PHAGOCYTOSIS OF LATEX BEADS BY *ACANTHAMOEBA*

II. Electron Microscopic Study of the Initial Events

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ABSTRACT

Electron microscopic studies confirm and extend the conclusions derived previously from a quantitative biochemical study of the phagocytosis of polystyrene and polyvinyltoluene latex beads by *Acanthamoeba* (1). Latex beads 1.305, 1.90, and 2.68 μ in diameter are ingested individually, with each bead tightly surrounded by a membrane derived from the plasma membrane. Latex beads 0.557, 0.264, 0.126, and 0.088 μ in diameter are accumulated at the surface of the ameba and then phagocytosed, with many beads tightly packed within one membrane-bounded vesicle.

INTRODUCTION

We have recently published (1) a quantitative biochemical study of the phagocytosis by *Acanthamoeba* of seven monodisperse preparations of latex beads 0.088–2.68 μ in diameter. The phagocytosis of latex beads of each diameter exhibited saturation kinetics, and the kinetic constants were the same for beads of all sizes when the concentration of beads was expressed on a mass (or volume) basis. The kinetic data were definitely not a function of the diameter of the beads, their surface area, or their concentration expressed as number of beads. Also, the very slow rate of uptake, from the medium, of glucose-^{14}C or albumin-^{131}I was not increased as a consequence of the phagocytosis of latex beads. These data led to the following hypotheses: (*a*) there exists an "optimal" size for phagocytic vesicles which is approximately the same for beads 0.088–2.68 μ in diameter; (*b*) one latex bead or more accumulates at the surface of an ameba until this "critical" volume is reached; (*c*) the concentrated mass of beads is then ingested by a highly selective process, so that the concomitant uptake of solute molecules is below the level of detection.

These general conclusions are supported by the electron microscopic observations reported in the present paper.

MATERIALS AND METHODS

Monodisperse preparations of polystyrene latex beads with diameters of 0.088, 0.126, 0.264, 0.557, and 1.305 μ and polyvinyltoluene latex beads with diameters of 1.90 and 2.68 μ were generously provided by Mr. L. J. Lippie of the Dow Chemical Co., Midland, Mich. According to the manufacturer, the various preparations consist of approximately 99% polymer, 0.5% sulfonate detergent, and 0.5% inorganics of unspecified nature. Identical results were

