PHAGOCYTOSIS OF LATEX BEADS BY *ACANTHAMOEBA CASTELLANII* (NEFF)

III. Isolation of the Phagocytic Vesicles and Their Membranes

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ABSTRACT

A method is described for the rapid and efficient isolation of phagocytic vesicles from large scale cultures of *Acanthamoeba castellanii* (Neff) that have been incubated with polystyrene latex beads. Cells were allowed to phagocytose latex beads for 30 min and then were homogenized, and the phagocytic vesicles were isolated by one centrifugation through several layers of sucrose. Identity and purity of the phagocytic vesicles were determined by electron microscopy, chemical analyses, and assays of acid phosphatase, α- and β-glucosidase, and reduced nicotinamide adenine dinucleotide dehydrogenase. When phagocytosis was allowed to occur for longer periods the phagocytic vesicles appeared to fuse with each other and perhaps with digestive vacuoles. The resultant vesicles which contained many beads were heavier than those which consisted of only one bead or a few beads with a closely applied membrane. Ultrasonication ruptured the isolated vesicles, and the membranes could then be isolated in 30–50% yield based on phospholipid analysis. These membranes were essentially free of acid hydrolases and, presumably, other soluble proteins, as was also indicated by their low ratio of protein to phospholipid. The membranes have been prepared both as closed vesicles and as open sheets.

INTRODUCTION

The phagocytosis of latex beads by *Acanthamoeba castellanii* (Neff) involves a complex series of integrated events resulting in the selective ingestion of one or more beads within a phagocytic vesicle. The development of a quantitative assay allowed the study (Weisman and Korn, 1967) of a number of biochemical parameters of the process. This, together with electron microscopic evidence (Korn and Weisman, 1967), led to the conclusion that beads of diameter 1.305 μ and larger are ingested individually, while beads of diameter 0.557 μ and less are accumulated at the surface of the amebas prior to their ingestion as a compact mass. In all cases the phagocytic vesicle is formed from the surface membrane that is closely apposed to the ingested particles. So selective is the phagocytic process that amebas can ingest the beads from a volume of medium 100 times their own volume with no detectable uptake of glucose-^{14}C or of albumin-^{131}I dissolved in the medium (Weisman and Korn, 1967). The amebas are very active and can readily ingest sufficient beads to account for 15% of their own volume. In so doing, the cells simultaneously ingest a very

high percentage of their surface membrane. These facts, together with the inert nature of the latex beads and the absence of a detectable surface coat on the plasma membrane of the amebas[1] (Bowers and Korn, 1968), make this an attractive system for studying the physiology, biochemistry, and morphology of phagocytosis.

In this paper we describe a procedure for the isolation of phagocytic vesicles, in essentially one centrifugal step, from an homogenate of amebas that have been allowed to ingest latex beads of diameter 1.099 μ. Following brief sonication, the membranes of these vesicles can be quickly isolated in good yield. We present evidence for the chemical, enzymatic, and morphological purity of the isolated phagocytic vesicles and the membranes derived from them.

MATERIALS AND METHODS

Incubations

Acanthamoeba castellanii (Neff, 1957) was grown in 1 liter shaking cultures on proteose peptone-glucose medium, pH 6.7, as described by Weisman and Korn (1966). For most experiments 7-day cultures which had reached a cell concentration of 0.5–1.0 $\times$ 10^6 cells/ml were used. Polystyrene latex beads of diameter 1.099 μ (Dow Chemical Co., Midland, Mich.) were briefly sonicated to disperse any clumps and were added directly to the culture flask at a final concentration of 1 mg/ml (approximately 1.5 $\times$ 10^9 beads/ml, or 2000 beads/cell). These conditions vary slightly from those used by Weisman and Korn (1967).

The rate of phagocytosis was quantitatively measured by the method of Weisman and Korn (1967). Aliquots of 5 ml were taken from the incubation mixture and added immediately to 5 ml of 4 $\times$ 10^{-4} M dinitrophenol which instantaneously stops phagocytosis. The cells were then washed free of excess beads by centrifugation at 500 g, and the ingested beads were extracted with dioxane and quantitated by the absorption at 259 mμ. In all experiments samples were taken at 0, 10, 20, and 30 min after the addition of the latex beads to the incubation flask. In some experiments additional samples were taken at hourly intervals up to 4 hr. In several experiments the amebas were washed free of excess beads after incubation for 30 min, resuspended in fresh growth medium, and reincubated for 37 hr. In those experiments samples for analysis were removed at 2, 4, 8, 14, 26, and 37 hr.

[1] Bowers and Korn (1968) did describe the presence of occasional surface invaginations (coated pits) which contain amorphous material of unknown composition on their external surfaces, but these specialized regions comprise less than 1% of the area of the cell surface.

Isolation of Phagocytic Vesicles

The amebas were harvested by centrifugation for 5 min at approximately 500 g and washed three times with cold 0.02 M Tris buffer, pH 6.8 (0.02 M Tris [hydroxymethyl]aminomethane hydrochloride adjusted to pH 6.8 with Tris base), to remove uningested beads. The cells from a 1 liter culture flask were brought to a volume of 10 ml with Tris, and 10 ml of 60% (w/v) sucrose was added. The amebas were homogenized by 10 up and down strokes of the tight pestle of a 20 ml Dounce homogenizer (Kontes Glass Co., Vineland, N.J.). The homogenate was kept in an ice bath at all times.

