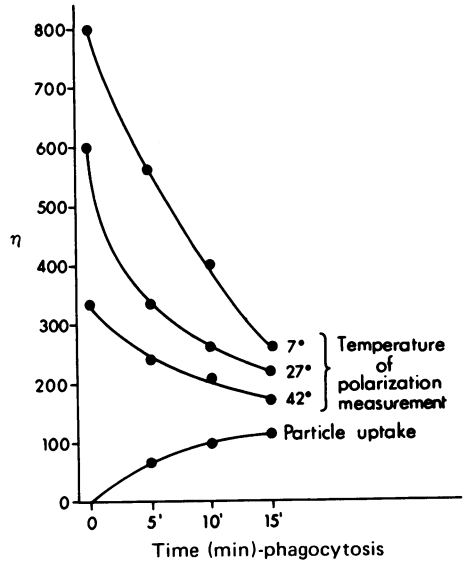

FIG. 2. Microviscosity, η, in centipoise of membranes isolated at progressive times during phagocytosis. The three upper lines show η calculated from depolarization of DPH at different temperatures in a single experiment. Particle uptake (lower curve) was quantified as before (3, 11) from uptake of dye-labeled emulsion and is expressed in relative units (not shown on ordinate).

perylene labeling (Fig. 1 *Lower*). Cytoplasmic granules in PMN showed a similar intense fluorescence. Identical patterns were seen with DPH, although its fluorescence fades with extreme rapidity. The intensity of fluorescence could be varied by altering the concentration or time of exposure to the probe, but the overall pattern of fluorescence was essentially the same under a range of conditions (labeling with 0.1–2 μm perylene or DPH for periods between 5 and 60 min).

Because of the extensive labeling, we first suspected that the probes actually stimulated the formation of intracellular vesicles by pinocytosis. Therefore, fibroblasts were fixed with 5% glutaraldehyde or 2% paraformaldehyde (15 min at 37°), which completely inhibits the internalization of surface-bound lectins that occurs by adsorptive endocytosis in fibroblasts (Fernandez and Berlin, unpublished data). The pattern of fluorescence due to DPH or perylene was not different between prefixed and unfixed cells.

These observations established that information on cytoplasmic membrane cannot be easily derived from the labeling of intact cells by nonpolar lipophilic fluorescent probes. Such probes in whole cells would report a weighted average reflecting their solubility in all membranes (peripheral and intracellular) and in other lipid-containing structures. Presumably the same cautions must be exercised with the use of probes with similar physical properties for electron spin resonance or nuclear magnetic resonance studies. Because we were primarily interested in alterations of the plasmalemma that might accompany phagocytosis, our studies were based entirely on measurements of isolated plasmalemmae labeled *in vitro*.

Differences in Microviscosity of Control Plasma Membranes Reported by Perylene and DPH. The microviscosity calculated from fluorescence depolarization measurements of DPH-labeled membranes isolated from nonphagocytizing cells was 375 ± 24 SEM centipoise. Interestingly, the calculated microviscosities obtained using perylene were consistently lower by 10–35% and were comparable to data previously reported for leukocyte membranes (16).

Because the viscosity-polarization standard curves for the two probes were obtained using the same standard oil, it is obvious that the observed differences in membrane microviscosities must reflect differences between their rotation in the isotropic standard oil and in the anisotropic membrane. Theoretical treatment of fluorescence depolarization by anisotropic molecular rotations (17) has shown that for planar molecules such as perylene, depolarization is brought about by motion that is an average of in-plane and out-of-plane rotations. In-plane rotations dominate. DPH, on the other hand, is not planar but cylindrical, and adsorption and emission dipoles are oriented along the extended axis of the molecule. In this case rotation about the axis will not lead to depolarization. In a biological membrane in which the hydrophobic interior of the membrane may show some degree of order, in-plane rotations of perylene (occurring parallel to paracrystalline fatty-acid chains) may be relatively less affected than the tumbling motion required of DPH. In any case, all of the changes reported here occurred in a qualitatively similar fashion for both perylene and DPH.

Table 1. Microviscosities of plasma membranes at 37° *

No colchicine		Colchicine added	
Control	Phagocytosis	Control	Phagocytosis
375 ± 24 (5)	176 ± 15 (5)	354 ± 15 (4)	345 ± 8 (4)

* Data in centipoise ± SEM. Number of observations in parentheses. Membranes were isolated and then labeled with DPH as described in the *text*. Cells were treated with colchicine during control incubation or during phagocytosis.

