IN VITRO INDUCTION OF LYSOSOMAL ENZYMES BY PHAGOCYTOSIS*

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(Received for publication 29 January 1970)

Recent morphologic and biochemical studies of macrophage lysosomes (1, 2) have shown that pinocytic vesicles are converted into secondary lysosomes in the centrosphere region of the cytoplasm by fusion with Golgi vesicles and/or preexisting secondary lysosomes. Environmental molecules which increase the rate of pinocytic activity result in both increased numbers of lysosomes and increased levels of lysosomal enzymes. The mechanism by which endocytosis stimulates the formation of macrophage lysosomes is still obscure. This report will concern the role of membrane interiorization and the nature of interiorized substrate on the control of lysosomal hydrolases.

Materials and Methods

Animals.—In all studies, 25–30 g male mice of the NCS strain (pathogen-free), maintained at The Rockefeller Univeristy, were employed.

In Vitro Cultivation of Mononuclear Phagocytes.—The cells from the peritoneal cavity of unstimulated mice were harvested in heparinized phosphate-buffered saline (PBS), pH 7.4, by techniques previously described (3). A 5 ml sample of cells (2.0–2.5 $\times$ 10^6/ml in Medium 199 and 20% newborn calf serum (NBCS) (Grand Island Biological Co., Grand Island, N. Y.) was dispensed to each 15 cm^2 T-flask and incubated for 60 min at 37°C. The flasks were then washed twice in Medium 199 to remove lymphocytes and reincubated in fresh Medium 199 containing NBCS at concentrations as high as 50%. In experiments using 30 cm^2 T-flasks, 10 ml of cell suspension was employed.

At the time of harvest, the tissue culture media was removed and the macrophage monolayers were rinsed 3 times with 5 ml portions of physiological saline. The cells were then removed from the flasks in 3.0 ml saline by six cycles of freeze-thaw treatment, transferred to polypropylene tubes and stored at −20°C until assayed.

Cells for morphologic study were cultivated on cover slips in Leighton tubes (3). 1.25% Glutaraldehyde in phosphate-buffered saline, pH 7.4, was applied for 10 min at 4°C for fixation.

Quantitation of Phagocytosis.—Phagocytosis was evaluated by direct count of intracellular particles by oil immersion phase contrast microscopy on glutaraldehyde-fixed cover slip preparations.

* This investigation was supported in part by Grant PF-418 from the American Cancer Society and Grant AI-07012, U. S. Public Health Service, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

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Fixation and Processing for Electron Microscopy.—Cultured cells were fixed and stained, according to prior reports (4) for ultrastructural observation.

Cytochemical Stain for Acid Phosphatase.—Macrophage monolayers in T-15 flasks were fixed in glutaraldehyde as described above. The fixative was decanted and 0.88 M sucrose added for 30 min at 4°C. followed by a brief distilled water rinse. Prewarmed Gomori (5) substrate, freshly prepared with β-glycerophosphate (Eastman Kodak Co., Rochester, N. Y.), was added, the flasks incubated for 15–30 min at 37°C., the cells rinsed and processed for electron microscopy.

Enzyme Assays.—β-glucuronidase and cathepsin D were assayed by methods previously described (3, 6). Acid phosphatase was assayed using alpha-naphthyl acid phosphate as substrate (7). In initial experiments both β-glycerophosphate and alpha-naphthyl acid phosphate were used as substrate, but the latter was preferred because of the increased sensitivity of the assay system. The relative activity of acid phosphatase among sets of flasks in a given experiment were identical, regardless of the substrate used.

All enzyme activities exhibited zero order kinetics under the assay conditions employed.

Protein.—Protein content was determined by the method of Lowry (8) using crystalline egg white lysozyme as the standard.

Starch Assay.—The method of McCready et al. (9) was used to assay for starch in macrophage homogenates. Correction was made for intracellular glucose content.

Photography.—Phase contrast photomicrographs were taken with the Zeiss Ultraphot II at a magnification of 1250 using 4 inch × 5 inch Versapan Galstar film.

RESULTS

General Considerations.—Although previous studies had shown that increased endocytic activity leads to enhanced lysosome formation, it was not possible to investigate the possible sites for control using the pinocytic system (1, 10). The requirements for the continuous presence of pinocytic inducer and the nonselective uptake of substrate by pinocytosis precluded the possibility of dissociation of each stage of the endocytic process. These difficulties were overcome by using defined, particulate substrates which were selectively interiorized over a short span of time. The general plan of these studies was to follow the enzymatic and morphologic response of cultured macrophages after the phagocytosis of naturally occurring and synthetic particles.

The Response to Erythrocytes.—

Morphology: Initial studies were conducted with aldehyde-treated sheep erythrocytes which were stable and lacked intrinsic hydrolase activity.

Thoroughly washed formaldehyde-treated sheep erythrocytes of 4.5 μ diameter (Difco Formocells, Difco Laboratories, Inc., Detroit, Mich.) were added to monolayers of macrophages in T-30 flasks cultivated for 24 hr in the presence of 50% NBCS in Medium 199. Phagocytosis was permitted to proceed for 1 hr at 37°C, after which the macrophages were reincubated in fresh media. A mean of 8–10 particles per macrophage were interiorized. By a 1 hr pulse of 7.0×10^6 erythrocytes/ml, more than 97% of the macrophages had ingested red cells.

The erythrocytes were rapidly ingested as shown in Fig. 1 a. 1 hr after

phagocytosis, the erythrocytes were seen as large phase-dense particles, segregated in the centrosphere region of the cytoplasm. The intracellular erythrocytes were slowly degraded as shown in Fig. 1 b and 1 c. By 12 hr, many were less phase-dense than at 1 hr. By 24 hr they had lost their characteristic appearance and the centrosphere region was filled with large phase-lucent vacuoles. By 48 hr, the cells which had ingested erythrocytes were morphologically indistinguishable from controls.

Enzymology: The lysosomal enzyme activities of macrophages after ingestion of sheep erythrocytes were next examined.

