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# ACTIVATION AND RELEASE OF LYSOSOMAL ENZYMES FROM ISOLATED LEUKOCYTIC GRANULES BY LIPOSOMES. A PROPOSED MODEL FOR DEGRANULATION IN POLYMORPHONUCLEAR LEUKOCYTES¶

Intracellular redistribution of lysosomal enzymes is accomplished by fusion of the lysosomal membrane with the membrane of the digestive vacuole.[1] Phagocytosis by polymorphonuclear (PMN) leukocytes offers a striking example of such membrane fusion leading to disruption of leukocytic granules followed by activation and discharge of lysosomal enzymes into the phagocytic vacuole, a process called degranulation.[2-6] Leukocytic granules do not seem to fuse with each other, nor do the granules rupture into the cytoplasm after engulfment of particulate material.[7-9] Since granules fuse only with the membrane of the phagocytic vacuole, it appears that the primary event leading to fusion occurs in that portion of the cell membrane surrounding the ingestum.

It is not clear which constituents (phospholipid and/or protein) of the cell membrane change during phagocytosis and participate in membrane fusion. In order to study the mechanisms involved in this interaction between lysosomal and vacuolar membranes, a cell-free system was established using isolated PMN granules and substituting liposomal model membranes for the vacuolar membrane. The use of liposomes was based on the observation of Bangham, *et al.*[10-12] that phosphatidyl choline dispersed in water formed bimolecular leaflets similar to biological membranes in their permeability to simple cations. The liposomes used in the present experiments were composed of phospholipids known to occur in rabbit PMN leukocytes.[13] Of this group of membrane-forming phospholipids those which possess negative charge were found to interact with leukocytic granules, producing disintegration of the granule structure as well as activation and release of lysosomal marker enzymes. It seems that the mode

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of action of liposomes differs from that of lysophosphatidyl choline, another agent known to disrupt granules.[14] A preliminary report of some of these experiments has appeared elsewhere.[15]

## MATERIALS AND METHODS

*PMN leukocytic granules.* Rabbit leukocytes were obtained as previously described[16,17] using acid-citrate-dextrose (ACD) solution for anticoagulation. Differential counts of the harvested cells revealed that more than 95 percent were heterophile leukocytes. Cells were disrupted by sonication (15 seconds, Branson Sonifier Model S110 equipped with a microtip, 5½ inches × ⅛ inch and operated at 3 amperes). Granules were then separated by differential centrifugation in 0.34M sucrose. A uniform suspension of granules was routinely obtained by gently resuspending of the 8200 × *g* pellet in 0.34M sucrose. All samples were maintained at 0-4° C. and isolated granules were used within 1-2 hours.

*Preparation of liposomes from phospholipids.* The phosphatidyl choline type of liposomes composed of chromatographically pure egg phosphatidyl choline (General Biochemicals), dicetyl phosphate (Nutritional Biochemical Corp.) and cholesterol (Sigma Chemical Co.) was prepared as described elsewhere.[18] Other phospholipids used to prepare liposomes were: phosphatidyl inositol (choline free) containing 7 percent phosphorus with a phosphorus:inositol ratio of 2:1 (Nutritional Biochemical Corp.); chromatographically pure phosphatidyl ethanolamine, and synthetic phosphatidyl choline as 1-alpha lecithin (dipalmitoyl) from General Biochemicals. Phospholipids were stored in chloroform at −20° C. Before use chloroform was evaporated under vacuum and then the phospholipid films were allowed to swell in water at room temperature for one hour. Suspensions were homogenized by sonication for 90 seconds in the Branson Sonifier.

*Experimental system.* A granule suspension of 0.2 ml. containing 0.5 mg. protein obtained from approximately 7 × $10^7$ cells was mixed with 0.1 ml. of a phospholipid suspension containing 0.5mM phosphorus, unless otherwise specified. The system was buffered to pH 5.0 either with 0.05M acetate buffer or 0.05M citrate-HCl buffer. The final volume of the incubation mixture was 0.5 ml. and the concentration of sucrose 0.25M. After incubation at 37° C. for 20 minutes enzyme substrate (diluted in buffered 0.25M sucrose) was added and the mixture incubated at 37° C. for 30 minutes. The reaction was then stopped by addition of either 0.2M glycine buffer pH 10.4 or 0.2M borate-KCl buffer pH 9.8. Insoluble matter was spun down at 20,000 × *g* for 20 minutes and light absorbence of the developed color in the clear supernatant was read in a Zeiss spectrophotometer. This procedure measured the free activity of both bound and soluble enzymes. When activity of soluble enzyme alone was measured, the incubation mixture was spun down at 20,000 × *g* for 20 minutes before addition of substrate, and then the supernatant was tested for enzyme activity.

*Enzyme assays.* The activity of beta glucuronidase was measured by the method of Gianetto and de Duve[19] with phenolphthalein glucuronide (Sigma Chemical Co.) as the substrate. Beta N-acetylglucosaminidase was assayed by the method of Woolen, *et al.*[20] with p-nitrophenyl beta N-acetyl-glucosaminide (Sigma Chemical Co.). Enzymatic assays were run with blanks containing all ingredients with the exception of substrates. Light absorptions of substrates themselves were very low and did not contribute substantially to the readings. Both enzymatic reactions proceeded linearly over the incubation period and proportionally with the concentration of lysed granules used

as the source of enzymes. The results were expressed as the percent of total activity found in an aliquot of granules treated with 0.1 percent Triton X-100 for 20 minutes at 0° C. Total enzyme activities of the granule preparations measured by the amount of hydrolyzed substrates were: 1.5 μM phenolphthalein/mg. of granule protein/1 minute for beta glucuronidase, and 0.05 μM p-nitrophenyl/1 mg. of granule protein/1 minute for beta N-acetylglucosaminidase.