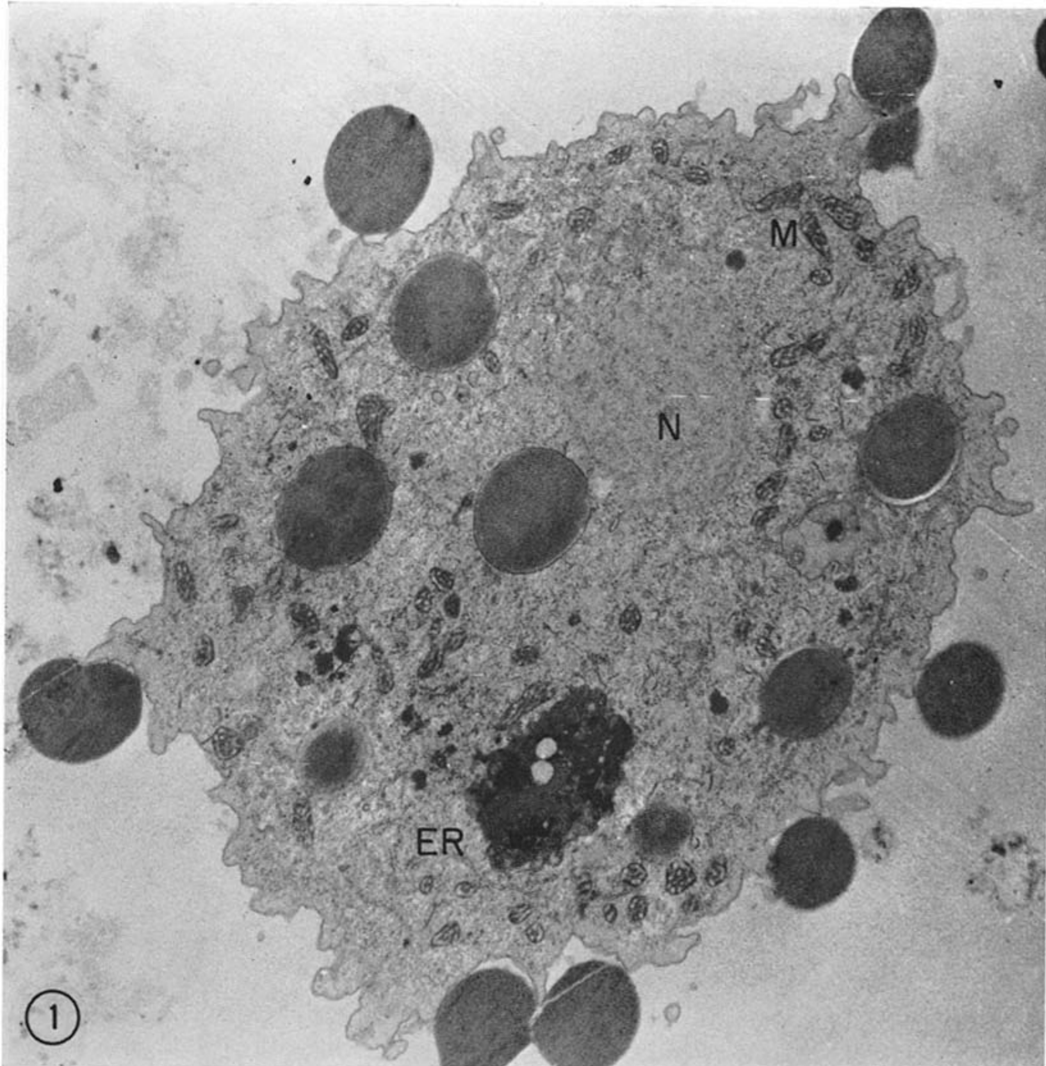

FIGURE 1 Cross-section of a cell actively phagocytosing 1.90-μ latex beads. In this experiment 3×10^6 amebae and 2.8×10^9 (11 mg) beads were incubated per milliliter of 1.5% proteose-peptone in 0.015 M phosphate buffer, pH 6.7, for 10 min at 30°. Nucleus (*N*), mitochondria (*M*), and endoplasmic reticulum (*ER*) are apparent. Cells were fixed in glutaraldehyde–osmium tetroxide and embedded in Maraglas. × 8100.

obtained with preparations that were exhaustively dialyzed and washed before use. The beads in each preparation are stated to be uniform in size, within standard deviations of about 1–3%.

Acanthamoeba sp (2) was grown in proteose–peptone–glucose medium, pH 6.7, in 1 liter shaking cultures as described by Weisman and Korn (3). Amebae were harvested after 6–8 days by centrifugation at 500 *g* and washed three times with 0.015 M phosphate buffer, pH 6.7. Amebae were incubated with the latex beads in 10 ml of either 0.1 M glucose or 1.5% proteose in 0.015 M phosphate buffer, pH 6.7. The concentration of amebae was 3×10^6 cells/ml, and the latex beads were usually present at a concentration of 1–1.5% (w/v). Incubations were performed in 50-ml Ehrlenmeyer flasks at 30° in a Dubnoff shaking water bath oscillating at 80 cycles/min.

To measure the amount of latex beads ingested by the amebae, duplicate 1 ml aliquots of the incubation mixture were added to 1 ml of 4×10^{-4} M 2,4-dini-

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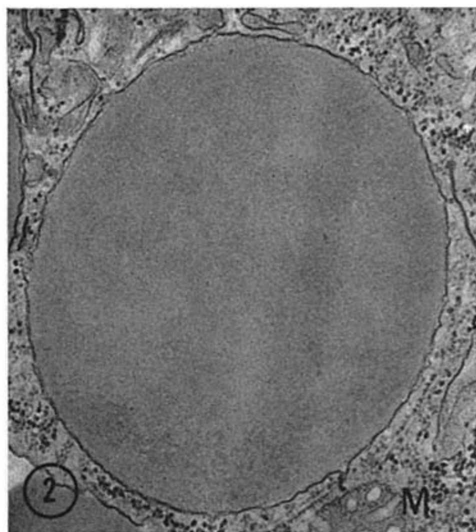

FIGURE 2 An ingested 2.68 μ latex bead within the phagocytic membrane. In this experiment 3×10^6 amebae and 1.1×10^9 (11 mg) beads were incubated per milliliter of 1.5% proteose-peptone. Note how closely the phagocytic membrane surrounds the bead. The numerous, dark granules are glycogen. Mitochondria (*M*). Fixation and embedding were as in Fig. 1. × 31,700.