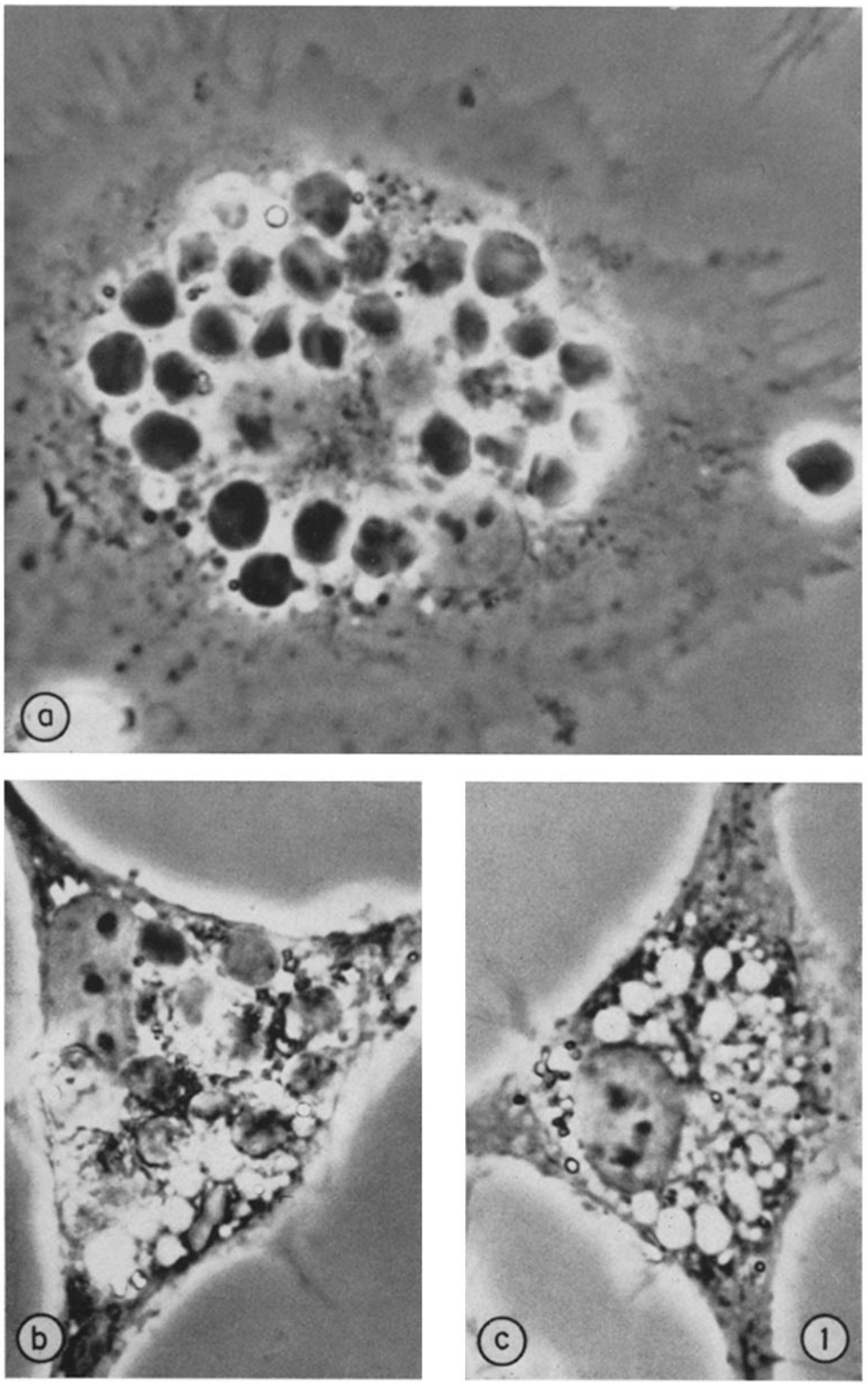
6 hr after phagocytosis, the acid phosphatase activity of cultivated macrophages was identical to that of controls (Fig. 2). By 12 hr, the activity in the experimental flasks was rising and by 24 hr reached a peak level which was 2–3 fold higher than controls. This slowly fell to control values by 48 hr. To determine if the enzyme response was limited to acid phosphatase or was representative of a more general phenomenon, the activities of two other lysosomal enzymes, β-glucuronidase and cathepsin D, were examined. A similar but less marked change also occurred for these two lysosomal enzymes. The increase in cell protein at 1 hr reflected the amount of erythrocyte protein phagocytized. This fell as the digestion of erythrocytes proceeded, but was still 20% higher than controls at 24 hr. The protein changes paralleled the morphologic observations which showed that the digestion of aldehyde-treated erythrocytes required longer than 24 hr.

The response of the lysosomal enzymes to the uptake of varying numbers of erythrocytes is shown in Fig. 3. Relative activities have been plotted with the control values at 24 hr assigned a value of 1.0. The maximal enzyme response occurred with red cell concentrations of 3–10 $\times$ 10^6/ml. A dose of 3.5–7.0 $\times$ 10^6 erythrocytes (RBC)/ml were used for subsequent experiments.

The influence of puromycin on the lysosomal enzyme response of phagocytizing macrophages: It was important to determine if the increase in lysosomal enzyme activity was dependent on continued protein synthesis. Previous experiments had shown that the hydrolase response induced by pinocytosis could be inhibited by puromycin or DL, parafluorophenylalanine (11). It was not possible to extrapolate these data to the phagocytic system, since pinocytosis itself was dependent on continued protein synthesis (12). For the present experiments puromycin was used as an inhibitor of protein synthesis.

Macrophages cultivated in 30% NBCS in Medium 199 for 24 hr were permitted to carry out phagocytosis of aldehyde-treated sheep RBC for 1 hr in the absence of puromycin. The remaining erythrocytes were removed and fresh media added containing puromycin in doses ranging from 0.2–5.0 μg/ml. The flasks were then harvested and assayed 24 hr after phagocytosis.

As shown in Fig. 4, puromycin at concentrations of 0.2–0.5 μg/ml had little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effect on any of the lysosomal enzymes. The acid phosphatase response to phagocytosis was inhibited by 50% in the presence of 1 μg/ml and completely inhibited by 2 μg/ml. A similar inhibition of β-glucuronidase and cathepsin D activity was observed at these doses. There was no measurable enzyme activity at puromycin doses of 5 μg/ml, but these have not been plotted, since a concomitant decrease in cell numbers occurred. Puromycin doses of 1–2 μg/ml did not, however, reduce the number of cells.

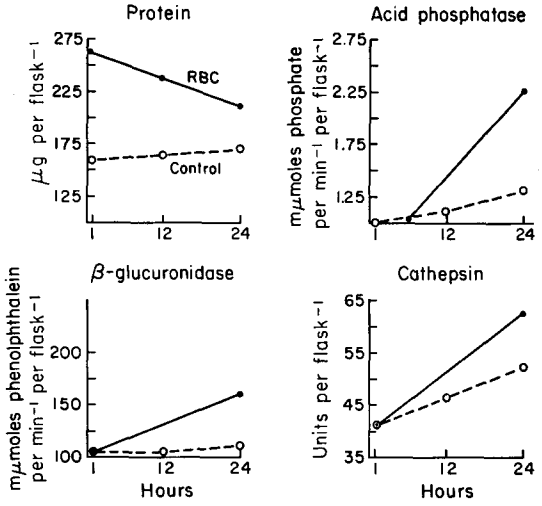


FIG. 2. Time course for changes in protein, acid phosphatase, β-glucuronidase, and cathepsin after phagocytosis of aldehyde-treated sheep erythrocytes. Phagocytic pulse from 0–1 hr.

The influence of pinocytosis on the hydrolase response to phagocytosis: It was important to determine if the enzyme increases after phagocytosis could be separated from or were dependent upon continued pinocytic stimulation provided by culture in media containing 30–50% NBCS. The contributions of phagocytosis and pinocytosis to lysosomal enzyme induction were evaluated by quantitating the postphagocytic response of macrophages cultured in low and high levels of serum. Prior experiments have shown that serum concentrations control the rate of pinocytosis in cultivated macrophages (1).

FIG. 1. a–c. Stages in the digestion of formaldehyde-stabilized sheep erythrocytes within macrophages. Phase contrast. × 2500. (a) 1 hr after phagocytosis, ingested erythrocytes are phase-dense and are clustered in the central cell body. The particles are highly refractile under phase and distinct phagosomes cannot be discerned. (b) 12 hr after phagocytosis Ingested red cells are less phase-dense and refractile and phase-lucent vacuoles appear in the cytoplasm. (c) 24 hr after phagocytosis. The perinuclear region of the cytoplasm is filled with phase-lucent vacuoles and distinct erythrocytes cannot be seen.

The β-glucuronidase and cathepsin activities of macrophages grown in 50% and 5% serum are shown in Fig. 3. In 50% serum the β-glucuronidase and cathepsin activities of controls increased slightly over the 24 hr period examined, whereas in 5% serum the enzyme activity decreased markedly. However, the increases in β-glucuronidase and cathepsin activity of the phagocytic cells were not altered by changes in serum concentration.

These data suggested that a continued pinocytic stimulus was not essential for the lysosomal enzyme increases that followed phagocytosis.