*Other methods.* The protein content of the trichloroacetic acid insoluble fraction of the granule preparation was determined by the method of Lowry, *et al.*[21] with crystalline bovine albumin (Armour Pharmaceutical Co.) as a standard. Phosphorus in the phospholipid preparations was determined by the method of Berenblum and Chain.[22]

*Preparation of material for electron microscopy.* Following incubation of granules and liposomes at 37° C. for 20 minutes and centrifugation at 20,000 × *g* for 20 minutes at 0° C. the resulting thin pellets were submerged in cold 1 percent glutaraldehyde in modified Tyrode's solution at pH 7.3[23] for 1½ hours, rinsed in buffer, and secondarily fixed in Millonig's phosphate-buffered 1 percent osmium tetroxide for 30 minutes. Specimens were then stained *en bloc* in an aqueous solution of 1 percent uranyl acetate for 18 hours prior to routine dehydration and embedment in American Araldite. Unsupported thin sections were stained in uranyl acetate and lead citrate and examined in a Philips 200 electron microscope.

Formation of liposomes by swollen phospholipids was also examined by negative staining. One drop of swollen phospholipid suspension was mixed with one drop of aqueous 2 percent potassium phosphotungstate[10] on a carbon-coated formar-covered grid. After being drained and dried, the mixture was examined by electron microscopy.

## RESULTS

### *Activation of lysosomal enzymes and disintegration of granules by phospholipids forming liposomes.*

Three phospholipids known to occur in rabbit PMN leukocytes, namely, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol[18] formed liposomes. The latter two were able to activate lysosomal marker enzymes and to cause structural disintegration of granules. As shown in Table 1, liposomes composed of egg phosphatidyl choline with and without cholesterol were inactive. However, incorporation of dicetyl phosphate, a long chain polyanion, allowed this type of liposome to activate lysosomal enzymes and to disrupt the granules. In contrast, synthetic phosphatidyl choline did not form liposomes under the conditions studied (room temperature) and had no effect on leukocytic granules. Dicetyl phosphate added to synthetic phosphatidyl choline produced no effect. Electron micrographs of granules and granules incubated with three types of liposomes are shown in Figures 5-8. Inserts in Figures 6, 7, and 8 show the appearance of negatively stained liposomes.

The activation of lysosomal enzymes in leukocytic granules by liposomes was dependent on the concentration of liposomes as measured by their phosphorus content. Figure 1 illustrates the activities of beta glucuronidase

TABLE 1. FORMATION OF LIPOSOMES, ACTIVATION OF LYSOSOMAL ENZYMES AND DISINTEGRATION OF GRANULES BY PHOSPHOLIPIDS

| *Phospholipids* | *Liposomes formation observed* | *Activation of lysosomal enzymes** | *Disintegration of granules observed* |
|---|---|---|---|
| Phosphatidyl ethanolamine (chromatographically pure) | + | + | + |
| Phosphatidyl inositol | + | + | + |
| Phosphatidyl choline (egg, chromatographically pure) | + | — | — |
| Phosphatidyl choline (egg) and cholesterol | + | — | — |
| Phosphatidyl choline (egg) with cholesterol & dicetyl phosphate | + | + | + |
| Phosphatidyl choline, dipalmitoyl (synthetic) | — | — | — |
| Phosphatidyl choline, dipalmitoyl (synthetic) & dicetyl phosphate | — | — | — |

+ yes
— no
* Increase of free activity of beta glucuronidase and beta N-acetyl glucosaminidase to at least 40 percent of total enzyme activity. Enzyme activity of granules incubated alone was 10 and 11 percent respectively.

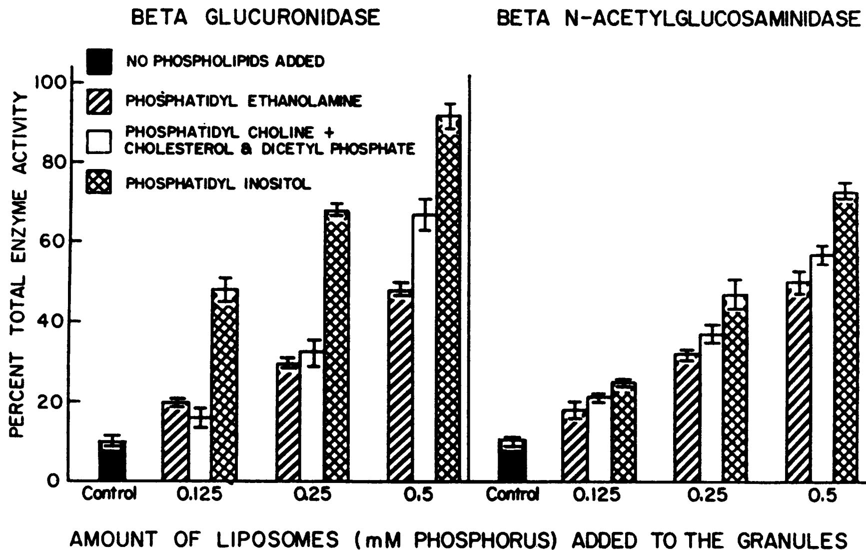



FIG. 1. The effect of varying concentrations of liposomes on activation of lysosomal enzymes in leukocytic granules at pH 5.0. Each bar represents the mean of three experiments performed in duplicate. Brackets shown are standard deviations.

and beta N-acetylglucosaminidase obtained with increasing concentrations of three types of liposomes. As noted, the phosphatidyl choline type of liposomes containing cholesterol and dicetyl phosphate, and liposomes formed by phosphatidyl ethanolamine produced almost equal activation of both marker enzymes. The highest activities of lysosomal marker enzymes were obtained in the presence of phosphatidyl inositol which in all concentrations activated more beta glucuronidase than beta N-acetylglucosamini dase.

Since liposomes added to granules activated lysosomal enzymes, an experiment was performed to ascertain whether liposomes directly influence the activity of soluble lysosomal enzymes. Enzymes were prepared in active, soluble form by treatment of granules with 0.25M glycine-HCl buffer at pH 3.0, and insoluble matter was removed by centrifugation at 90,000 $\times$ *g* for 90 minutes. When liposomes were added to such a preparation of lysosomal enzymes, the activities of beta glucuronidase and beta N-acetylglucosaminidase assayed at pH 5.0 remained unchanged. Thus, the activation of lysosomal enzymes by liposomes occurred only through the interaction of liposomes with intact granules.