Phagocytosis Is Associated with a Decrease in Microviscosity of the Cytoplasmic Membrane. Cytoplasmic membranes were isolated from PMN at various intervals during phagocytosis and the microviscosity was determined. As shown in Fig. 2, the microviscosity decreased progressively with phagocytosis. At 37°, after 15 min of phagocytosis, the decrease in microviscosity was approximately 50% (Table 1). The viscosity change required particle ingestion. Thus, viscosity was not altered by incubation of cells with emulsion in the presence of 5 mM EDTA, which blocked phagocytosis (1). This viscosity change could not be accounted for in terms of a direct interaction of BSA or emulsion with the plasma membrane. Measurements of the contamination of membrane fractions by emulsion using oil Red O as a marker showed that the emulsion could contribute a maximum of 10% to the observed microviscosity change. Further, microviscosity was not changed either by incubation of cells with BSA alone or by incubation of isolated membranes from control or phagocytic cells with emulsion at 37° under the same conditions as intact cells.

Finally, the microviscosity change was not a unique response to one particular particle because phagocytosis of polystyrene beads, which did not require BSA, also led to a decrease in membrane microviscosity (30–50%, four experiments).

Effect of Colchicine on Membrane Microviscosity. As shown previously (1) colchicine at 5 μm had no effect on the rate or extent of phagocytosis of oil emulsion by rabbit PMN. Addition of 5 μM colchicine to membranes that had been isolated from control or phagocytic cells did not affect their microviscosities. However, when cells were allowed to phagocytize in the presence of colchicine, the characteristic decrease in membrane microviscosity did not occur. Fig. 3 shows a representative experiment. A slight decrease in microviscosity was detectable even with colchicine treatment in this experiment and in one of five others. The mean difference between nonphagocytizing PMN and colchicine-treated phagocytizing

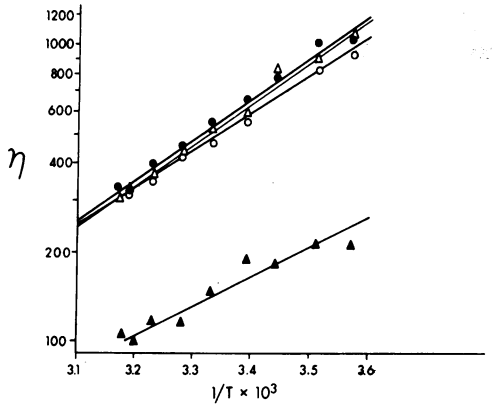

FIG. 3. A semilogarithmic plot of the microviscosity, η, in centipoise against the reciprocal of the absolute temperature. A representative experiment. DPH was the fluorescent probe. Phagocytosis was interrupted after 20 min by centrifugation. Several points should be noted: (*i*) plasma membrane viscosity is markedly decreased after phagocytosis (▲), (*ii*) colchicine in the absence of phagocytosis does not affect microviscosity (△), and (*iii*) the presence of colchicine during phagocytosis abolishes the decrease in viscosity (○). Control (●).

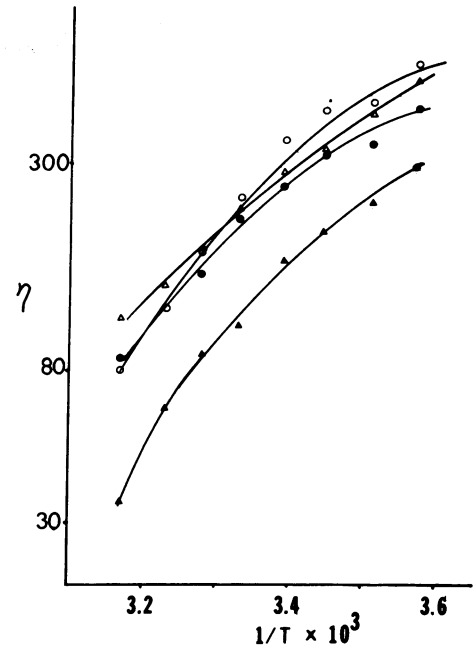

FIG 4. Semilogarithmic plot of microviscosity, η, of liposomes made from extracts of plasma membranes against the reciprocal of the absolute temperature. Data from a single experiment are shown. Scatter from liposomes made from DPH-labeled membranes was measured at 430 nm. This wavelength did not excite DPH but could be used to excite perylene, which was introduced subsequently for polarization determinations. (●) Control; (△) control with colchicine; (▲) phagocytosis; (○) phagocytosis with colchicine.

PMN was not significant ($P > 0.5$). Table 1 is a summary of the data. The decrease in microviscosity associated with phagocytosis of polystyrene beads was also abolished by colchicine (two experiments).

The specificity of the colchicine effect was examined with the photoderivative lumicolchicine. Lumicolchicine does not affect microtubules although it affects certain other membrane functions that are sensitive to colchicine, the nucleoside transport system, for example (18, 19). Incubation of isolated membranes or phagocytizing cells prior to membrane isolation with 10 μM lumicolchicine had no effect on membrane microviscosity.