10 ml of this suspension of disrupted cells in 30% sucrose were placed at the bottom of a centrifuge tube for the No. 30 rotor of the Spinco Model L centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) and were then overlayed with approximately 10 ml each of 25, 20, and 10% sucrose (w/v) and centrifuged for 90 min at 78,480 g. The phagocytic vesicles accumulated at the interface between the 10 and 20% sucrose layers (see Results) from which they were carefully collected after removal of the overlying 10% sucrose layer. This procedure takes advantage of the fact that the beads have a density of 1.05. In some experiments the suspension of phagocytic vesicles was diluted with an equal volume of 0.02 M Tris, and the phagocytic vesicles were collected as a pellet by centrifugation for 15 min at approximately 5000 g.

Isolation of Membranes

Two slightly different procedures were used for the isolation of membranes from the phagocytic vesicles. In one procedure, 10-ml aliquots of a suspension of phagocytic vesicles in 10% sucrose were ultrasonicated either for six 10-sec pulses or for two 30-sec pulses in the Branson Sonifier Model S-75 (Branson Instruments, Inc., Stamford, Conn.) at maximum intensity with 1 min intervals in between each pulse. The suspension was kept in an ice-water bath throughout the procedure. The suspension was then diluted with an equal volume of distilled water and centrifuged for 15 min at approximately 5000 g to remove the latex beads and any unruptured vesicles. Membranes were collected as a firm pellet when this supernatant solution was centrifuged for 60 min at 105,400 g in the Spinco No. 40 rotor.

In the alternate procedure, the phagocytic vesicles were pelleted and resuspended in 0.02 M Tris prior to ultrasonication as described above. The supernatant from the centrifugation at 5000 g was centrifuged for 30 min at 38,800 g in the Spinco No. 30 rotor to recover the membranes as a pellet.

Electron Microscopy

Prior to fixation, samples from the sucrose gradient centrifugation were diluted with water and pelleted by centrifugation. Whole cells and cell fractions were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate, pH 6.8, containing 1 mM calcium chloride for 45 min at 4°C. The specimens were washed several times with phosphate buffer and postfixed in 1% osmium tetroxide in the same buffer for 1 hr at 4°. The samples were then washed with water and dehydrated at 4° with a graded series of ethanol solutions up to 95% followed by two changes of absolute ethanol at room temperature. Propylene oxide could not be used because it dissolves the latex beads (Korn and Weisman, 1967). The specimens were soaked for several hours at room temperature in a solution containing equal parts of embedding mixture and absolute ethanol, were infiltrated in embedding mixture overnight at 10°C, and were then placed in fresh embedding mixture in B.E.E.M. capsules (Better Equipment for Electron Microscopy, Bronx, N.Y.) for polymerization at 60°C for 24–48 hr. The embedding mixture contained 26.25 g Epon 812 (Shell Chemical Co., New York, N.Y.), 7.90 g dodecenylsuccinic anhydride (Ladd Research Industries, Inc., Burlington, Vt.), 15.85 g Nadic methyl anhydride (Allied Chemical Co., Morristown, N.J.), and 0.75 g benzyl dimethylamine (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) (Luft, 1961). Thin sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome and were picked up on uncoated copper grids. The sections were stained for 5 min each in 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963) and were examined in an RCA EMU 3G electron microscope at 50 kv.

Enzyme Assays

All assays were performed on aliquots that were frozen immediately after their isolation and which were thawed just before their addition to the assay system. Acid phosphatase was assayed as described by Rosen et al. (1966), utilizing β-glycerophosphate as substrate. Controls were incubated either with heat-inactivated enzyme or in the presence of 2×10^{-3} M sodium fluoride as inhibitor. Nucleotidase activity was measured according to the method of Wallach and Kamat (1966). In addition to 5′-ATP, 5′-AMP, 5′-GMP, 5′IMP, 5′-UMP, and 2′,3′-cyclic AMP were employed as substrates. Heat-inactivated enzyme was used as a control. For both the acid phosphatase and nucleotidase assays, the release of inorganic phosphate was measured by the method of Chen et al. (1956).

Glucosidase activity was assayed according to Hestrin et al. (1955). Both α- and β-paranitrophenyl glucoside were used as substrates, and the release of paranitrophenol was measured spectrophotometrically at 410 mμ. Again, heat-inactivated preparations served as controls.

Reduced nicotinamide adenine dinucleotide dehydrogenase (NADH-dehydrogenase = DPNH diphorase) was assayed spectrophotometrically by measuring the decrease in absorbance at 340 mμ due to the oxidation of NADH. Readings were made at 15-sec intervals for 5 min at room temperature according to the method of Wallach and Kamat (1966).

Chemical Assays

Protein was measured by the method of Lowry et al. (1951). A slight turbidity due to the presence of latex beads was removed by filtering the final solution through a Millipore filter of pore size 0.45 μ.