Response to Nondigestible Particles: Polyvinyl Toluene, Polystyrene, and Starch.—It was now possible to selectively interiorize large amounts of defined substrate without a major contribution from the culture medium. This allowed

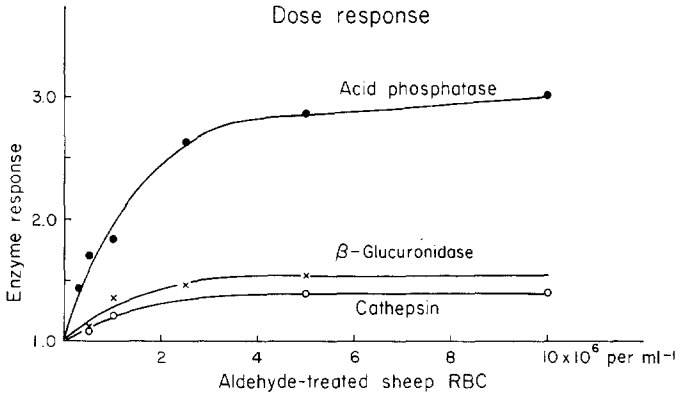


FIG. 3. The dose-response relationship of macrophage acid phosphatase, β-glucuronidase, and cathepsin to phagocytosis of erythrocytes. Ordinate: relative peak enzyme activity with control value at 24 hr = 1.0. Abscissa: initial erythrocyte concentration used for phagocytosis.

us to investigate the contribution of each of several steps of the endocytic process to lysosomal enzyme induction.

The first step in endocytosis is the interiorization of the plasma membrane to form the phagocytic or pinocytic vacuole membrane. To determine if membrane interiorization was a sufficient stimulus by itself, it was necessary to dissociate interiorization from the subsequent intracellular events. One approach was to provide the macrophage with a particle it could ingest, but not digest. In addition, the particle had to be nontoxic, easily ingested, and of sufficient size so that an equivalent amount of membrane would be interiorized. Polyvinyl toluene particles (PVT) of 3.5 μ met these requirements.

Thoroughly washed PVT particles (Dow Chemical Co., Midland, Mich.) were added to macrophages cultivated for 24 hr in 30% NBCS. A 1 hr pulse with 7×10^6 PVT particles/ml resulted in the uptake of 6–8 particles per macrophage. After phagocytosis, the medium was changed to one containing 5% NBCS, incubated for 24 hr at 37°C, and harvested. Another group of flasks were handled in an identical fashion but were incubated in 30% NBCS for an additional 24 hr.

The polyvinyl toluene particles were readily ingested and produced no morphologic evidence of toxicity to the macrophages (Fig. 6 a). PVT was seen as highly refractile phase-lucent particles which were segregated in the centrosphere region of the cytoplasm in a manner identical to that for RBC.

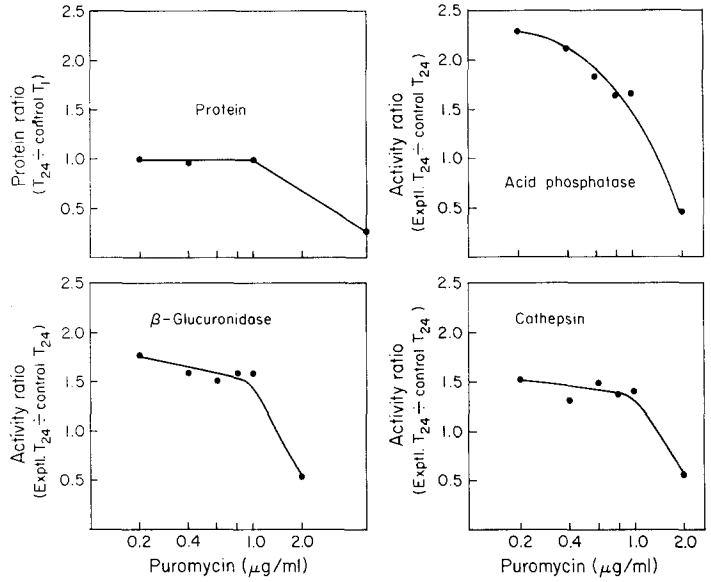


FIG. 4. The effect of puromycin on protein and lysosomal enzyme response 24 hr after phagocytosis of erythrocytes.

There was no increase in acid phosphatase, β-glucuronidase, or cathepsin activity after phagocytosis of the polyvinyl toluene particles. The acid phosphatase activity at 24 hr (Fig. 7) was identical to that for controls.

Although there was no morphologic evidence of toxicity to the macrophage, it was important to determine if subtle changes had occurred which might have prevented a response to endocytic stimuli. This was tested by providing the PVT-treated cells with a pinocytic stimulus by culturing in 30% NBCS (Fig. 7). Both the control and PVT-treated cells responded in an identical fashion with an increase in lysosomal hydrolases. The failure of the macro-

phages to demonstrate acid hydrolase response after PVT uptake was, therefore, not due to any functional impairment of the cells.

To determine if the inability of PVT to induce lysosomal enzymes was representative of a more general phenomenon, the response to polystyrene particles (Difco, 0.8 μ diameter) was examined. Polystyrene was also ineffective as an inducer of lysosomal enzymes. Since PVT and polystyrene are both hydrophobic, it was of interest to examine the enzyme response to a nondigestible hydrophilic particle. Nondigestible *Amaranthus* starch, kindly supplied by Dr. Manfred Karnovsky, Harvard University, was found to be ineffective as an inducer of lysosomal enzymes. During the 48 hr period after

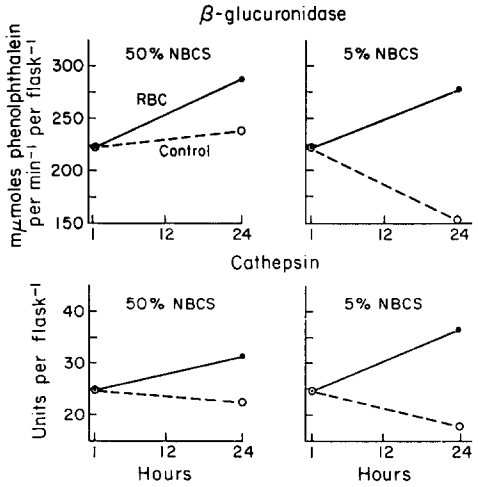


FIG. 5. The effect of serum concentration on β-glucuronidase and cathepsin response to phagocytosis of erythrocytes. After phagocytosis from 0–1 hr cells were either maintained in 50% NBCS or switched to 5% NBCS for 24 hr.

phagocytosis of the starch there was no change in intracellular starch content, indicating that the starch was neither digested nor excreted.

These results indicated that membrane interiorization per se was not related to lysosomal enzyme production and suggested the importance of a later step in the endocytic process.

The Fusion of Phagosomes with Preexisting Macrophage Secondary Lysosomes.—The next step in the endocytic process is the fusion of the phagosome with primary and secondary lysosomes. The morphologic correlate of this event is degranulation, manifested by a decrease in numbers of discrete lysosomes. To determine if fusion with secondary lysosomes occurred after phagocytosis, degranulation was evaluated by direct counts of secondary lysosomes.

Macrophages were grown for 24 hr in 50% NBCS in Medium 199 to produce large and numerous secondary lysosomes. 2 hr after phagocytosis of RBC or PVT particles, the number of particles ingested and the number of secondary lysosomes per macrophage were counted by phase microscopy.

Degranulation was found to be proportional to the number of particles ingested (Fig. 8). There was no difference in degranulation between the PVT- and RBC-treated cells. Although semiquantitative at the best, this study suggested that secondary lysosome-phagosome fusion was the same after PVT or RBC ingestion.

As an additional qualitative evaluation of fusion, the transfer of histochemically demonstrable acid phosphatase into phagosomes was examined after the uptake of RBC and nondigestible particles.