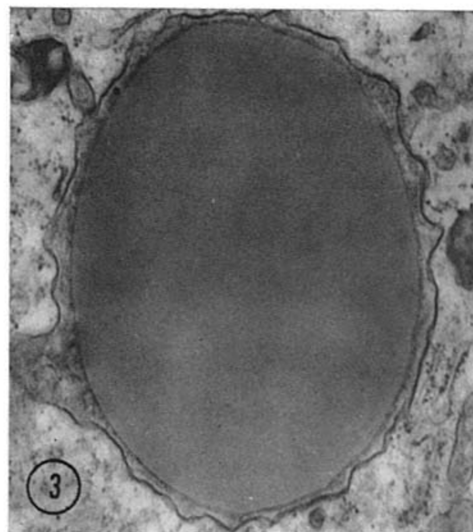

FIGURE 3 A phagocytic vesicle containing a 1.90 μ latex bead. In this instance there is some space between the vesicle membrane and the bead which is filled by amorphous material of unknown origin. This is from another section of the same experiment as Fig. 1. × 28,900.

trophenol which instantly stops phagocytosis (1). The cells were washed free of excess beads by centrifugation from 0.015 M phosphate buffer, pH 6.7, three times at 500 *g* for 5 min. The ingested beads were then extracted with dioxane (4) and the concentration of polystyrene or polyvinyltoluene was determined by the absorption at 259 or 267 mμ, respectively (1).

Samples (2 ml) for electron microscopy were usually added directly to the fixative without removal of excess beads. If the cells were collected and washed free of excess beads before fixation, no beads could be observed in the process of ingestion. In some experiments the amebae were fixed in 5% glutaraldehyde in Millonig's buffer (5) for 1 hr at 0° and postfixed in 1% osmium tetroxide in the same buffer for 45 min at 0°. In other experiments the amebae were fixed in 1% potassium permanganate in 0.1 M cacodylate buffer for 45 min at 0°, washed briefly with water, and postfixed in 1% uranyl acetate in water for 1 hr at 0°. The fixed cells were washed briefly with water and dehydrated through 50, 70, and 95% ethanols for 15 min each at 0° followed by three changes of absolute ethanol at room temperature. Samples were embedded in either Maraglas (Polysciences Inc., Rydal, Pa.) or Durcupan (Fluka AG, Basel, Switzerland). The Maraglas embedding mixture contained 68 ml Maraglas 655, 20 ml Cardolite NC513, 10 ml dibutyl-

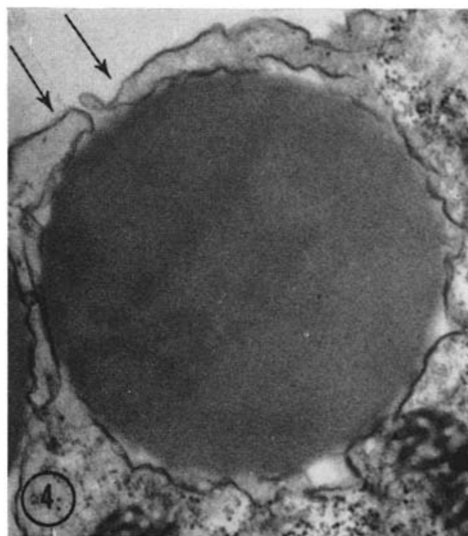

FIGURE 4 A phagocytic vesicle just in the process of closing around a 2.68 μ latex bead. Note how the medium is almost entirely excluded from the vesicle and that the advancing tips (arrows) appear to just meet. This is the same experiment as in Fig. 2. × 22,000.