Because of the solubility of polystyrene in chloroform-methanol and its subsequent interference with the analytical procedures, lipids were extracted by stirring each fraction with 15 volumes of absolute ethanol at room temperature for several hours. Control experiments had demonstrated that this method extracted all of the lipid that could be extracted by the standard procedure employing 19 volumes of chloroform:methanol, 2:1. The ethanolic solution was evaporated to dryness under reduced pressure, and the lipids were dissolved in chloroform-methanol which was then washed according to the procedure of Folch et al. (1957). The chloroform layer was evaporated to dryness, and the lipids were dissolved in chloroform and separated by chromatography on 1 g of silicic acid (Unisil; Clarkson Chemical Co., Williamsport, Pa.) into neutral lipids (eluted with 20 ml of chloroform) and phospholipids (eluted with 20 ml of methanol). Phospholipids were ashed to inorganic phosphate (Ames and Dubin, 1960) which was measured by the method of Chen et al. (1956). Triglycerides were measured by a slight modification of the ester assay of Rapport and Alonzo (1955). Steroids were measured by the Liebermann-Burchard reaction (Moore and Baumann, 1952) and by gas-liquid chromatography at 250°C on a column of 3% OV-17 (Applied Science Laboratories, State College, Pa.) (Smith and Korn, 1968). The latter analysis was essential because sterol derivatives, that were not detected by the colorimetric assay, were present.

Nucleic acids were extracted with either trichloroacetic acid (Schneider, 1957) or perchloric acid (Ceriotti, 1955) and measured by the absorption at 260 and 280 mμ. Pentose was determined by the orcinol reaction (Ceriotti, 1955), and deoxypentose by the diphenylamine procedure (Schneider, 1957).

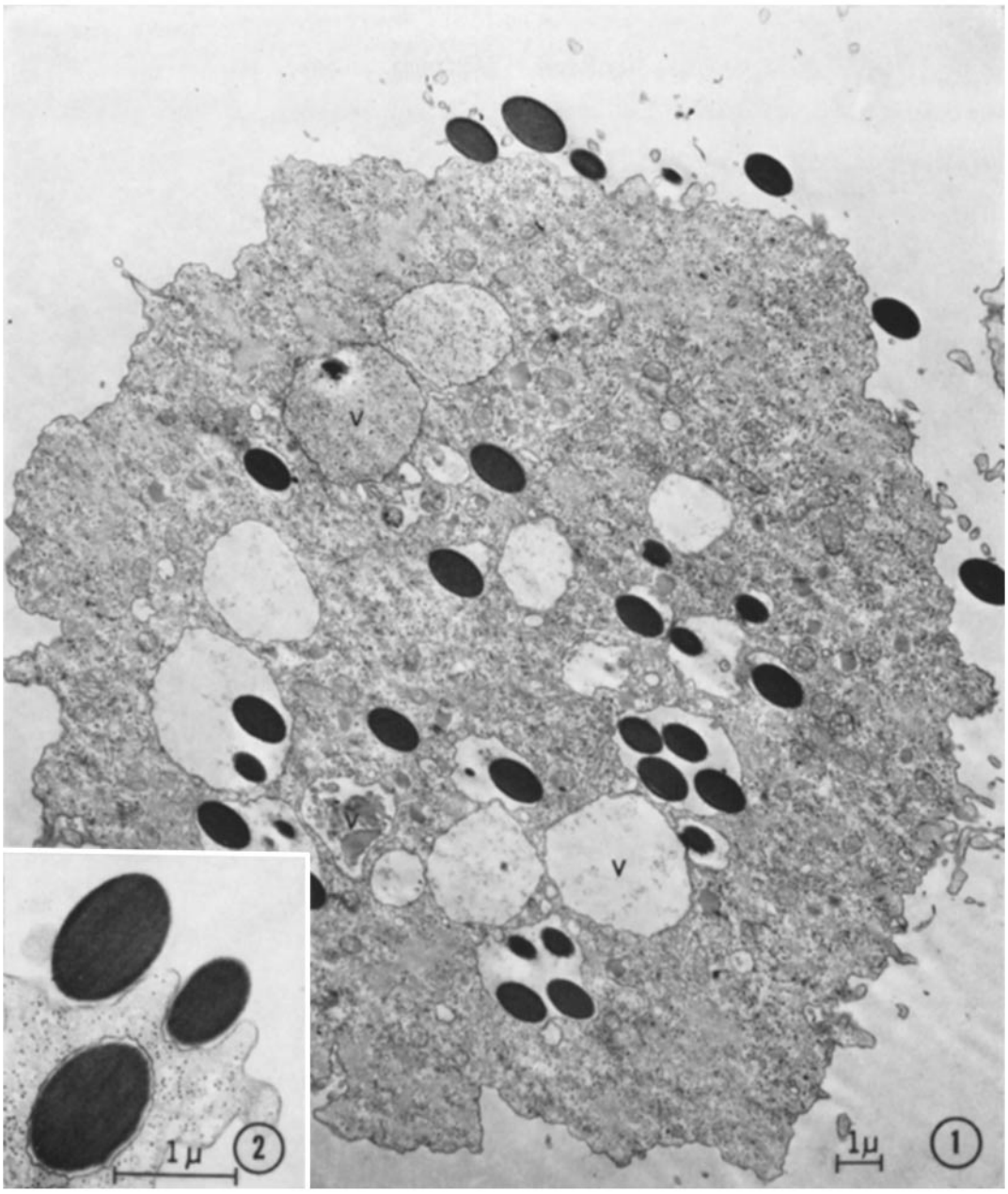


FIGURE 1 A typical cross-section of an *Acanthamoeba* following 30 min incubation with 1.099-μ beads. Note numerous large digestive vacuoles (*v*), some with a relatively empty appearance, others enclosing flocculent material, glycogen, or dense membranous material. Several beads may be seen surrounded by tightly apposed limiting membranes. Groups of beads may also be noted within larger vacuoles indistinguishable from digestive vacuoles. × 6,600.