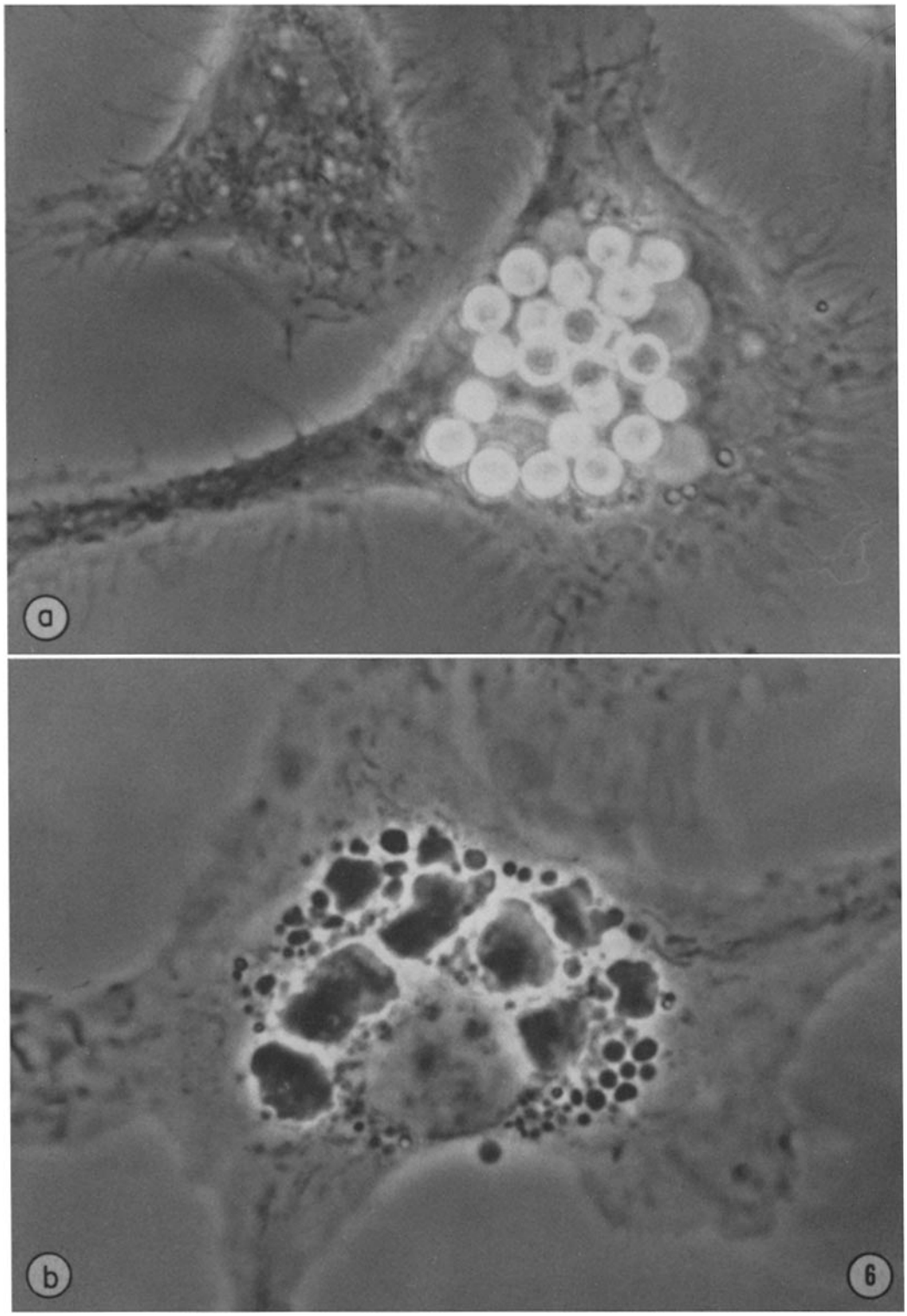
Macrophages were grown in T-15 flasks for 24 hr in Medium 199 with 50% NBCS. 2 hr after phagocytosis of RBC or PVT particles, the cells were fixed in 1.25% glutaraldehyde in phosphate-buffered saline, stained for acid phosphatase, and processed for electron microscopy as described in Materials and Methods.

Fig. 9 illustrates a typical cell stained for acid phosphatase 2 hr after phagocytosis of PVT particles. The lead reaction product was uniformly present in the large dense granules. It exhibited a particulate appearance distributed over the matrix, as well as the membrane of the granules.

The phagosomes were visible as large emtpy vacuoles as the polyvinyl toluene was removed during the embedding procedure. The reaction product was present along the inner face of nearly every phagosome membrane. Secondary lysosomes were frequently observed in various stages of fusion with the phagosome. A similar distribution of reaction product was observed in the RBC phagosomes as shown in Fig. 10. There was no obvious difference between the number of acid phosphatase-positive phagosomes after phagocytosis of PVT or RBC. The markedly different enzymatic response to PVT or RBC could not be explained on this basis.

The Response to Aggregated BGG.—Since the studies thus far had suggested that digestion or some process beyond this stage was responsible for lysosomal enzyme induction, it was of interest to determine what compounds could provide the required stimulus. These studies were initiated by examining the enzyme response after phagocytic ingestion of a particulate protein.

Particulate bovine gamma globulin (fraction II, Pentex Inc., Kankakee, Ill.) was prepared by heat denaturation for 5 min at 70°C in phosphate-buffered saline, pH 7.4. The precipitate was centrifuged at 500 *g* for 15 min and washed 3 times to remove any remaining soluble and very small particulate protein. Immediately before use, the heat-denatured BGG was dispersed by brief sonication. It was then added in a concentration of 250 μg/ml to cultivated macrophages for a period of 5 hr. A prolonged exposure was required to achieve substrate interiorization sufficient to be readily visualized by light microscopy. The monolayers were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then washed and overlaid with fresh 30% NBCS in Medium 199, reincubated at 37°C, and harvested at varying intervals.

The heat-denatured BGG was taken up as small particles of approximately 1 μ diameter. Intracellular aggregation of the ingested material produced irregularly shaped phase-dense granules as seen in Fig. 6 b.

The results of quantitative experiments similar to those described for RBC phagocytosis are shown in Fig. 11. Phagocytosis of BGG proceeded from 0–5

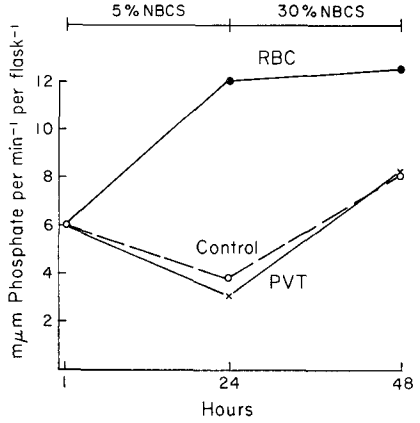


FIG. 7. Comparison of macrophage acid phosphatase response to phagocytosis of polyvinyl toluene particles and erythrocytes.

hr. The acid phosphatase activity of BGG-treated cells 12 hr after the onset of phagocytosis was 50% greater than that for control cells and by 24 hr had returned to control values. The time at which the peak enzyme response occurred was related to the length of exposure to BGG. Cells exposed to BGG for 12 hr, instead of 5 hr, showed a prolonged rise in acid phosphatase activity, with peak activity still present 24 hr after the onset of phagocytosis (shown by the x in Fig. 11). A similar response was observed with cathepsin activity, although the increase was less marked than for acid phosphatase. These experi-

FIG. 6 a, b. Cultivated mouse macrophages shortly after the ingestion of polyvinyl toluene particles (3.5 μ) and aggregated bovine gamma globulin. Phase contrast. (a) Uniform, highly refractile polyvinyl toluene particles clustered within a well spread cell, × 2500. (b) Irregular phagosomes containing heat-aggregated bovine gamma globulin. The particles are initially ingested as 1 μ spheres and are subsequently segregrated within large phagocytic vacuoles as a result of repeated membrane fusions. × 2800.

ments indicated that uptake of protein alone was a sufficient stimulus for lysosomal enzyme induction.