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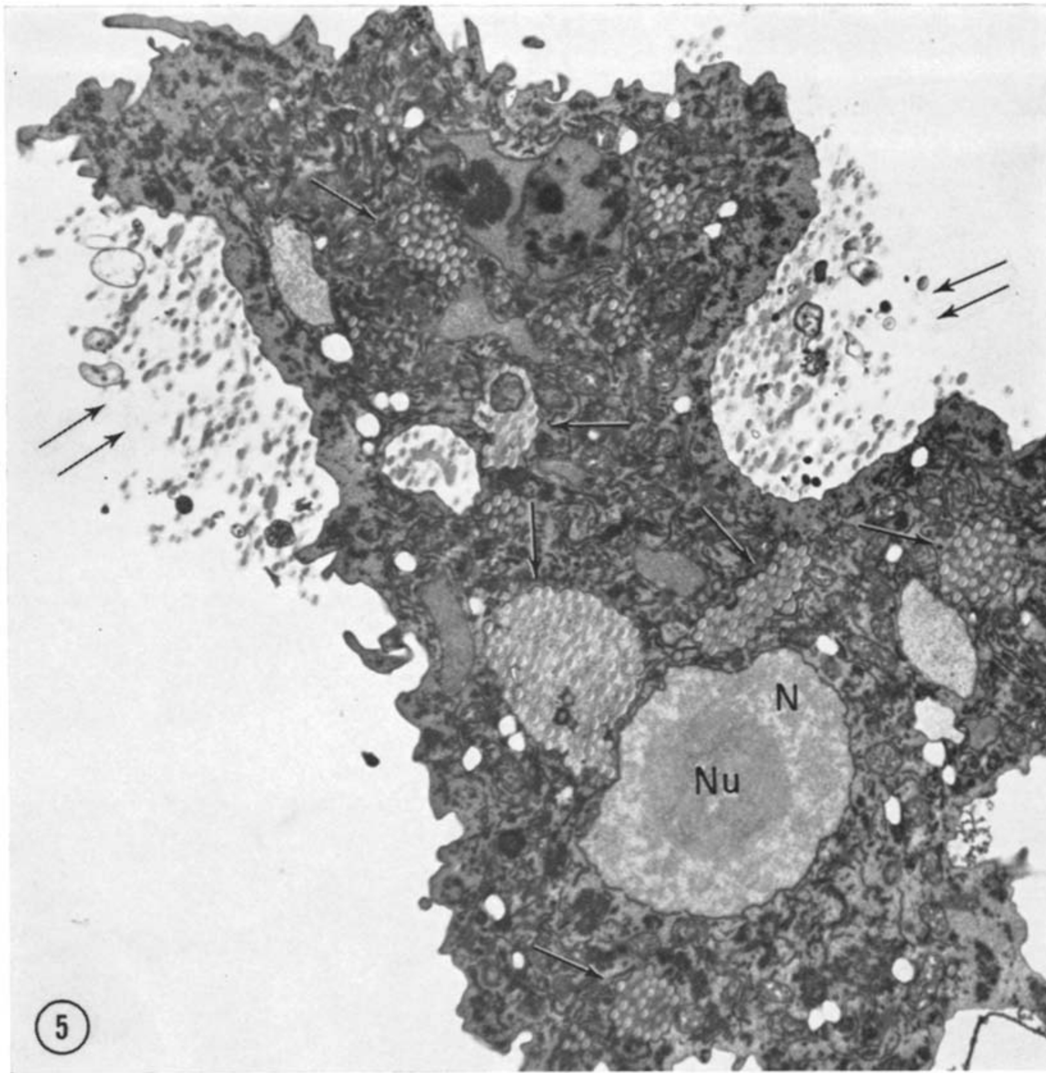

FIGURE 5 A section of an ameba in the process of phagocytosis of 0.264-μ latex beads. Note the several phagocytic vesicles (arrows) within the cell, each of which contains many beads, and the two areas of the cell surface (double arrows) where beads are accumulating and are in the process of ingestion. The nucleus (*N*) and nucleolus (*Nu*) are shown in this section. This picture should be contrasted to that in Fig. 1. In this experiment 3×10^6 amebae and 1.2×10^{11} beads (1.2 mg) were incubated per milliliter of 0.1 M glucose in 0.015 M phosphate, pH 6.7. Fixation was in permanganate–uranyl acetate and embedding in Durcupan. $\times$ 6800.

phthalate and 2 ml benzyldimethylamine. The Durcupan mixture contained 5 ml component A, 11.7 ml component B, 1.1 ml component C, and 0.3 ml component D. The fixed, dehydrated amebae were left overnight at 5° in 50% mixture of ethanol and embedding material. Two more equilibrations were carried out overnight at 5° in the embedding mixture alone. The samples were then placed in fresh embedding mixture in Beem capsules (Better Equipment for Electron Microscopy, Bronx, N.Y.) and polymerized at either 60° for 3 days (Maraglas) or at 42° for 18 hr (Durcupan). Thin sections were cut on a Servall Porter-Blum MT-2 microtome with a glass or diamond knife. Sections were picked up on 300-mesh,