FIGURE 2 Higher magnification of the cell periphery during active phagocytosis of polystyrene beads. 1-μ beads are typically engulfed singly by fusion of the tightly applied plasma membrane. Very little of the external milieu is engulfed during this phagocytic process. Glycogen particles may be seen scattered throughout the cytoplasm. × 17,100.

RESULTS

General Description of the Phagocytic Process

The rate and extent of phagocytosis are functions of a number of variables including the concentration of cells and beads, the degree of encystment, the age of the culture, the temperature of incubation, and the composition of the medium (Weisman and Korn, 1967). Under the conditions of the present experiments, the rate of uptake of polystyrene latex beads of diameter 1.099

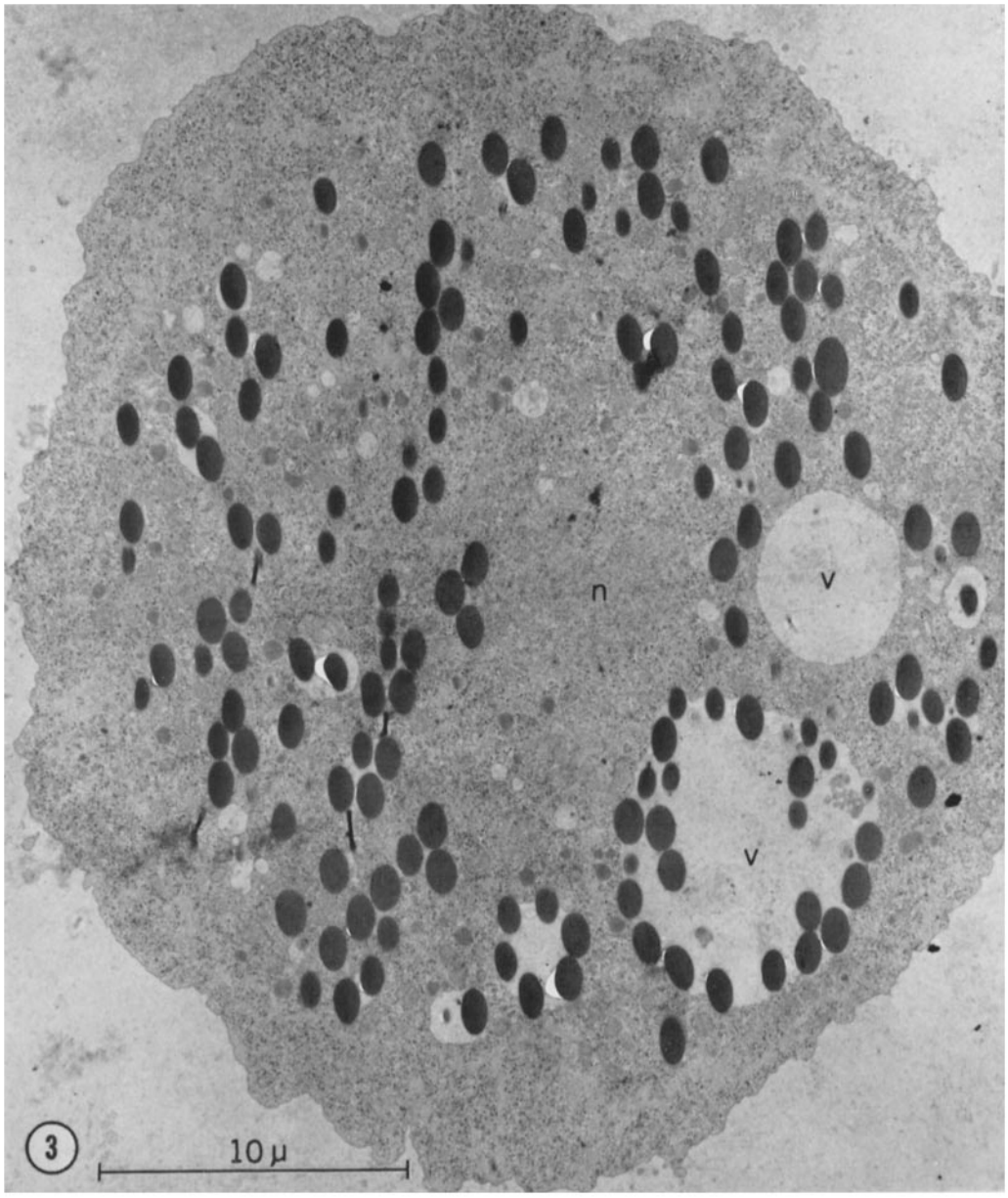


FIGURE 3 *Acanthamoeba* incubated with 1.099-μ diameter beads for 60 min. While the majority of beads remain scattered throughout the cytoplasm in small membrane-enclosed vesicles, some of the large digestive vacuoles contain considerable numbers of beads (*v*) 1 hr after initiation of phagocytosis. Nucleus (*n*). × 4,200.