Microviscosities Determined in Extracted Lipids from Control and Phagocytic Membranes. Liposomes were made from isolated lipids of control and phagocytic membranes. Liposomes from phagocytic membranes showed a decrease in microviscosity with phagocytosis qualitatively similar to that observed with intact membranes (Fig. 4). Lower absolute values were observed. The decrease in microviscosity was not detectable when the probe was introduced into extracted lipids from cells that phagocytized in the presence of colchicine.

DISCUSSION

We have previously shown that phagocytosis induces alterations in the density of lectin receptors on the plasmalemma of PMN (1) while appearing to spare membrane transport functions (2). Because the distribution of concanavalin A receptors over the surface before phagocytosis is inherently uniform, it is suggested that there is selective inclusion of receptors into the endocytic vesicle. By analogy, the changes in microviscosity reported here may reflect a similar selective internalization of specific lipids. Changes in content of fatty acid, cholesterol, the phospholipid head groups, or glycolipids could all produce the observed changes in microviscosity. Initial analyses of membrane phospholipids by two-dimensional thin-layer chromatography indicate errichment of phosphatidylserine and depletion of phosphatidylethanolamine in the plasmalemma after phagocytosis.

Other workers have suggested an enzymatic remodeling of membrane lipids during phagocytosis. For example, Smolen and Shohet (20) and Mason *et al.* (21) showed that the proportion of saturated fatty acids was increased in membranes of phagocytic vesicles as compared to plasma membranes of control *nonphagocytizing* cells. Possible explanations included an increased mobilization of saturated fatty acids from neutral fat (22) and destruction of unsaturated fatty acids by epoxidation and peroxidation secondary to the generation of superoxide and hydrogen peroxide (23). However, these processes would act to *increase* microviscosity, while our data clearly establish that the microviscosity of plasma membrane is *decreased* after phagocytosis.

All of these membrane alterations resulting from phagocytosis (decrease in microviscosity, depletion of lectin receptors, and preservation of membrane transport) are colchicine-sensitive. These changes could be independent. However, we propose a working hypothesis that accommodates all of the observations in a single model. In our view microtubules induce a cooperative process in which key membrane proteins, perhaps membrane-bound tubulin itself (24), are first engaged and concentrated by the microtubules. These protein(s) may be associated with specific lipids which in turn favor the association of other specific proteins, and so on. In this way, phagocytosis may induce a propagated microtubule-dependent regional phase change. A region of more condensed, or less fluid, lipid corresponds to the membrane region that envelops the phagocytized particle. The region is internalized and removed from the cytoplasmic membrane, leaving a residual cytoplasmic membrane of low microviscosity. Partitioning of lectin receptors into the condensed region and transport proteins into the low-viscosity residual plasmalemma could be specified by their affinity for regional lipids.

The concept of lateral phase separation has been developed in studies of model systems. For example, Shimshick and McConnell (25) used a spin label probe to show that at certain

temperatures binary mixtures of phosphatides coexist as solid and liquid phases. There is also evidence that membrane enzymes are activated by specific lipids and are probably surrounded by a boundary of relatively immobilized lipid. Vanderkooi *et al.* have shown this for cytochrome oxidase (26) and Träuble and Overath have indicated that roughly 20% of the membrane phospholipid in bacteria may be bound in this fashion (27). Kleeman and McConnell (28) have shown that at critical temperatures at which liquid and solid regions of the membrane coexist proteins separate into the liquid regions, as we suggest for transport proteins during phagocytosis. At the same time, one can envision that other proteins such as lectin receptors could associate specifically with the solid phase (for example, those with saturated fatty acids in their immobilized lipid boundary).

It is interesting that recent work by Papahadjopoulos, Poste, and their colleagues (29, 30) and by Huang and Pagano (31) have emphasized the potential importance of lateral phase separations for the membrane fusion of phospholipid vesicles with plasmalemma. The development of a lateral phase separation during phagocytosis might similarly promote the excision of a condensed phase into a phagocytic vesicle and the simultaneous resealing by fusion of the residual plasma membrane. Calcium appears to induce a phase separation in phosphatidylserine-containing vesicles (29). However, neither 10 mM calcium nor EDTA affected the microviscosities of isolated membranes from control or phagocytic PMN.

According to our model, phagocytosis appears to lead to the actual physical separation of less fluid regions from the cytoplasmic membrane. However, lateral phase separations may occur under conditions where membrane is not actually separated. For example, binding of concanavalin A to receptors on PMN could induce a small regional condensation of membrane lipids that may be essential to the induction of a variety of cytoplasmic events, including microtubule assembly (32, 33). There is evidence from spin-label studies that mitogenic lectins induce a rapid increase in membrane fluidity (34), suggesting solution of the spin label in a noncondensed phase. Further, Romeo *et al.* (35) have shown that the contact of intact PMN with polystyrene beads brings about an immediate increase in fluorescence intensity of 1-anilino-8-naphthalene sulfonate. These rapid changes could correspond to initial steps in the development of large regional phase separations.

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