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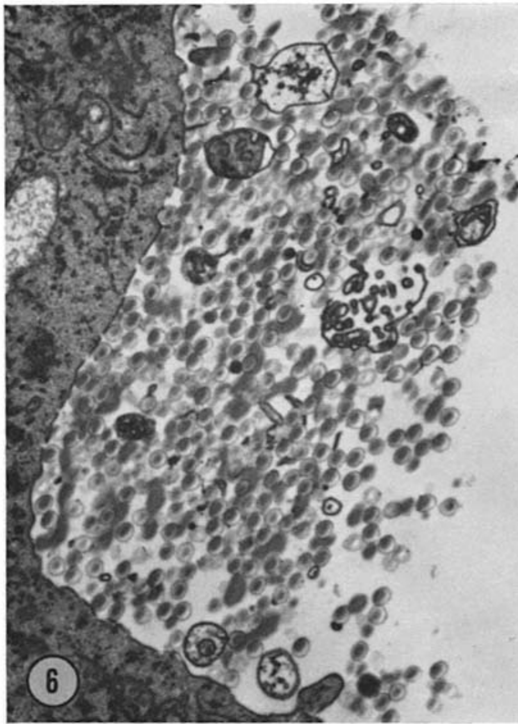

FIGURE 6 Latex beads (0.264 μ) accumulating at the surface of the cell. The beads are several hundred fold more concentrated than they were in the incubation medium. Concentration of beads at the cell surface is seen only when amebae are actively phagocytosing. This is the same experiment as that described in Fig. 5. × 8900.

unsupported copper grids, stained with lead citrate (6), and examined in an RCA-EMU 3G electron microscope at 50 kv.

The solubilities of polystyrene and polyvinyltoluene in organic solvents create certain difficulties. Propylene oxide cannot be used in the embedding procedure since it will completely or partially extract the latex beads from the cells. Beads that are bound to, but not yet inside, the cells are especially susceptible. Ethanol seems to have little or no effect on the latex beads. Epoxy resins at room temperature are also good solvents for the latex beads, but this problem can be diminished by working at low temperatures. Even so, the beads, especially the smaller ones, are often partially or totally dissolved during the embedding procedure. The solubility of the latex beads in organic solvents has not been commented on by some workers who have used them in studies of phagocytosis (7) but has been mentioned by Overman (8).

RESULTS AND DISCUSSION

Fig. 1 shows a low magnification view of the cross-section of an ameba fixed during the uptake of 1.9-μ latex beads. Micrographs of amebae ingesting beads with diameters of 2.68, 1.90, and 1.305 μ are indistinguishable except on the basis of the size of the bead. In all cases each bead is phagocytosed singly. The phagocytic membrane usually surrounds the bead very closely as shown in Fig. 2, but sometimes there is a little space between the membrane and the bead as in Fig. 3. The selectivity of the uptake of an individual bead is clearly seen in Fig. 4. No evidence has been found for specialized areas for phagocytosis at the cell surface. As many as 10 beads with diameters 2.68 μ

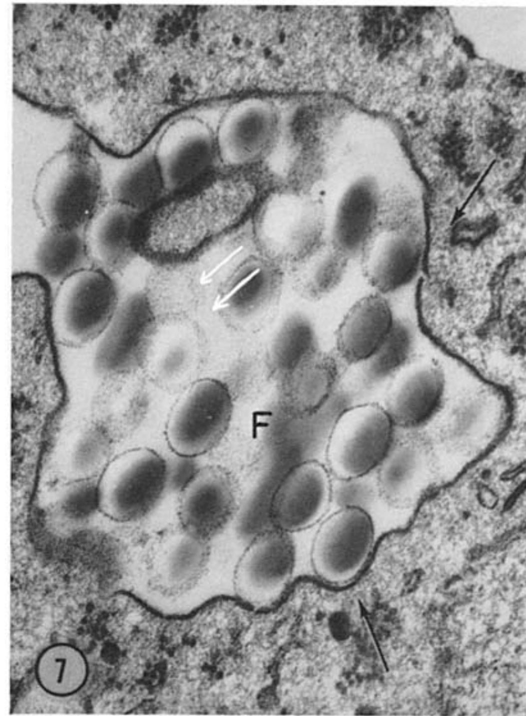

FIGURE 7 Phagocytic vesicle in the process of formation around 0.264 μ latex beads. The characteristic trilaminar appearance of the plasma membrane can be seen (arrows). The amorphous material surrounding the beads is very clear (white arrows). Some of the beads have been partially dissolved and fused (*F*) by the action of the embedding plastic. In this experiment 3×10^6 amebae and 1.2×10^{11} (1.2 mg) beads were incubated per milliliter of 1.5% proteose-peptone for 15 min. Fixation and embedding as in Fig. 5. × 39,100.