μ was fairly constant for at least 30 min. At that time 10–20% of the beads which were added to the culture medium had been phagocytosed with an average uptake of 200–500 beads per cell. The rate of uptake diminished at about 1 hr, and phagocytosis essentially stopped after several hours.

As was anticipated from the results of Korn and Weisman (1967), electron microscopic observations indicated that the beads were, at least for the most part, ingested individually (Figs. 1–3). During active phagocytosis numerous microprojections occurred at the surface of the ameba. These were often seen in contact with the beads when the fixative was added directly to the incubation medium. The projections may become the sheets of cytoplasm that tightly enclose the beads as they are taken into the ameba.

In several experiments the amebas were pelleted gently after incubation for 30 min with beads and then resuspended in fresh medium without beads. Samples were taken for electron microscopy at 1, 1.5, 2, 4, 8, 26, and 37 hr after addition of the new medium. Progressive aggregation of the beads into larger vacuoles was observed, presumably due to the fusion of individual phagocytic vesicles with each other and, perhaps, with preexisting digestive vacuoles. The larger vacuoles often included flocculent material similar to that seen in digestive vacuoles (Bowers and Korn, 1968). The ultimate fate of the beads within the large vacuoles has not been elucidated by this study. Numerous aggregates of beads were still present within these vacuoles 37 hr after phagocytosis had occurred.

The fusion of the phagocytic vesicles is a continuous process which has been observed as early as 10 min after the initiation of phagocytosis. The accumulation of beads in larger vesicles is not extensive, however, until at least 2 hr after phagocytosis has begun. It should be emphasized that after 1 hr of incubation most of the ingested beads still appear to lie within the phagocytic vesicles with limiting membranes closely apposed to the surfaces of the beads (Fig. 3).

Isolation of Phagocytic Vesicles

Cells were homogenized, and the homogenate was fractionated by sucrose gradient centrifugation, as described in Materials and Methods, to obtain six fractions as illustrated in Fig. 4. The fractions were characterized by visual and electron microscopic observation as follows.

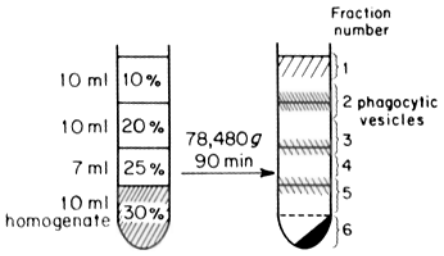


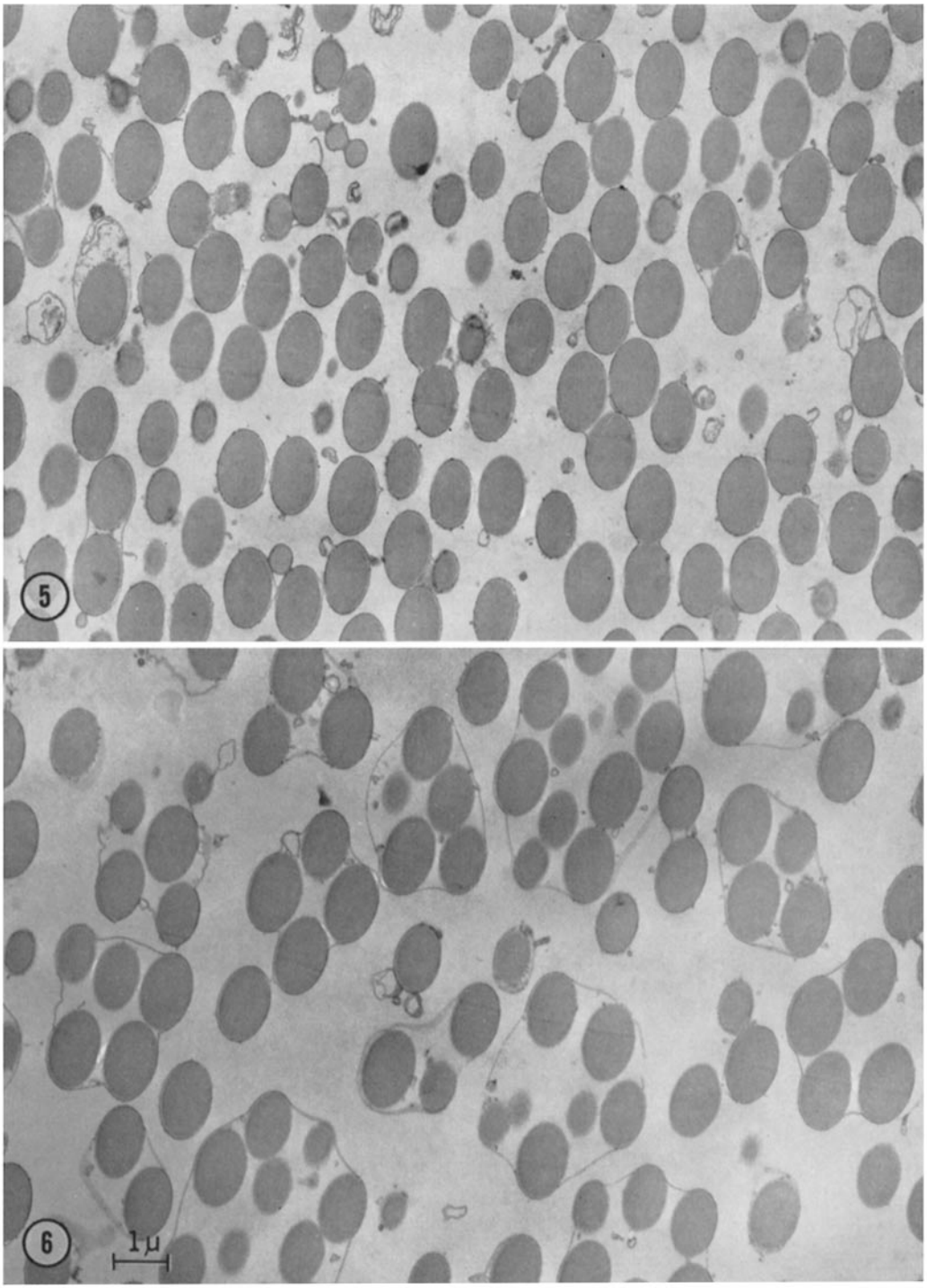
FIGURE 4 Schematic diagram of the procedure for isolating phagocytic vesicles surrounding 1.099-μ diameter polystyrene beads. A cell homogenate is fractionated by discontinuous sucrose gradient centrifugation in a No. 30 Spinco rotor.