*Effect of pH on interaction of liposomes with granules*

Because pH in PMN leukocytes decreases during phagocytosis[24] it has been suggested that this fall in pH may contribute to the lysis of granules.[7] Therefore experiments were undertaken to examine the effect of pH on the interaction of granules and liposomes. Granule-liposome suspensions were incubated at different pH values. Buffers employed were of very low ionic strength (I = 0.008) in order to eliminate the known effect of ionic environment on lysosomal membrane.[19] After centrifugation of the incubation mixture, the supernatants were assayed for soluble enzyme activity after adjustment of pH to 5.0, and the pellets were examined by electron microscopy. In the absence of liposomes, pellets obtained after incubation of granules at pH values from 7.0 to 3.5 consisted of intact granules similar to the control preparation shown in Figure 5. In contrast, morphological analysis of pellets obtained after incubation of granules with liposomes at pH 7.0 as well as pH 5.0 (Figs. 8 and 9) revealed that granules were disintegrated. However, as shown in Figure 2, the activity of soluble beta glucuronidase released by the liposomes composed of phosphatidyl choline, cholesterol, and dicetyl phosphate was lowest at pH 7.0, rose twofold at pH 5.0 and reached maximal values at pH 3.5. The release of soluble enzyme by phosphatidyl inositol was greater, but exhibited a similar pattern: an increase of soluble enzyme as pH decreased. In control experiments, granules incubated alone released a small amount of active enzyme under

the conditions studied until the pH was lowered to 3.5 when the activity of soluble beta glucuronidase reached 45 percent of total actvity. It seems apparent on the basis of these combined results that the interaction of liposomes with granules took place over a wide pH range, but solubilization (release) of active enzyme was pH dependent and proceeded when pH dropped to 5.0 and below.

Lysophosphatidyl choline (Nutritional Biochemical Corp.) in concentration of 0.15 mM phosphorus was also tested. As previously observed, this monoacyl phospholipid did not form liposomes but its membrane-lytic activity caused disruption of bimolecular leaflets of phospholipids[10] as well as disintegration of granules and activation of lysosomal enzymes.[14] In contrast to the results obtained with liposomes, the activity of soluble enzyme released by lysophosphatidyl choline was essentially similar from pH 7.0 to 3.5 (Fig. 2).

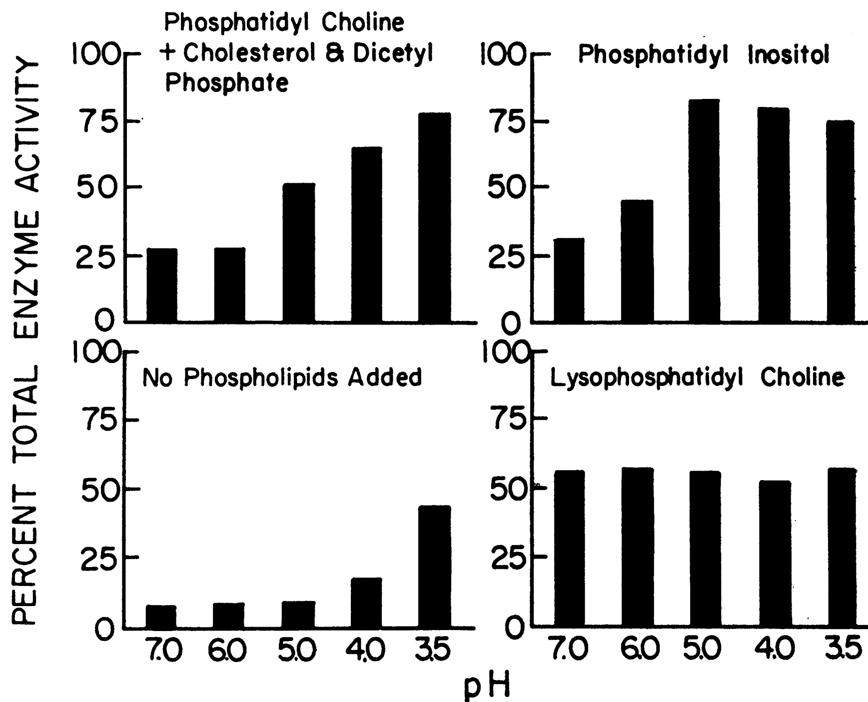



FIG. 2. The release of soluble beta glucuronidase from leukocytic granules by liposomes and lysophosphatidyl choline at varying pH. The following buffers (final I = 0.008) were used: tris-maleate, pH 7.0; tris-maleate, pH 6.0; acetate, pH 5.0; acetate, pH 4.0; glycine-HCl, pH 3.5.

### *Granule-liposome interaction and inhibitors of lipolysis*

To elucidate whether the interaction between granules and liposomes was mediated by phospholipid breakdown products (lysocompounds) which could be formed from the added liposomes, the effect of the following substances was studied: (a) sodium fluoride, an inhibitor that blocks lipolytic enzymes in PMN leukocytes[25] and in consequence prevents formation of phospholipid breakdown products (lysocompounds); and (b) albumin, which binds lysophosphatidyl choline and abolishes its lytic effect on granule membrane.[14,26] As seen in Figure 3, sodium fluoride added in final concentrations of 0.1M to a system containing granules and liposomes did not alter the activation of beta glucuronidase. This finding suggests that in the system described, leukocytic phospholipase is not responsible for the observed phenomen. Crystalline rabbit albumin (Nutritional Biochemical Corp.) added to the liposomes in a final concentration of 5 mg/ml. did not significantly inhibit activation of lysosomal beta glucuronidase by the phosphatidyl choline or the phosphatidyl inositol type of liposomes. However, in control experiments the same concentration of albumin completely abolished the activation of beta glucuronidase by lysophosphatidyl choline and intact granules were seen by electron microscopy (Fig. 11). Lysophosphatidyl choline alone produced profound disintegration of granules with almost total loss of membrane structure (Fig. 12).