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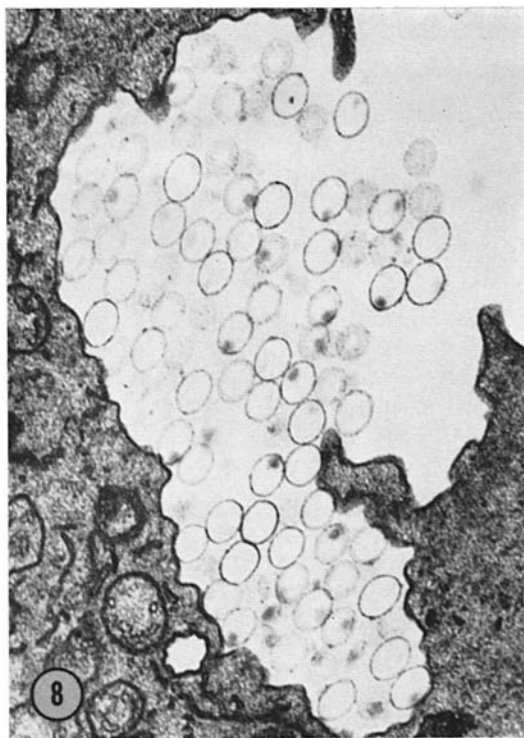

FIGURE 8 A phagocytic vesicle in the process of formation around beads with diameters of 0.264 μ. In this instance all of the polystyrene has been dissolved during the embedding procedure and the amorphous material that coats the beads is left as a ghost. In this experiment 3×10^6 amebae and 1.2×10^{12} (1.2 mg) beads were incubated per milliliter of proteose–peptone. Fixation was in permanganate–uranyl acetate and embedding in Durcupan. Ethanol was not used. $\times$ 20,600.

have been seen in the process of uptake in a cross-section that contained 14 ingested beads.

In contrast to the above description, latex beads with diameters of 0.577 μ and smaller are always seen to accumulate at the cell surface prior to their ingestion. This is shown at low magnification in Fig. 5 and at higher magnification in Fig. 6. Once the beads have reached a volume appropriate for uptake the highly concentrated mass is ingested, surrounded closely by the phagocytic membrane (Fig. 7).

If inert polystyrene and polyvinyltoluene latex beads accumulate at the surface of amebae, which have no visible extraneous slime coat, it is reasonable to assume that the amebae may secrete a substance that serves to bind the beads to the cell. No unequivocal evidence for this assumption has yet been obtained, but the smaller beads (Figs. 6 and 7) always seem to be surrounded by an amorphous layer. The amorphous material is most clearly seen where the polystyrene latex has been dissolved during the embedding procedure. Partial dissolution of the latex beads is apparent in Fig. 7, and the polystyrene has been completely dissolved in the area shown in Fig. 8. The material that had surrounded the beads is left behind.

The results of several types of control experiments are important. The amorphous material is seen better after fixation in the permanganate–uranyl acetate than after fixation in osmium tetroxide, and its contrast is further enhanced by lead staining. The results are the same when washed and dialyzed beads are used and when the incubations are carried out in 1.5% proteose–peptone or in 0.1 M glucose. Beads have never been found in hundreds of sections of amebae from experiments in which uptake of beads did not occur. These included experiments in which amebae and latex beads were incubated together in the presence of 2,4-dinitrophenol, which blocks uptake, and experiments in which the amebae and latex beads were added separately to the fixative. Therefore the beads are actively bound to the cells, and bound beads are much better preserved during the procedures of fixation and embedding than are preparations of pure beads.