The Response to Amino Acid Homopolymers.—It next was of interest to determine if digestible polymers composed of a limited number of L-amino acids would be effective inducers of lysosomal enzymes. For this purpose we employed aggregates of homopolymers of poly-*l*-glutamic acid and poly-*l*-lysine.

Equimolar solutions of poly-*l*-glutamic acid (average mol wt 50,000) and poly-*l*-lysine (average mol wt 47,000 Yeda, Miles Research Laboratories) dissolved in PBS, pH 7.4, in a concentration of 1000 μg/ml were slowly mixed with stirring at room temperature, forming a copious white precipitate. The precipitate was then chilled to 4°C for 30 min, washed 4 times in PBS in the cold centrifuged at 500 *g* for 15 min between washes, and suspended to volume in phosphate-buffered saline.

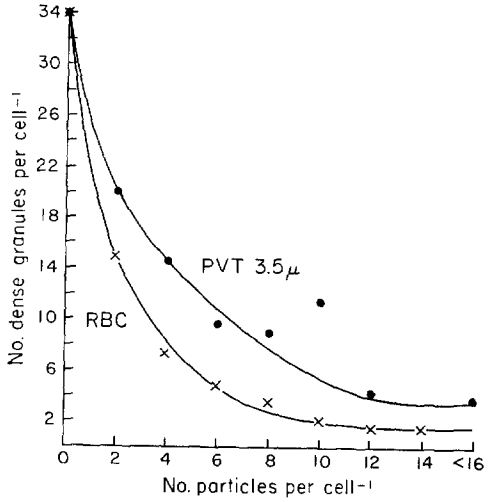


FIG. 8. Degranulation of macrophages after phagocytosis of erythrocytes and polyvinyl toluene particles.

The aggregates were dispersed just before using by brief sonication. The resulting stable nontoxic aggregates of approximately 1 μ diameter were then added to macrophage monolayers for a period of 2 hr, after which the cells were washed and fresh medium added.

The aggregates of poly-*l*-glutamic and poly-*l*-lysine were readily phagocytized, forming irregularly shaped phase-dense particles. Within 2 hr, clear vacuoles began to form around the interiorized aggregates. The vacuoles continued to enlarge up to 48 hr after phagocytosis, forming the enormous vacuoles seen in Fig. 12. The vacuoles decreased in size very slowly beyond 48 hr, but did not completely disappear by 96 h.

The aggregates of poly-*l*-glutamic acid and poly-*l*-lysine proved to be effective inducers of lysosomal enzymes as shown in Fig. 13.

Acid phosphatase activity was 50–60% greater than control values 24 hr after phagocytosis and returned to control baseline values by 48–72 hr.

The formation of large vacuoles was an unexpected response to the ingestion of the polyamino acid aggregates. It was of some importance, therefore, to determine the nature of these vacuoles by electron microscopy. If they were autophagic, for example, cell material and not the phagocytized aggregates may have been responsible for the observed enzyme induction.

Electron micrography of macrophages after ingestion of poly-*l*-glutamic acid: poly-*l*-lysine aggregates are shown in Fig. 14 a. The aggregates are seen as the very electron dense spheres in various stages of interiorization at 2 hr. In the newly formed vacuole, the phagosome membrane was closely adherent to the ingested aggregates. By 24 hr (Fig. 14 b) greatly enlarged membrane-bounded vacuoles containing only the aggregated polymers were observed. There was no evidence of accumulation of cytoplasmic organelles, suggesting autophagy, in any of the vacuoles observed.

If partial digestion of endocytosed material were a requirement for lysosomal enzyme induction, it would follow that nondigestible polymers would not be effective inducers. This was examined by determining the macrophage response after uptake of poly-*d*-glutamic acid; poly-*d*-lysine aggregate was prepared in the same way as the L-isomers aggregates.

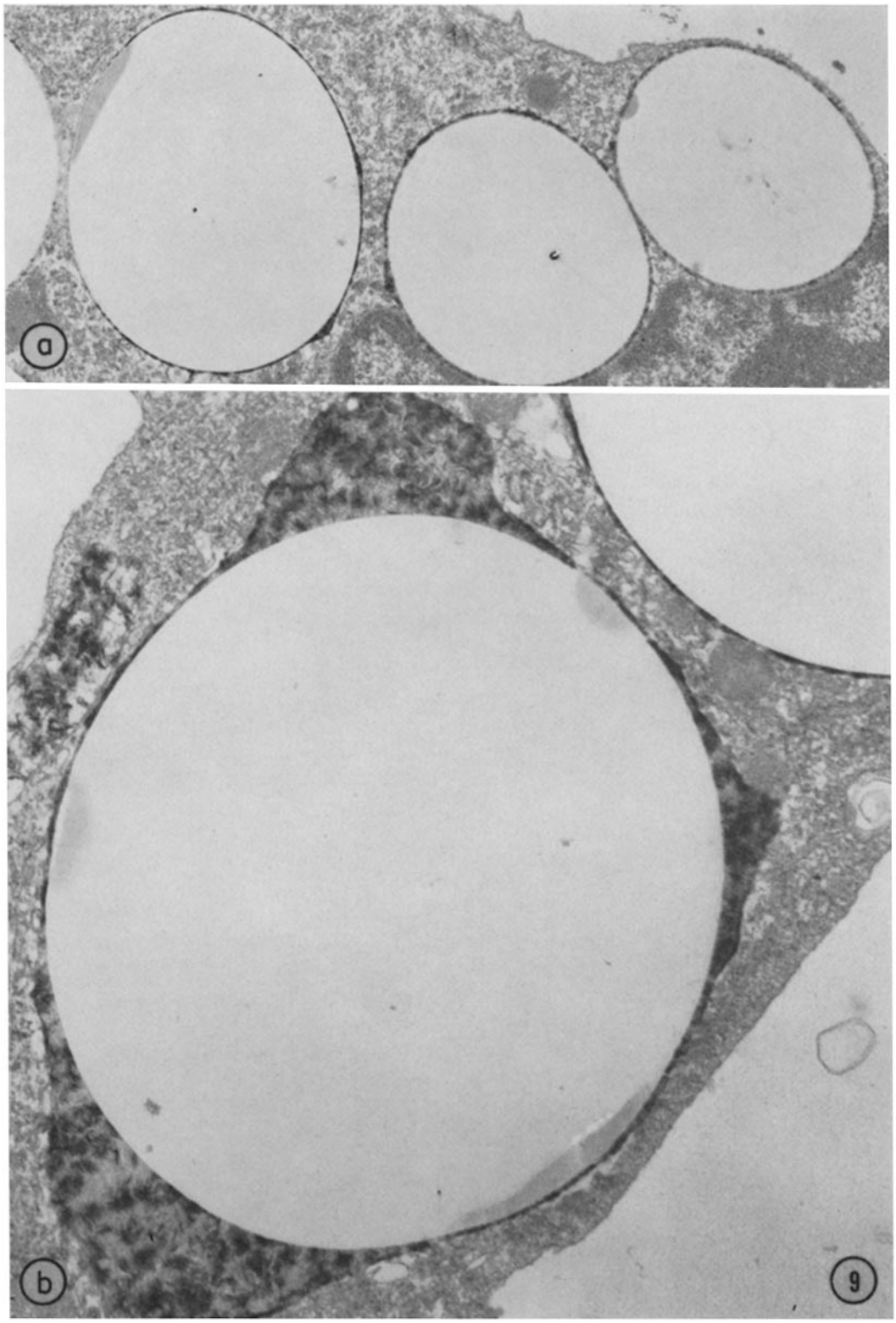
The poly-*d*-glutamic acid; poly-*d*-lysine aggregates produced vacuolation indistinguishable from that of the L-isomers. However, the vacuoles did not diminish in size after 48 hr but remained large for at least 96 hr.