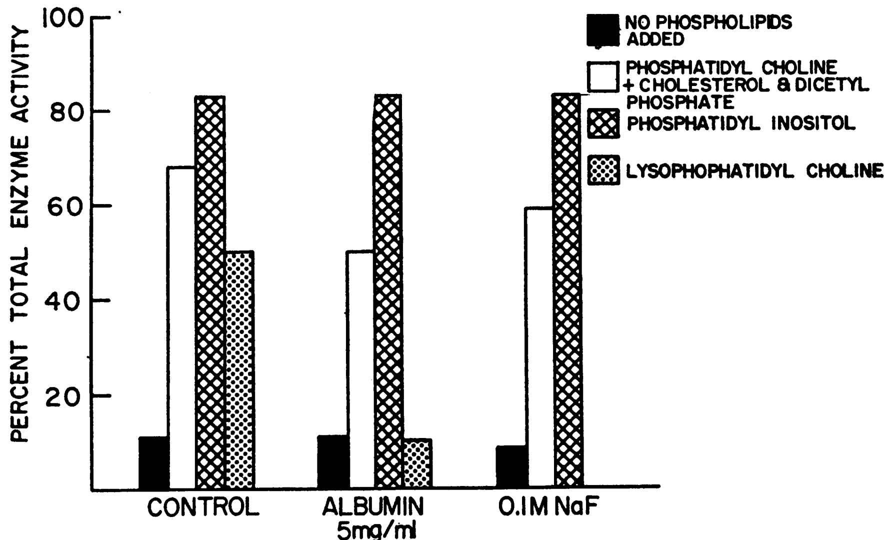



FIG. 3. The activation of beta glucuronidase in leukocytic granules by liposomes and lysophosphatidyl choline at pH 5.0 in the presence of sodium fluoride and albumin.

*Role of basic protein in the granule-liposome interaction*

Since negatively charged phospholipids possess the ability to form complexes with cationic proteins,[27] experiments were designed to determine whether this property of phospholipids plays a role in their interaction with leukocytic granules. Liposomes were preincubated with a polycationic protein, protamine sulfate (grade 1, Sigma Chemical Co.) for 10 minutes and granules were then added. The liposome-protamine complex did not activate beta glucuronidase or beta N-acetylglucosaminidase (Fig. 4) or disrupt the granules as seen by electron microscopy (Fig. 10). In contrast, lysophosphatidyl choline preincubated with protamine retained its activating effect (Fig. 4). Control experiments showed that protamine sulfate complexed with liposomes did not prevent activation of lysosomal enzymes by Triton X-100. In addition, protamine sulfate did not inhibit active lysosomal enzymes solubilized by other treatment (acid lysis of granules at pH 3.0).

## DISCUSSION

The cell-free system used in the present study was designed to simulate the functional complex involved in the degranulation of PMN leukocytes during phagocytosis. The experimental model included the following elements: (a) the isolated granules which represent those discharging their

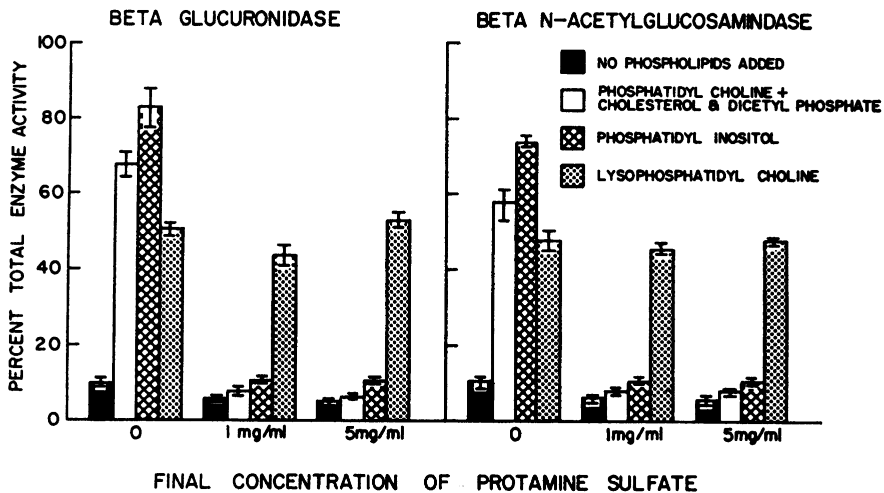



FIG. 4. The effect of protamine sulfate on activation of lysosomal enzymes in leukocytic granules by liposomes and lysophosphatidyl choline at pH 5.0. Each bar represents the mean of three experiments performed in duplicate. Brackets shown are standard deviations.

bactericidal and enzymatic contents into the phagocytic vacuole;[4,5,7–9,16] (b) the liposomal model membranes composed of swollen phospholipids which may represent the lipid portion of the vacuolar membrane. All three types of phospholipids used for preparation of liposomes—phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol—are present in rabbit PMN leukocytes.[13] The latter phospholipid has been found in the leukocytic cell membrane,[28] and it is known that the vacuolar membrane is at least partially derived from the surface cell membrane.[8,9] Furthermore, phospholipid turnover is markedly increased during phagocytosis;[29] (c) the acid pH used in the experimental system was purposely chosen because phagocytosis is accompanied by a fall in intracellular or intravacuolar pH to at least 5.0[24,30,31] Such pH provided by very low ionic strength buffers during a relatively short incubation period did not affect the stability of control granule preparations as measured by activity of lysosomal marker enzymes and the appearance of granules by electron microscopy.

The study reported here demonstrates that some phospholipids in the form of liposomal model membrane possess high affinity for the membrane of PMN granules. This interaction leads to disintegration of the granule structure and to activation and release of lysosomal enzymes.

There are at least two characteristics of the phospholipids that correlate with their interaction with granule membrane: (a) the ability to form liposomes, and (b) a negative charge. With the exception of lysophosphatidyl choline, all phospholipids that caused activation of lysosomal enzymes and disintegration of granules also formed liposomes. Lysophosphatidyl choline, as monoacyl phospholipid, does not form liposomes but wedge-shaped molecules of this compound penetrate bimolecular membranes causing their instability and releasing segments of phospholipids which are seen as discrete discs.[10] Diacyl phospholipids such as egg phosphatidyl choline when added to water form liposomes that behave as bimolecular membranes. However, *synthetic* phosphatidyl choline failed to form liposomes under the conditions studied and failed to activate lysosomal enzymes. A comparison of this synthetic compound with egg phosphatidyl choline revealed that the former contains only saturated fatty acids (dipalmitoyl residue) and the latter has at least one unsaturated fatty acid residue.[32] It is known that the size and shape of phosphatidyl choline micelles depend on the nature of their fatty acid residues.[33] Thus, the degree of saturation of the fatty acids of phosphatidyl choline affects its ability not only to form liposomes but to interact with the granule membrane.