Fraction 1, the upper half of the 10% sucrose layer, contained a cloudy white emulsion of neutral fat droplets.

Fraction 2, the layer at the 10–20% interface, contained most of the polystyrene latex beads. When diluted with water the beads were readily sedimented (see Materials and Methods) as a compact pellet suitable for electron microscopic, chemical, and enzymatic analyses. Most of the pellet consisted of one or a few beads tightly enclosed within a membrane (Fig. 5). A small percentage of the beads, located at the bottom of this pellet, were in groups of 2–15 beads (in thin section) within a single vesicle (Fig. 6). The distribution of beads within Fraction 2 was similar for samples prepared from amebas incubated with beads for 30 min or 2 hr. Approximately 10% of the beads were not surrounded by membrane in most experiments. These may represent beads that were not phagocytosed. Very little contaminating material was present in the vesicle fraction. An occasional mitochondrion or fragment of rough surfaced endoplasmic reticulum could be discerned.

Fraction 3 included both the 20% sucrose layer and material at the interface between the 20 and 25% sucrose layers. The solution was visually clear. The cloudy interface contained a few large membrane-enclosed aggregates of beads similar to those found at the bottom of the pellet prepared from Fraction 2, except for the presence of more electron-opaque, flocculent material within them. These may represent digestive vacuoles which have fused with the phagocytic vesicles. Profiles of rough surfaced endoplasmic reticulum and some glycogen particles were also present in this fraction.

Fraction 4, the visually clear 25% sucrose layer,



FIGURES 5 and 6 Micrographs from the top (Fig. 5) and bottom (Fig. 6) of a single pellet derived from the phagocytic vesicle fraction. Note closely applied vesicle membranes, particularly in the upper part of the pellet. An occasional mitochondrion and a few fragments of rough-surfaced endoplasmic reticulum represent the only identifiable contaminants in this fraction. × 9,000.

contained a few membrane fragments, mitochondria, and glycogen particles.

Fraction 5, material at the interface between the 25 and 30% sucrose layers, contained large cell fragments and digestive vacuoles that were rendered buoyant by the presence of polystyrene latex beads.

Fraction 6, the pellet at the bottom of the tube and the overlying 30% sucrose layer, included cysts, unbroken trophozoites, mitochondria, digestive vacuoles (all in the pellet), and small membrane fragments, free ribosomes, glycogen particles, and soluble protein (in the 30% sucrose).

From the visual and microscopic observations it was apparent that most of the phagocytosed beads banded in Fraction 2. This is confirmed by the quantitative data in Table I which summarizes the results of one typical experiment. Flask A was incubated without beads. Flask B was incubated with beads for 30 min, and the cells were washed and homogenized. Flask C was incubated with beads for 30 min, then the cells were washed and incubated for an additional 90 min without beads before homogenization. The three batches of cells were then fractionated into the usual six fractions, each of which was analyzed for polystyrene (beads) and protein. After incubation of the cells with beads for 30 min, 70% of the beads were isolated in Fraction 2, and most of the remainder were in Fractions 5 and 6. When cells were incubated for 2 hr, Fraction 2 still contained about 50% of the beads, but more appeared in Fraction 5 apparently because of the increased fusion of vesicles and the presence of dense material in the vesicles. Fraction 2 from the control cells was virtually free of protein, but Fraction 2 from the cells that had ingested beads accounted for about 3% of the total protein of the homogenate. This protein was derived from the membrane and content of the phagocytic vesicles.

Distribution of Enzymes

From the electron microscopic observations and the protein and polystyrene analyses, it was apparent that Fraction 2 consisted of phagocytic vesicles and little else. It was desirable to characterize the fraction further and to determine its purity by enzymatic analyses. All six fractions were analyzed for two enzymes, acid phosphatase and β-glucosidase, which were expected to be in the phagocytic vesicles, and one enzyme, NADH-dehydrogenase, usually associated with the endoplasmic reticulum and mitochondrial outer membrane (Sottocasa et al., 1967).