The D-polymers proved to be ineffective inducers of lysosomal enzymes (Fig. 13).

DISCUSSION

The striking heterophagic activity of mononuclear phagocytic cells is fundamental to their participation in defense reactions and tissue reorganization. Lysosomal enzymes play a central role in the digestive activity of these cells. The current demonstration that in vitro phagocytosis of digestible proteins and polyamino acids induces an increase in lysosomal enzyme activity may have direct relevance to in vivo events occurring at sites of inflammation.

The cellular response to nondigestible particles has been previously examined with whole animal systems. Meijer (13) found that intraperitoneal injection of carbon, polyvinylpyrrolidone (PVP), or dextran produced a 50–100% increase in mouse hepatic acid phosphatase activity but none in the spleen. Goldberg (14) found IV Fe-dextran produced no increase in guinea pig hepatic acid phosphatase but a 100% increase in mouse hepatic acid phosphatase. It is difficult to interpret these in vivo studies since it is unlikely that phagocytosis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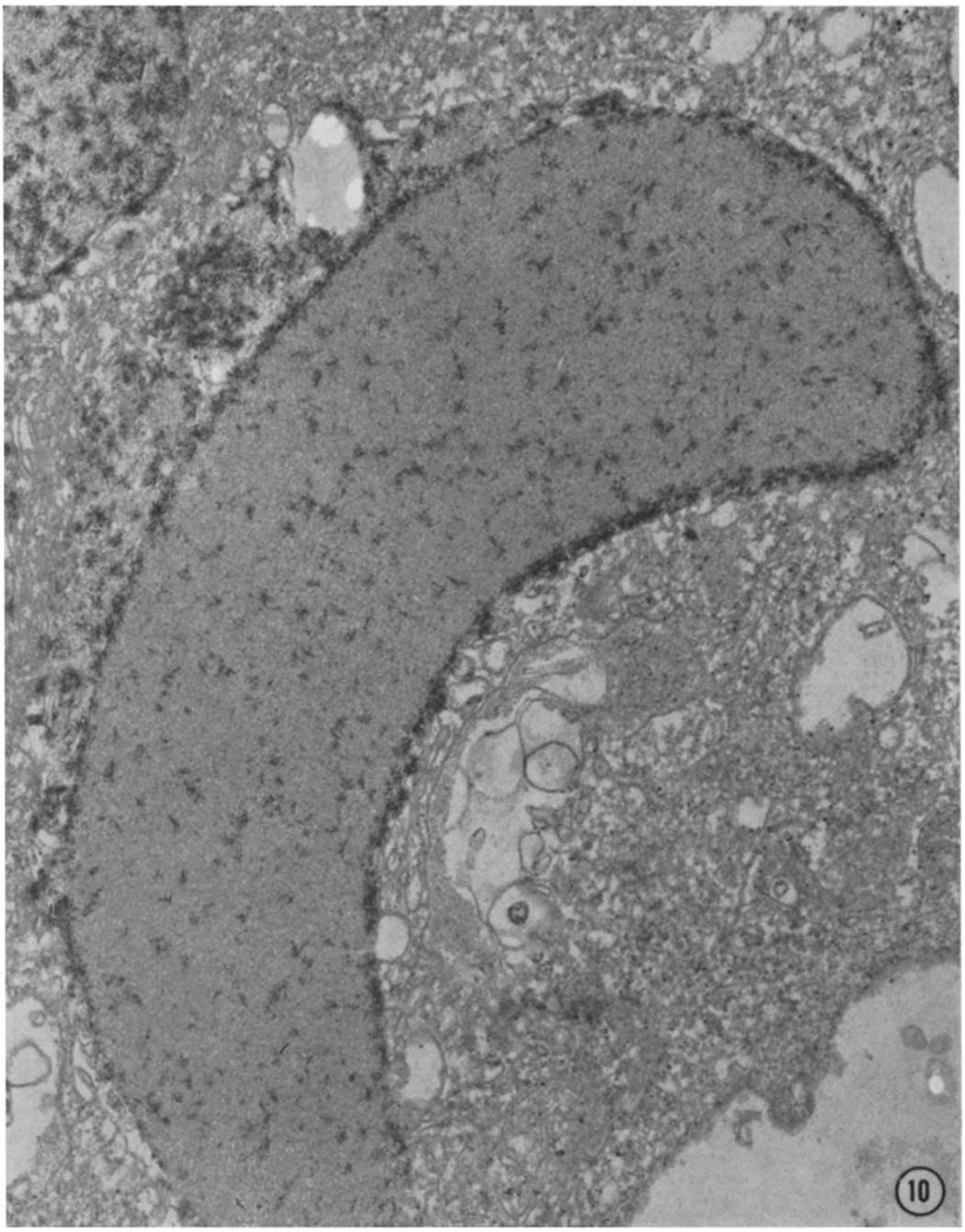

FIG. 9 a,b. The localization of acid phosphatase in phagocytic vacuoles containing polyvinyl toluene (PVT) particles. 2 hr after ingestion, the dense lead reaction product is seen within and outlining the phagosomes. Most of the PVT has been extracted during the embedding procedure leaving otherwise empty vacuoles with tightly apposed membranes. (a) × 10,000. (b) × 25,000.

Fig. 10. The distribution of acid phosphatase in a phagocytic vacuole containing an erythrocyte. The preparation was fixed with glutaraldehyde 2 hr after phagocytosis had occurred. The dense leading reaction product outlines the phagosome. × 21,000.

of a nondigestible particle is the only variable. Production of an inflammatory response may have altered the cell population either by proliferation or exogenous cell infiltration, producing relative increases in acid hydrolase-rich phagocytic cells (15). The additional possibilities that the foreign materials altered the pinocytic rates of parenchymal and/or phagocytic cells, that the nondigestible particles were complexed to digestible protein when ingested, and that the autophagy induced by foreign materials makes any explanation of whole animal results difficult (10, 16). Similar difficulties beset interpretation of Maack's (17) findings that lysozyme administered intravenously results in increased cathepsin and acid ribonuclease activity of rat kidney but has no effect on acid phosphatase activity.

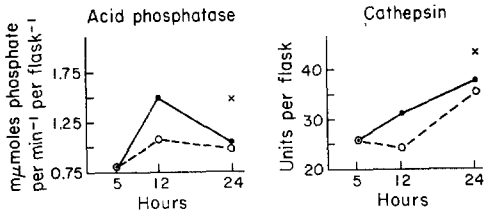

FIG. 11. Time course for changes in acid phosphatase and cathepsin after phagocytosis of aggregated bovine gamma globulin. ○, control in 20% NBCS; ●, phagocytic pulse from 0 to 5 hr; X, phagocytosis from 0 to 12 hr.

Phagocytosis and pinocytosis are variant forms of endocytosis apparently identical in all processes subsequent to the stage of membrane interiorization. The current work has demonstrated that phagocytosis can induce lysosomal enzyme formation by a mechanism operative beyond the state of interiorization. It follows, then, that pinocytosis may well induce lysosomal enzyme function by the same mechanism.