The role of negative charge in the liposome-granule interaction is apparent from a comparison of egg phosphatidyl choline which has a neutral charge and phosphatidyl inositol which has a negative charge.[34] Although

phosphatidyl choline formed liposomes, it did not activate lysosomal enzymes or disrupt granules; phosphatidyl inositol did. The addition of a long chain polyanion such as dicetyl phosphate to phosphatidyl choline changes its net charge to negative[11] and the resulting liposomes were able to activate lysosomal enzymes and induce disintegration of granules.

The activation of lysosomal enzymes in granules by liposomes was not always followed by their subsequent release in soluble form. The present studies showed that after the membrane breaks, some portion of the active lysosomal enzymes remain bound or absorbed to membrane fragments and are sedimentable at 20,000 × $g$. Disruption of granules by liposomes was followed by weak solubilization of enzyme at pH 7.0 but the amount of soluble enzyme increased when pH decreased to 5.0 or below. Thus, acidic pH is particularly important in solubilization (release) of active enzyme in this system. It is of interest that the pH in the phagocytic vacuole may reach 3.0.[31] Solubilization of beta glucuronidase by lysophosphatidyl choline represented a different pattern and was not dependent on a decrease of pH because the amount of soluble enzyme released by this compound at pH 7.0 was as great as at pH 3.5. This fact indicates that changes in the lysosomal membrane induced by lysophosphatidyl choline may be different from those produced by liposomes.

It is noteworthy that pellets prepared from granules incubated alone at acidic pH and very low ionic strength consisted of intact granules, and very little debris was present to indicate that any granule disruption had occurred. In previous experiments performed under different conditions, clearing of granule suspensions observed at acid pH was interpreted only as indicating granule lysis.[16] Since the present findings show that half of the lysosomal marker enzymes were in the supernatant after incubation at pH 3.5, it is not clear whether substrate became more accessible to enzyme under such conditions, whether enzymes were released from undisrupted granules, or finally, whether the granules remaining intact after incubation represented a fraction that is more resistant to acid.

Two possible explanations for the mechanism by which liposomes activate lysosomal enzymes and produce disintegration of leukocytic granules are: (a) phospholipid breakdown products such as lysosphosphatidyl choline, formed by the action of lipolytic enzymes, e.g., phospholipase on liposomes, might penetrate the lysosomal membrane causing its disruption; or (b) steric changes in the lysosomal membrane may be induced by direct interaction between liposomes and granules. The following evidence suggests that PMN leukocytic phospholipase is not responsible for the observed phenomenon. In the present experiments when the substrates for this enzyme, e.g., egg phosphatidyl choline and synthetic dipalmitoyl phos-

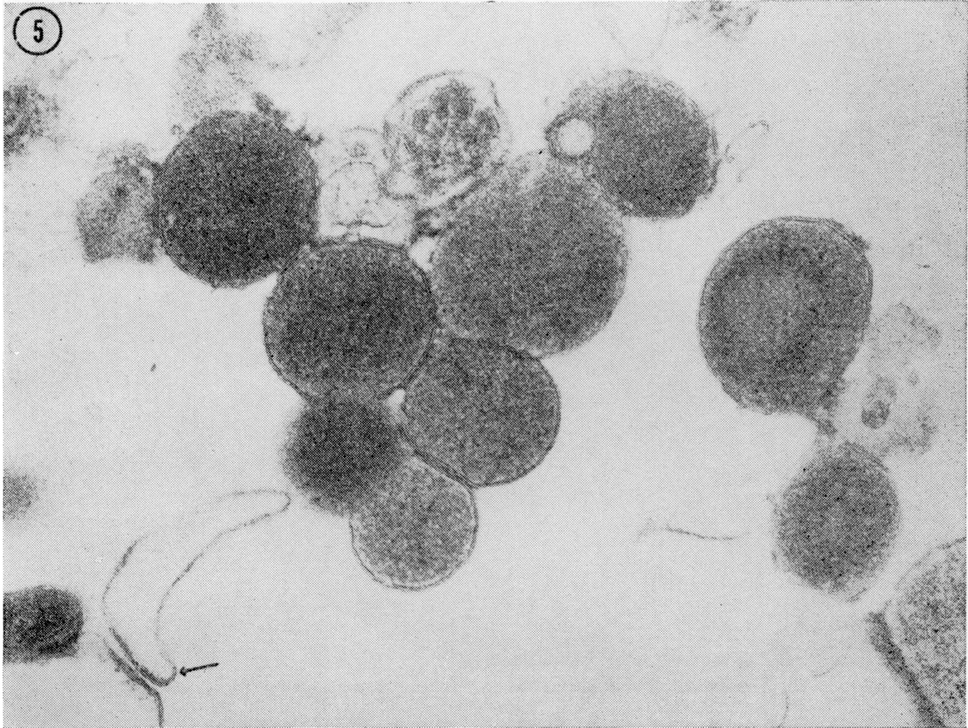



FIG. 5. Control preparation of granules, incubated at pH 5. Many typical rabbit PMN leukocyte granules, with intact membrane, may be identified. Granules were found singly and in small clusters. Mitochondria were the only other organelle recognized in control preparations. Fragments of membranes were present (arrow), but no structures resembling liposomes were noted. × *75,000*