Micrographs of sections of latex beads alone are difficult to obtain. In most attempts the beads were either totally dissolved or formed a molten mass in which individual beads were indistinguishable. Occasionally, sections of individual beads have been obtained, and these have never shown any amorphous coat. This is true even when the preparations have been fixed and stained in the usual manner. Three experiments were carried out in which beads were entrapped in agar after fixation in permanganate–uranyl acetate but before dehydration. In one of these experiments the latex beads were surrounded by amorphous material similar to that seen on beads undergoing phagocytosis. In the two other identical experiments the beads show no extraneous layer. It may be that this material is a contaminant of the beads but it seems equally likely that in this one control experiment the beads were coated with agar. In any event, it is certain

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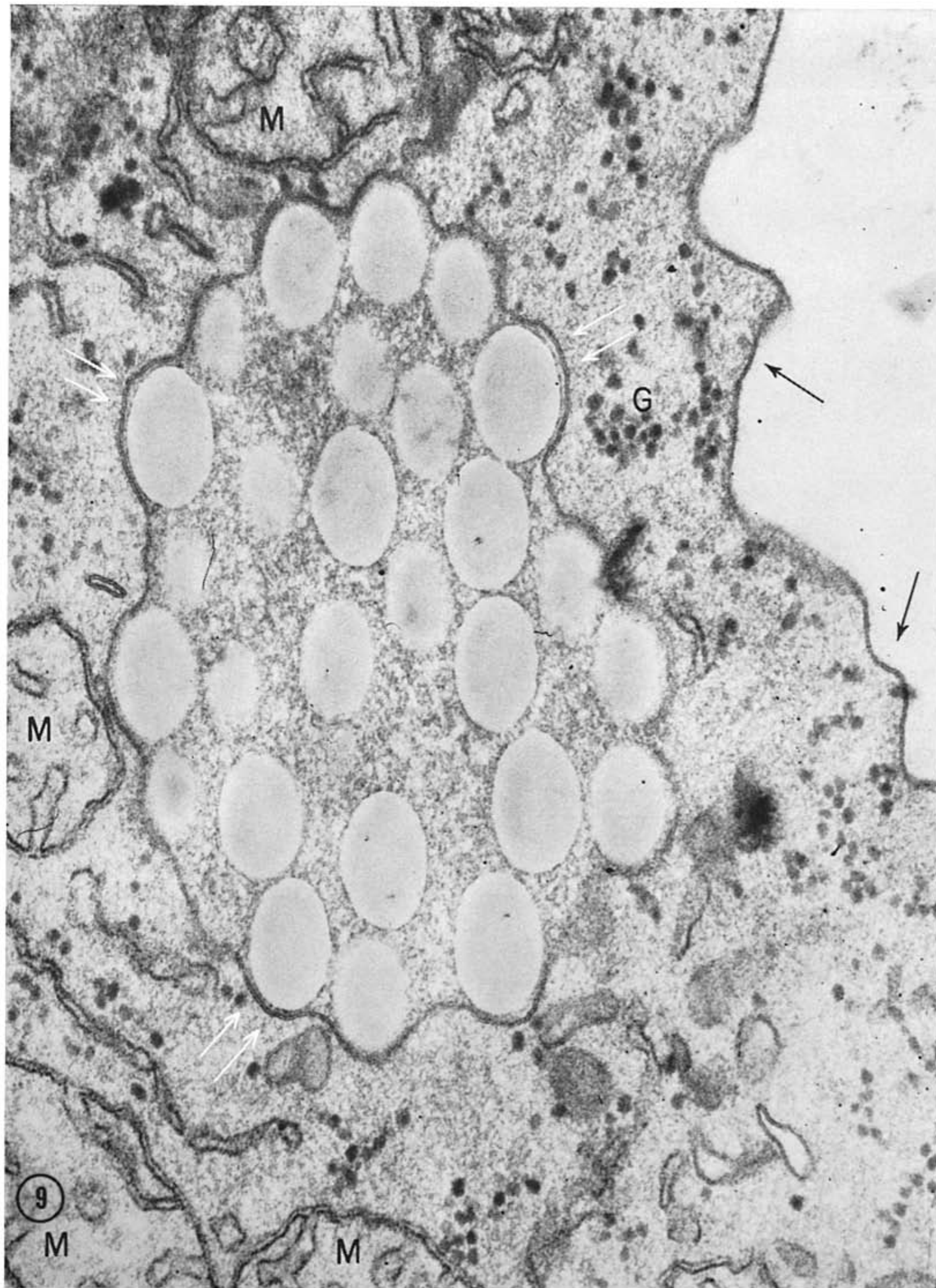

FIGURE 9 A phagocytic vesicle containing 0.264-μ latex beads. Note the typical trilaminar appearance of the plasma membrane (arrows) and the identical image of the vesicle membrane (white arrows). In this case, although the membrane surrounds the beads rather closely, there is appreciable amorphous material of unknown origin also in the vesicle. Mitochondria (*M*), glycogen (*G*). 3×10^6 amebae and 1.2×10^{11} beads with diameter of 0.264-μ were incubated per milliliter of 1.5% proteose–peptone. Fixation in permanganate–uranyl acetate and embedding in Maraglas. $\times$ 132,000.