The observation that puromycin inhibited the enzyme increase after phagocytosis suggested that new enzyme synthesis was required. This interpretation is consistent with previous observations from this laboratory that lysosome

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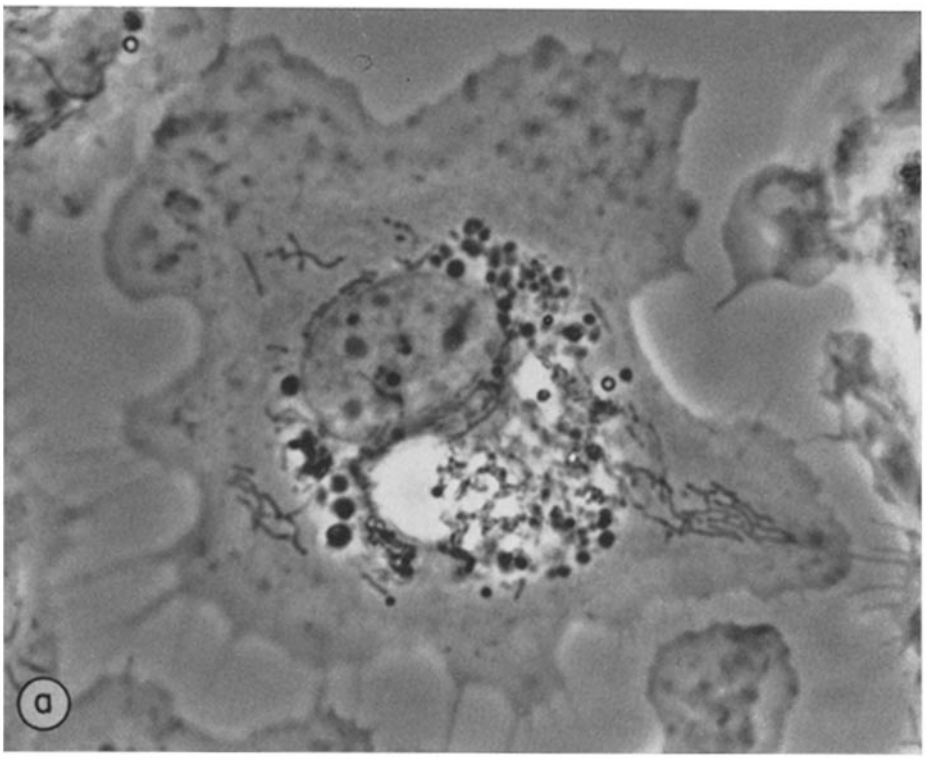
Fig. 12 a,b. The appearance of macrophages after the ingestion of poly-*l*-glutamic: poly-*l*-lysine coacervates. Phase contrast. × 2800. (a) 2 hr after the phagocytosis of coacervates. Numerous phase-dense granules of varying size and shape are present in the perinuclear area. Two large phase-lucent vacuoles have already formed. These contain aggregates of the homopolymers and arise from the fusion of phagosomes and preexisting secondary lysosomes. The cell is otherwise well spread and normal in appearance. (b) 48 hr after the phagocytosis of coacervates. A cell with two huge phase-lucent vacuoles containing aggregates. These fill most of the central cell body and are bounded by refractile lipid droplets and nuclei. Phase dense secondary lysosomes are not present and the cell is otherwise well spread.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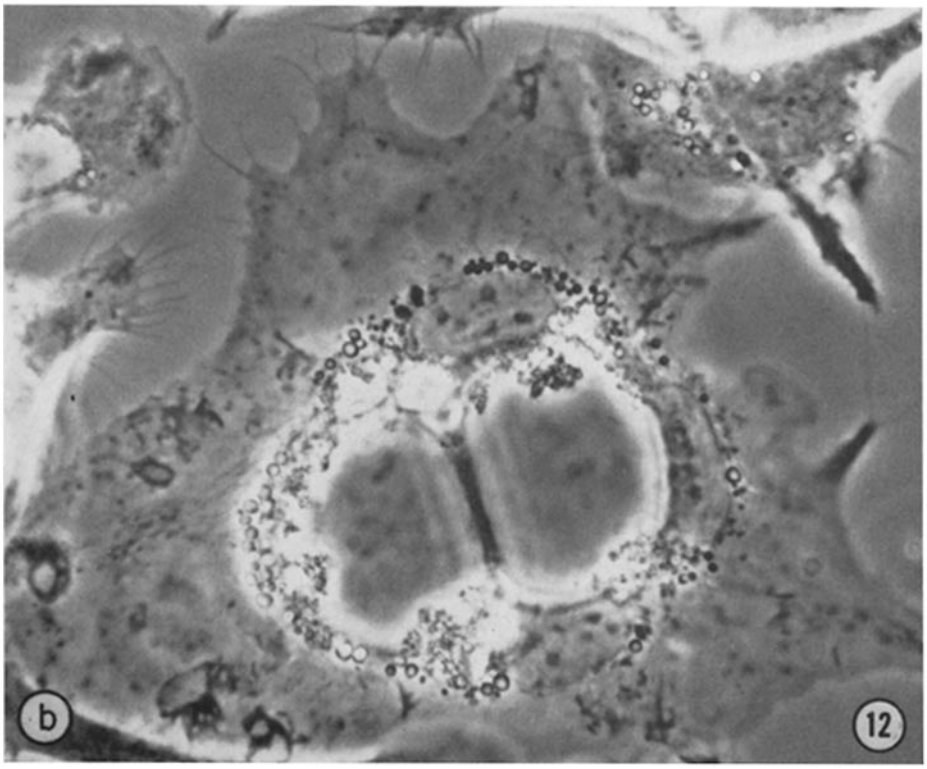
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formation induced by pinocytosis was dependent on new protein synthesis (2, 11). It seems unlikely that enzyme activation or removal of inhibition of preexisting enzyme could account for the increased activity of all three of the enzymes examined. The additional possibility that the changes in lysosomal enzyme levels after phagocytosis may, in part, represent altered rates of degradation must be considered. Shimke et al. (18) have shown that the administra-