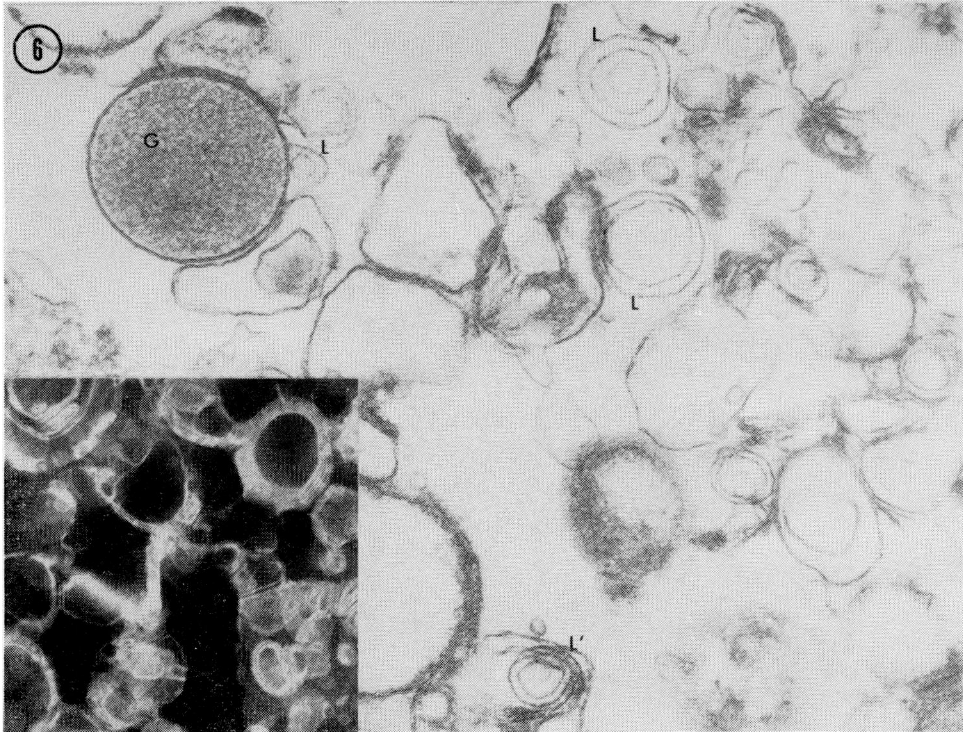



FIG. 6. PMN leukocyte granules incubated with liposomes composed of phosphatidyl choline, cholesterol, and dicetyl phosphate at pH 5. Rare intact granules (G) were seen. Many concentrically lamellated structures (L), presumably liposomes formed from the added phospholipid, were present. Although trilaminar unit membrane was obvious around the granules, the lamellae of the liposomes often appeared single rather than trilaminar. It is not clear whether segments of trilaminar unit membrane in some liposomes ($L^1$) are derived from the added phospholipid or pre-existing cell membranes. × *83,000*

FIG. 6. (*Insert*) Negative stain of liposomes formed by phosphatidyl choline, cholesterol and dicetyl phosphate. Liposomes are seen as spheroidal, multilamellar structures, which presumably correspond to the concentrically lamellated spherules observed in sectioned material (L in Fig. 6). × *83,000*

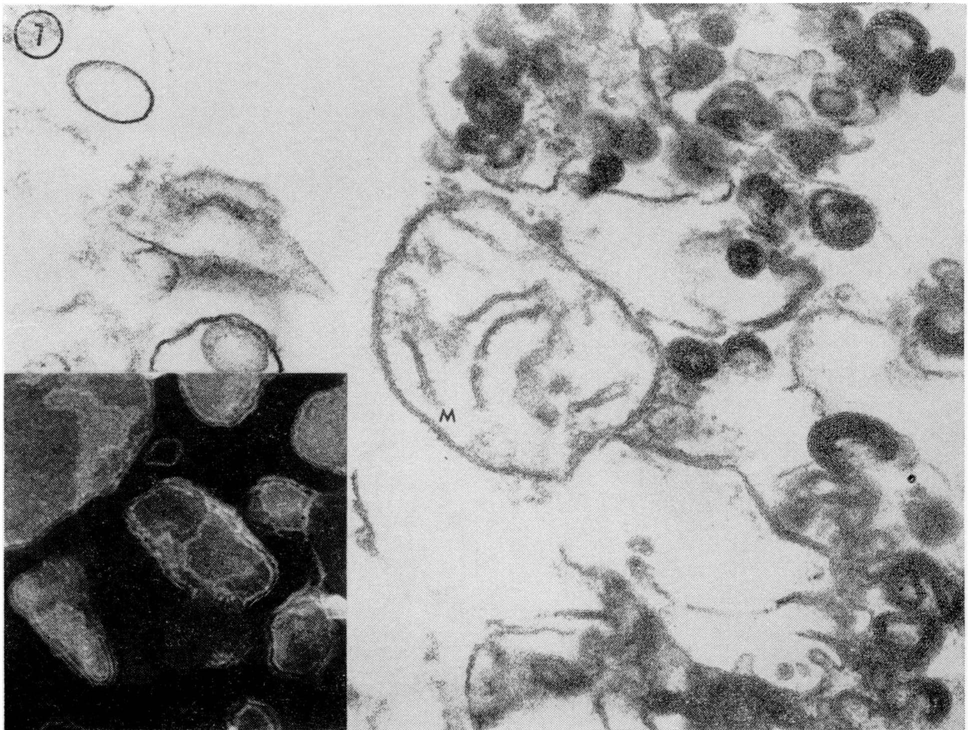



FIG. 7. Pellet obtained after incubation of granules with phosphatidyl ethanolamine at pH 5.0. Membrane fragments and mitochondria (M) were seen in these preparations as well as occasional intact granules. Numerous liposomes were identified and were composed of concentric, closely packed and evenly spaced lamellae, which formed a thumbprint configuration. × *71,000*

FIG. 7. (Insert) Negative stain of phosphatidyl ethanolamine. Two or three of the outer lamellae of each liposome are evident. The more central lamellae of the liposomes were not visualized, presumably due to failure of penetration of the negative stain. This explanation accounts for the difference in the number of lamellae seen in these liposomes and those examined in sectioned material. × *106,000*