No detectable acid phosphatase was present in Fraction 2 derived from the amebas incubated without beads, but Fraction 2 contained 20–25% of the total acid phosphatase of the homogenate

TABLE I

Distribution of Polystyrene Latex Beads and Proteins Among Cell Fractions Isolated by Discontinuous Sucrose Gradient Centrifugation

Fraction	Volume			Polystyrene			Protein		
	A	B	C	A	B	C	A	B	C
		ml			*mg*			*mg*	
1	7.7	7.8	6.5	0	1.3	0.7	0.01	0.09	0.01
2	6.5	8.6	8.8	0	32.2	22.6	0.01	2.63	1.87
3	8.9	9.0	9.1	0	0.8	1.5	0.04	0.30	0.24
4	4.3	3.7	4.2	0	0.3	0.2	0.43	0.75	0.67
5	5.0	6.5	6.0	0	4.4	14.9	18.2	46.8	47.5
6	3.2	1.5	1.5	0	6.6	1.9	53.5	32.9	27.2
Total	35.6	37.1	36.1	0	45.6	41.8	72.19	83.47	77.49

Flask A was incubated for 30 min without latex beads. Flask B was incubated for 30 min with latex beads. Flask C was incubated for 30 min with latex beads; the cells were washed free of uningested beads and were reincubated for 90 min. Each flask contained 7.7×10^8 cells in a volume of 900 ml. The amebas were homogenized, and the fractions were obtained as described in Materials and Methods and as illustrated in Fig. 4. The contents of each flask were divided equally among three centrifuge tubes. The data are given for one such tube.

of amebas incubated with beads (Table II). When amebas were incubated for an additional 90 min after the ingestion of beads, somewhat more acid phosphatase was found in Fraction 5, which is consistent with the presence in that fraction of large digestive vacuoles that contain beads. The phosphatase activity was nonspecific, hydrolyzing ATP, AMP, GMP, IMP, UMP, and 2′,3′-cyclic AMP in addition to β-glycerophosphate. In all cases the optimal pH was below pH 5, and activity was very low above pH 7. The apparent loss of total acid phosphatase activity during phagocytosis may reflect a release of the enzyme into the medium as noted by Cohn and Wiener (1963), the presence of a specific inhibitor of acid phosphatase such as that described in the digestive vacuoles of *Chaos chaos* by Rothschild (1966), or some inaccuracies in measuring the very high activity in Fraction 6 of the control cells.

Very similar results were found for β-glucosidase (Table II). No activity was found in Fraction 2 from the control cells, but about 15% of the total enzyme was in Fraction 2 prepared from the cells that had phagocytosed beads. Also, after 2 hr there appeared to be a shift of enzyme from Fraction 6 to Fraction 5. The optimal pH for this enzyme was also below pH 5. The same distribution of activity was found when β-paranitrophenylgalactose or α-paranitrophenylglucose was utilized as substrate instead of β-paranitrophenylglucose.

A very different distribution was found for NADH-dehydrogenase (Table II). Only about 1% of the total enzymatic activity appeared in Fraction 2. These differences are more clearly shown by comparing the specific activities of the three enzymes in the six fractions derived from homogenates of amebas which had been incubated with latex beads (Table III). The specific activity of acid phosphatase was at least 5 times greater in Fraction 2 than in Fraction 6 where most of the total activity occurred. Similarly the specific activity of β-glucosidase was 3–5 times greater in Fraction 2 than in Fraction 6. For the latter enzyme the specific activity of Fraction 3 was also high, although the total amount of enzyme in that fraction was very low (Table II). In contrast to the two acid hydrolases, the specific activity of NADH-dehydrogenase was always lower in Fraction 2 than in Fraction 6.

The data in Tables I–III were all taken from one preparation. The distributions of protein and acid phosphatase have been confirmed in three other preparations, and the distribution of β-glucosidase and NADH-dehydrogenase have been confirmed in two of these. In these latter two experiments no NADH-dehydrogenase was found above Fraction 4. The enzymatic analyses, therefore, confirm the electron microscopic evidence that Fraction 2 consisted of phagocytic vesicles and very little else.

Several preparations have been analyzed for

TABLE II

Distribution of Enzymes Among Cell Fractions Isolated by Discontinuous Sucrose Gradient Centrifugation

	Acid phosphatase			β-Glucosidase			NADH-dehydrogenase		
Fraction	A	B	C	A	B	C	A	B	C
		units			*units*			*units*	
1	0.01	0	0	0	0	0	0	0	0
2	0	3.22	2.46	0	6.2	4.4	0	0.11	0.28
3	0	0.06	0.31	0	0.9	0.9	0.03	0.09	0.01
4	0	0.02	0.01	0	0.4	0.1	0.06	0.08	0.08
5	1.45	3.41	4.73	1.6	7.8	11.2	2.65	4.65	4.05
6	16.8	7.88	4.57	37.0	25.1	14.3	16.4	7.72	8.01
Total	18.3	14.6	12.1	39	40.4	30.9	19.2	12.7	12.4

These data were obtained from the same experiments as those in Table I.