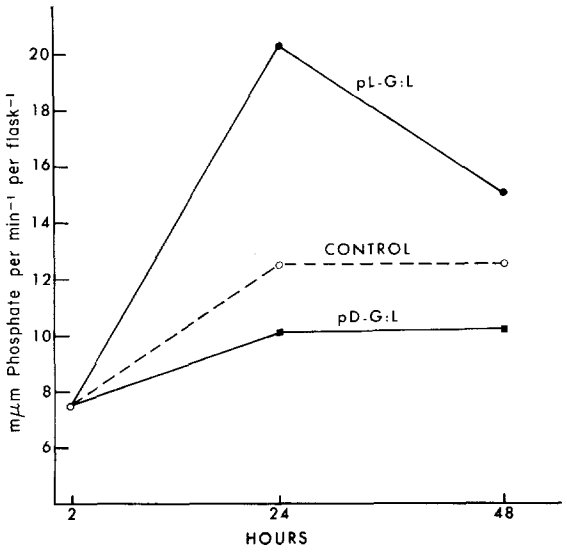
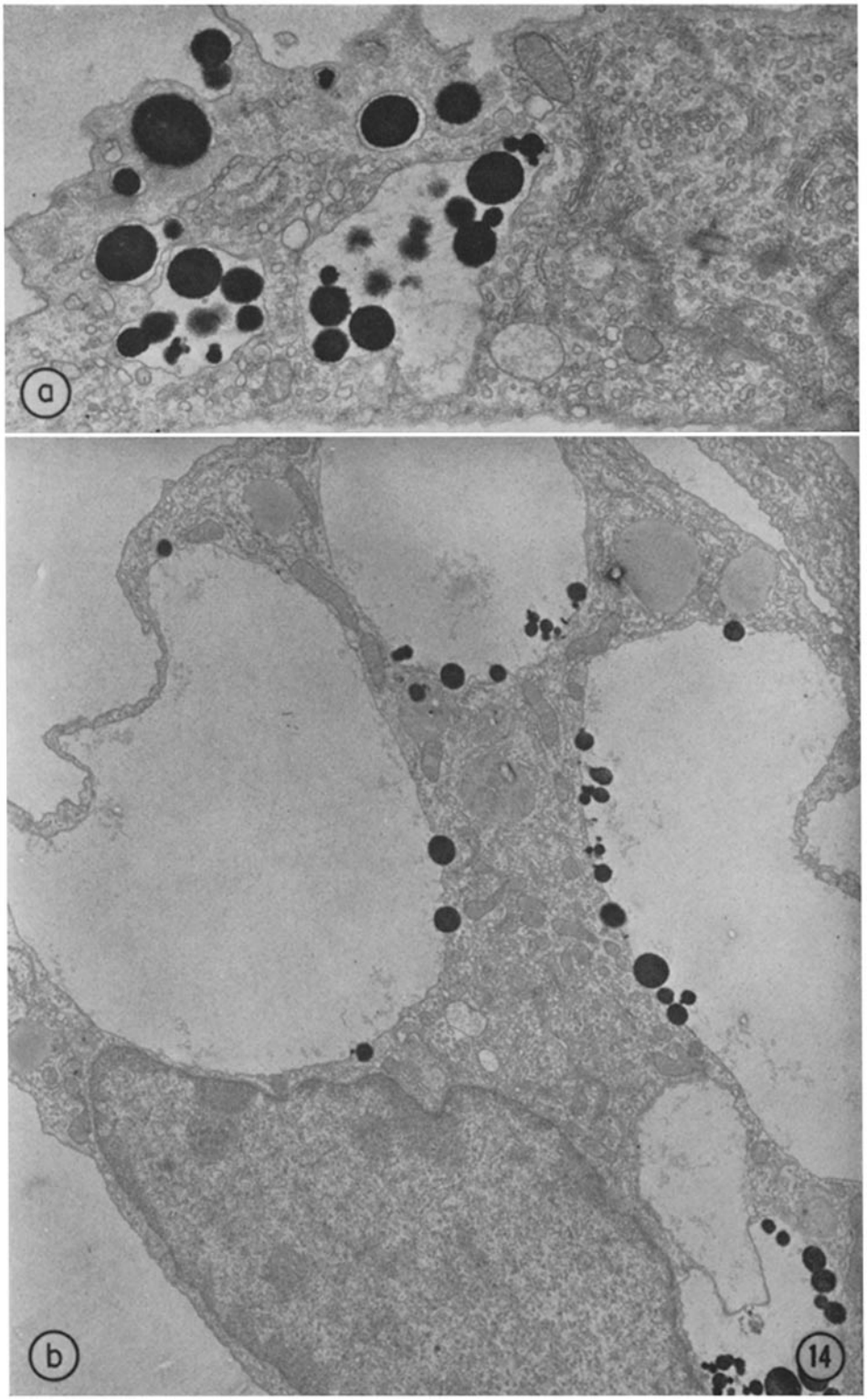


FIG. 13. Comparison of macrophage acid phosphatase response to phagocytosis of poly-*l*-glutamic acid:poly-*l*-lysine aggregates and poly-*d*-glutamic acid: poly-*d*-lysine aggregates.

tion of tryptophan reduced the rate of degradation of rat liver tryptophan pyrrolase by forming a complex that was no longer subject to degradation. The administration or iron has a similar effect on the degradation of ferritin protein (19). It is unlikely that a substrate protection mechanism is operative in the present case in which substrates induce enzymes not involved in their hydrolysis.

Fig. 14 a,b. The ultrastructure of cultivated macrophages which have ingested poly *l*-glutamic: poly *l*-lysine coacervates. (a) 2 hr after the addition of the coacervates. The homopolymer aggregates appear as electron-dense spheres present on the cell surface and within cytoplasmic phagosomes. The majority are initially taken up as single particles which subsequently fuse to yield complex phagosomes. × 17,800. (b) 24 hr after phagocytosis. Large, electron-lucent vacuoles have formed which contain spherules which are most often attached to the limiting membrane. Other cytoplasmic organelles are found between the vacuoles in an excellent state of preservation. × 8000.


a
b
14

The penetration of pinocytic vacuoles to the centrosphere region may lead to discharge of a few lysosomes which may in turn trigger the new synthesis of acid hydrolases. The difference in response to the various particles could then be accounted for by differences in rates of fusion between phagosomes and preexisting lysosomes. Fusion was evaluated directly by electron microscopic examination of acid phosphatase transfer from lysosome to phagosome, and indirectly by correlating the disappearance of lysosomes with the extent of phagocytosis. No differences in fusion were observed after phagocytosis of digestible and nondigestible particles. In both sets of experiments, however, the fusion of secondary lysosomes was the prinicipal process being evaluated, not the fusion of primary lysosomes. The current results, then, suggest that fusion of the secondary lysosomes and endocytic vacuole is not the stimulus for lysosomal enzyme induction, but do not rule out the possibility that fusion of primary lysosome and endocytic vacuole may act as the control site. Studies of possible differences in fusion requirements and rates of fusion of primary and secondary lysosomes with endocytic vacuoles would clearly be helpful in this regard.

The possibility that cell digestion or processes occurring beyond this stage are responsible for lysosomal hydrolase induction must be considered. The negative response to the nondigestible PVT, polystyrene (PST), and starch particles and the positive response to the digestible RBC and purified protein are consistent with such a mechanism. This becomes an even more attractive hypothesis in view of the ability of poly-*l*-glutamic acid; poly-*l*-lysine aggregates and the inability of poly-*d*-glutamic:poly-*d*-lysine aggregates to induce lysosomal enzymes.

It is difficult to imagine that intralysosomal polypeptides could exert a controlling influence on further lysosomal enzyme production. Further, it is clear from previous studies that peptides larger than 230 daltons have difficulty escaping from the lysosome (20). Munro (21) has shown that rates of liver protein synthesis are quite sensitive to the supply of exogenous amino acids. The prinicipal amino acid effect is at the level of translation although the rate of breakdown of some proteins is also affected. Information regarding the breakdown of poly-*l*-glutamic acid or poly-*l*-lysine by the intact macrophages, the nature and intracellular distribution of such products, and their ultimate fate may be helpful in understanding the site(s) for control over lysosomal enzyme formation.

SUMMARY

The in vitro induction of lysosomal enzymes by phagocytosis was demonstrated in cultivated mouse peritoneal macrophages. The contribution of each of several steps in the endocytic process to enzyme induction was examined. The enzymatic response after the uptake of equal numbers of erythrocytes (RBC) and nondigestible particles were compared. Phagocytosis of RBC pro-

duced a marked increase in the levels of acid phosphatase, β-glucuronidase, and cathepsin D. Puromycin (1 μg/ml) inhibited the enzyme response. In contrast, phagocytosis of polyvinyl toluene, polystyrene, and insoluble starch particles produced no increase in macrophage lysosomal enzymes, although fusion of phagosomes with preexisting lysosomes occurred normally. The endocytic stimulus to synthesis of inducible lysosomal enzymes, therefore, occurred at or beyond the stage of digestion. Purified protein (bovine gamma globulin) aggregates and homopolymer coacervates of poly-*l*-glutamic acid:poly-*l*-lysine were effective inducers of lysosomal acid phosphatase, β-glucuronidase, and cathepsin D, whereas homopolymers of the same D-amino acids were ineffective as inducers. Both the quantity of phagocytized substrate and its rate of enzymatic hydrolysis appear to control the level and persistance of lysosomal hydrolases.

The authors acknowledge the contribution of Dr. James G. Hirsch and Dr. Samuel Silverstein for the electron micrographs and Mrs. Barbara Altman and Mrs. Eileen Parks for their excellent technical assistance.

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