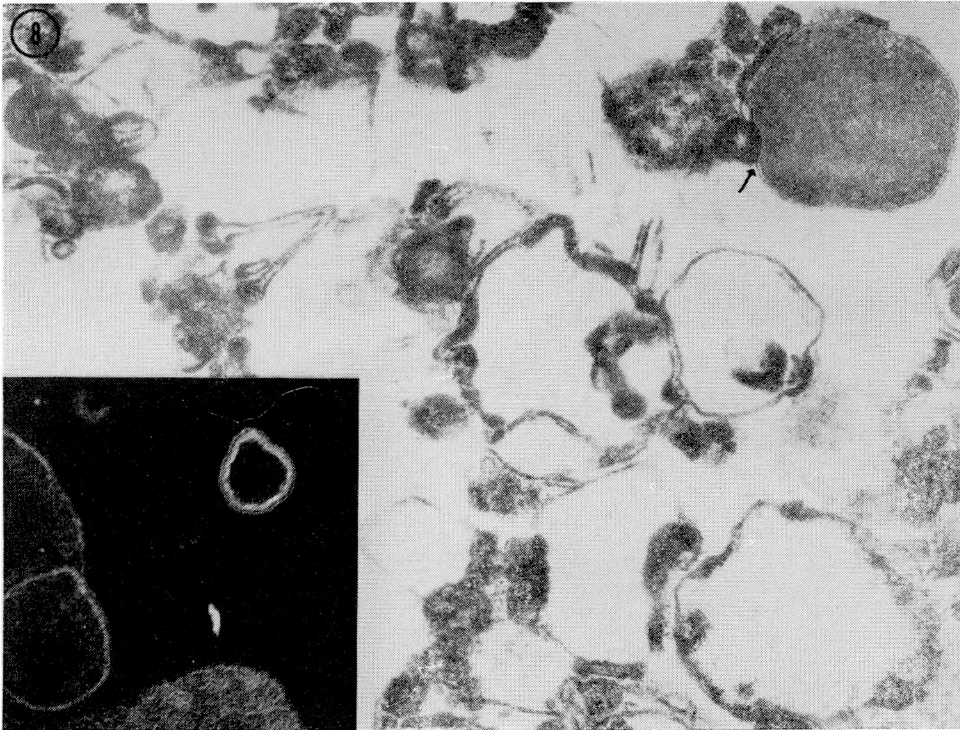
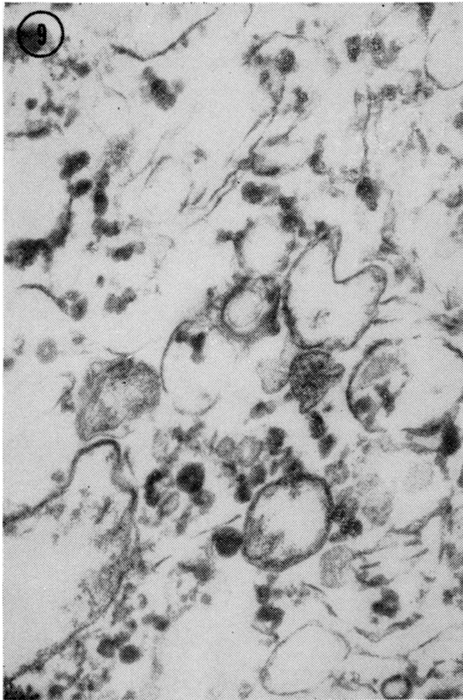


FIG. 8. Pellet obtained after incubation of granules with phosphatidyl inositol at pH 5.0. The pellet consisted of liposomes, membrane fragments, mitochondria, and rare granules. Liposomes of phosphatidyl inositol were seen as small, concentric, multilamellar structures which sometimes were observed to abut on the membrane of the rare remaining granules (arrow) and other membrane fragments. × *58,000*

FIG. 8. (*Insert*) Negative stain of phosphatidyl inositol. Outer lamellae of the liposomes are evident. The failure to demonstrate the central lamellae is presumably due to non-penetrance of the negative stain. × *113,000*



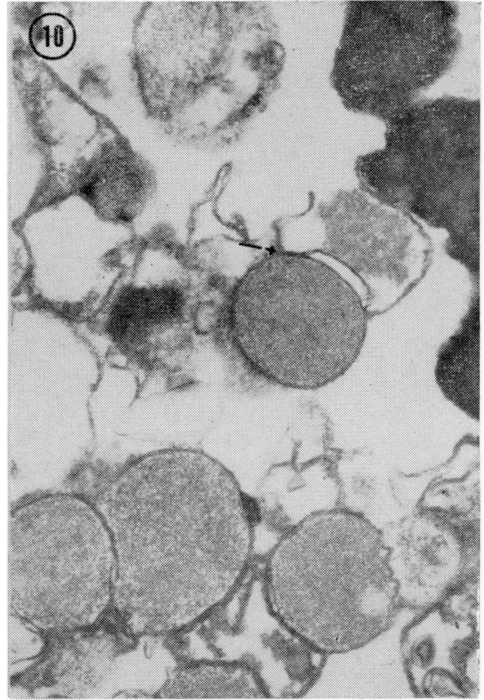
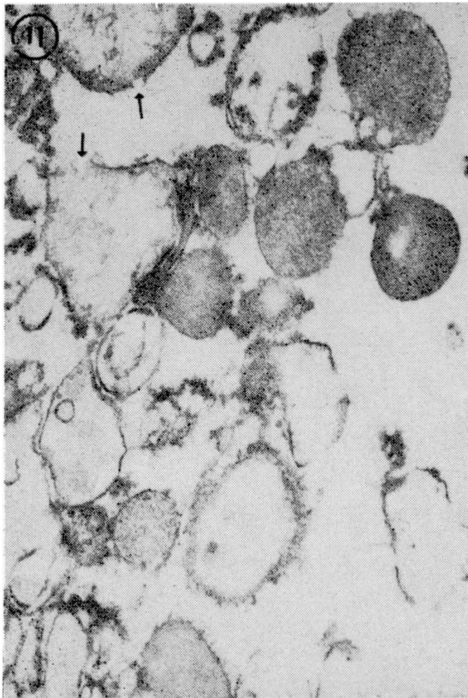




Fig. 9. Pellet obtained after incubation of granules with phosphatidyl inositol at pH 7.0. Very few granules remained intact. The appearance of this pellet did not differ substantially from the pellet formed after incubation of granules and phosphatidyl inositol at pH 5.0 (Fig. 8), although much more enzyme activity was sedimentable with this pH 7.0 preparation (Fig. 2). × *50,000*

Fig. 10. Pellet obtained after incubation of granules with a mixture of phosphatidyl inositol and protamine sulfate. Many intact granules were noted. The large crystalloid aggregates (upper right) seen in this preparation were also noted when only protamine sulfate and phosphatidyl inositol were mixed, and apparently result from complexing of protamine sulfate and phospholipid. Discrete, spheroidal phosphatidyl inositol liposomes such as in Figure 8 were not seen. In some sites extreme proximity of two segments of unit membrane produces a Y-like configuration (arrow). Similar apposition of membranes was noted in all preparations in which granules were incubated with protamine sulfate (with and without added phospholipid), and was not present in control preparations (compare with Fig. 5). × *53,000*