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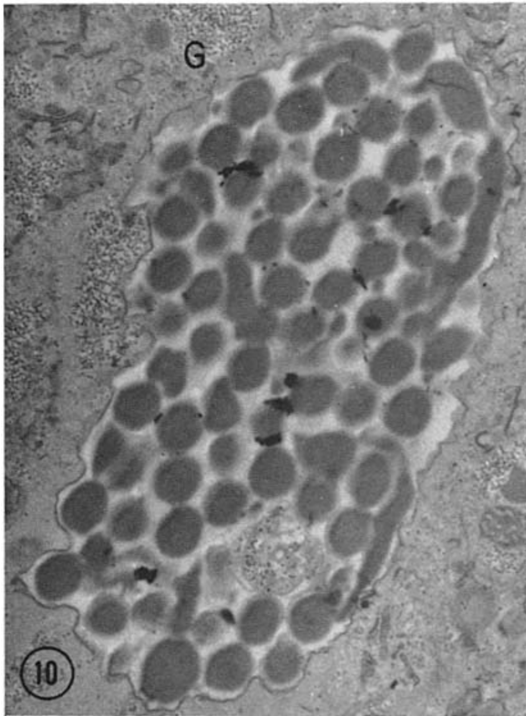

FIGURE 10 A phagocytic vesicle containing many beads with diameters of 0.557 μ. In this case the vesicle is rather fully packed with beads and seems to contain little else. Glycogen (*G*). This is the same experiment as Fig. 11. $\times$ 13,000.

that much more evidence must be obtained before it can be concluded that the amorphous material surrounding the latex beads in the micrographs represents a substance secreted by the amebae.

Individual vesicles containing small beads are shown in Figs. 9 and 10. The number of beads within any one vesicle can be very large. If the vesicle pictured in Fig. 10, for example, is assumed to be a sphere, it contains a minimum of 525 beads. That number of beads was present in $2.3 \times 10^4 \mu^3$ of incubation medium which is a volume almost six times as great as the mean cell volume (4000 μ^3) (1). The volume of the phagocytic vesicle, assuming it to be a sphere, is about 65 μ^3. The beads, then, were concentrated about 350 times at the cell surface before ingestion.

DISCUSSION

The electron microscopic evidence presented in this paper provides morphological support for the major conclusions drawn from the previous kinetic experiments (1). There is an optimal size for formation of the phagocytic vesicle that is satisfied by one bead with diameter of 1.305, 1.9, or 2.68 μ. Smaller beads are accumulated at the surface of the cell until they reach the critical volume, at which point they are ingested collectively within one vesicle. In all cases the vesicle is formed in such a way as to exclude most of the medium. The fact that an optimal size exists for the phagocytic vesicle may be a reflection of some macromolecular substructure in the plasma membrane or in the contractile mechanism by which the vesicle is formed.

There seems little doubt that the accumulation of the smaller latex beads is due to an active process of the amebae. Aggregations of beads were not detectable in the preparations at the time of addition to the incubation flasks, and those beads that were not ingested remained as individual particles. Clumps or aggregations of beads have never been seen in flasks in which phagocytosis was inhibited by anaerobiosis, dinitrophenol, or cyanide (1).

Brandt and Pappas (9) have reported that during the uptake of ferritin and thorium dioxide by *Pelomyxa carolinensis* the particles were bound to the hairlike projections of the surface membrane. The plasma membrane of *P. carolinensis* is covered with a thick layer of mucopolysaccharide that may serve as the binding substance. In the present experiment beads accumulated at a surface that has neither hairlike projections nor a visible extraneous coat. The process is similar to the binding of bacteria at the surface of *Hartmannella* sp observed by Ray (10). In both cases the amebae may secrete a substance that functions like the slime coat normally present on *P. carolinensis.*

The latex beads may have a slight negative charge, perhaps due to the anionic detergent. It seems unlikely that this is involved in the uptake process (1), and the latex beads are otherwise chemically inert. Therefore, the stimulus to all the events of phagocytosis is probably the mechanical contact between particles and ameba.

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