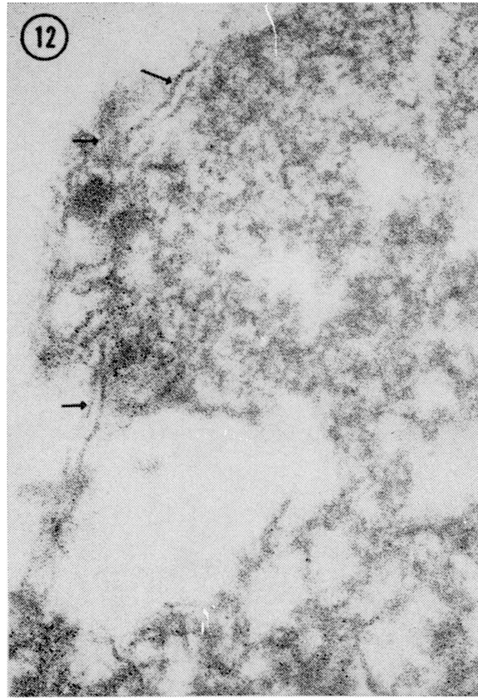


Fig. 11. Pellet obtained after incubation of granules with a mixture of lysophosphatidyl choline and albumin. Many granules were present, although there seemed to be more membrane discontinuity (arrows) than in control preparations. The relatively normal structure of granules and membrane in this preparation is in marked contrast to the striking membrane lysis produced when lysophosphatidyl choline is incubated with granules alone (Fig. 12). × *49,000*

Fig. 12. Pellet obtained after incubation of granules with lysophosphatidyl choline. The specimen consisted of amorphous debris, and no granules of mitochondria were seen. In comparison with the preparation of granules incubated with liposomes (Figs. 6, 7 and 8), there is very little remaining membrane structure (arrows.) × *115,000*

phatidyl choline[25] were used, activation and release of lysosomal enzymes from granules was not noted. Furthermore, the activity of PMN phospholipase is inhibited by 0.05 sodium fluoride,[25] while this compound did not significantly inhibit the activation of lysosomal enzymes in the present system. Although albumin binds and inhibits phospholipid breakdown products such as lysophosphatidyl choline, it had no effect on the activation of lysosomal enzymes by liposomes. In addition, electron microscopic analysis of remaining pellets revealed that the appearance of granule disintegration produced by lysophosphatidyl choline differed from that caused by liposomes. Thus, it seems reasonable to assume that phospholipid breakdown products were not responsible for activation of lysosomal enzymes in the presence of liposomes and that the latter probably act by a different mechanism.

The second mechanism by which liposomes might activate lysosomal enzymes involves the direct interaction between liposomes and proteins of the granule membrane. The stability of lysosomal membranes depends on proteins as well as phospholipids[1] and compounds which are able to interact with proteins of the granule membrane may alter its stability. Since phospholipids form complexes with proteins[35] and particularly with basic proteins,[27] it is possible that negatively charged liposomes interact with a protein in the granule membrane. The formation of a phospholipid-protein complex may induce steric changes in the lysosomal membrane causing its labilization and subsequent activation of lysosomal enzymes. The fact that protamine sulfate when added to the liposomes abolished their ability to interact with granules indicates that liposomes may have affinity for a protein of the granule membrane.

Based on these observations in a cell-free system, the following steps in the process of degranulation of leukocytes during phagocytosis are proposed:

1. During the development of the phagocytic vacuole the leukocytic cell membrane undergoes changes which result in unmasking of internal polar groups of phospholipids. This unmasking process might be induced either by steric changes due to attachment of ingested particles, or by the addition of phospholipid building blocks (micelles) to fragments of newly synthesized vacuolar membrane.

2. The unmasked, negatively charged polar groups representing specific phospholipid molecules in the vacuolar membrane are able to interact with a protein portion of the lysosomal membrane leading to membrane fusion, discharge of granule contents into the phagocytic vacuole, and activation of lysosomal enzymes.

3. Release (solubilization) of active enzymes from granule fragments in the phagocytic vacuole follows a drop of pH to 5.0 or below.

4. Subsequently activated lysosomal phospholipase may generate lyso-compounds which enhance further degranulation.

The ability of negatively charged liposomes to activate latent lysosomal enzymes and disrupt PMN leukocytic granules is a new biological property of membrane-forming phospholipids. This fact raises the possibility that *specific* phospholipid groups may have a role in membrane fusion phenomena and activation of lysosomal enzymes in other types of cells.

## SUMMARY

A cell-free system containing isolated PMN leukocytic granules and liposomal model membranes has been designed to study factors responsible for the fusion of lysosomal and vacuolar membranes during phagocytosis. Only liposomes composed of negatively charged phospholipids, namely phosphatidyl inositol and phosphatidyl ethanolamine, caused activation of lysosomal marker enzymes, beta glucuronidase, and beta N-acetyl glucosaminidase and produced disruption of granules. Liposomes formed by egg phosphatidyl choline were inactive unless dicetyl phosphate, a long chain polyanion, was added. When granules were incubated with liposomes at pH 7.0 structural disintegration occurred, but lysosomal enzymes remained largely sedimentable with the membrane fragments until decrease of pH from 7.0 to 5.0 or below solubilized the active enzymes.

The granule-liposome interaction did not appear related to the formation of phospholipid breakdown products. The activation of lysosomal enzymes by liposomes was not affected by sodium fluoride, an inhibitor of lipolytic enzymes in leukocytes or by albumin in concentrations which inhibited the granule-disrupting activity of lysophosphatidyl choline. The ability of liposomes to activate lysosomal enzymes was abolished when liposomes were complexed with protamine sulfate, a basic protein known to interact with phospholipids.

These data suggest that the mechanism of interaction of liposomes with leukocytic granules may involve the formation of a complex between phospholipids and a protein in the granule membrane. This interaction may lead to steric changes in the membrane, its disruption and activation of lysosomal enzymes. Subsequent release of these enzymes in soluble form requires a drop in pH. It is postulated that unmasked, negatively charged phospholipid groups from the phagocytic vacuole may interact with the lysosomal membrane during phagocytosis to initiate degranulation.

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