Units: 1 unit of acid phosphatase is the amount of enzyme that will hydrolyze 1 μmole β-glycerophosphate/min. 1 unit of β-glucosidase is the amount of enzyme that will hydrolyze 1 μmole β-paranitrophenylglucoside/min. 1 unit of NADH-dehydrogenase is the amount of enzyme that will utilize 1 μmole NADH/min.

TABLE III

Specific Activity of Enzymes in Cell Fractions Isolated by Discontinuous Sucrose Gradient Centrifugation

Fraction	Acid phosphatase			β-glucosidase			NADH-dehydrogenase		
	A	B	C	A	B	C	A	B	C
	units/mg protein			*units/mg protein*			*units/mg protein*		
1	(0.81)*	0.03	0	0	0	0	0	0	0
2	0	1.23	1.32	0	2.4	2.4	0	0.04	0.15
3	0	0.20	1.27	0	2.9	3.8	0.68	0.31	0.02
4	0	0.03	0.01	0	0.5	0.2	0.13	0.11	0.12
5	0.08	0.07	0.10	0.1	0.2	0.2	0.15	0.10	0.09
6	0.31	0.24	0.17	0.7	0.8	0.5	0.31	0.23	0.29

These data are derived from the same experiment as those in Tables I and II. Enzyme units are as in Table II.

* This erroneously high value was not repeated in three other experiments. It is due to the errors involved in measuring very small amounts of acid phosphatase (Table II) and protein (Table I).

protein, phospholipid, sterol, glycerides, RNA, and DNA. The weight ratio of protein to phospholipid varied between 4 and 6 which is higher than might be expected for membranes (Korn, 1969), and undoubtedly reflects the presence of soluble proteins within the phagocytic vesicle (see below). The molar ratio of sterol to phospholipid was reproducibly between 0.72 and 0.78, a value typical of plasma membranes (Korn, 1969). About 60% of the sterol was detectable by the Liebermann-Burchard reaction and was confirmed by quantitation of ergosterol and dehydrostigmasterol by gas-liquid chromatography. Those two sterols were present in the same ratio, about 2:3, as that found for the sterols of the whole cell (Smith and Korn, 1968). Two additional compounds were detectable by gas-liquid chromatography which are not present in extracts of the whole amebas. These have been tentatively identified as benzenoid derivatives formed by dehydrogenation and rearrangement of ergosterol and dehydrostigmasterol during the isolation of the vesicles. Proof of their structure will be presented elsewhere. The molar ratio of glyceride to phospholipid varied between 0.7 and 2.3. It is not certain whether the glycerides are true components of the phagocytic vesicle or represent contamination by neutral lipid. The weight ratio of RNA to protein was between 0.004 and 0.01, and DNA was not detectable.

Isolation of the Membrane Surrounding the Phagocytic Vesicle

Phagocytic vesicles were disrupted by ultrasonication, and the membranes were isolated by high-speed centrifugation after removal of the latex beads and unbroken vesicles by low-speed centrifugation (see Materials and Methods). The results of two such experiments are shown in Table IV. In one, the fractions were analyzed for protein and acid phosphatase (as a marker for the soluble contents of the phagocytic vesicle), and in the other for protein and phospholipid (as a marker for the membrane). These experiments also demonstrated that a somewhat purer preparation of phagocytic vesicles can be obtained by diluting Fraction 2 and sedimenting the vesicles at low speed prior to ultrasonication. All of the acid phosphatase and 70–90% of the protein were recovered under these conditions (Table IV, Experiment 1 control, low-speed pellet). Such preparations were entirely free of NADH-dehydrogenase.

When vesicles were subjected to ultrasonication, about 50% of the acid phosphatase was inactivated. Of the remainder, most was recovered in the low-speed supernatant indicating the release of the soluble proteins from within the vesicle. The low-speed pellet contained all of the latex beads and undisrupted vesicles. The membrane pellet obtained by high-speed centrifugation contained about 11% of the original protein and 30% of the original phospholipid. This fraction had very

TABLE IV

Distribution of Protein, Phospholipid, and Acid Phosphatase During the Isolation of Membranes from Phagocytic Vesicles

Experiment	Fraction	Protein		Acid phosphatase		Phospholipid: sonicated
		Control	Sonicated	Control	Sonicated	
		mg		*units*		*μmoles*
1	Phagocytic vesicles	0.84		0.80		
	Low-speed pellet	0.74	0.22	0.77	0.01	
	Low-speed supernatant	0.12	0.62	0.03	0.26	
	High-speed supernatant	0.10	0.41	0.00	0.03	
	High-speed pellet	0.01	0.19	0.01	0.01	
2	Phagocytic vesicles	13.8				3.84
	Low-speed pellet	9.3	5.0			1.53
	Low-speed supernatant	—	9.4			—
	High-speed supernatant	0	4.9			0.94
	High-speed pellet	0.5	1.5			1.19

In experiment 1 the phagocytic vesicles were sonicated for two 30-sec intervals, diluted with an equal volume of water, and centrifuged at 5000 *g* for 15 min. The supernatant solution from the low-speed centrifugation was centrifuged for 60 min at 105,400 *g*. Protein and phosphatase values represent the yield from approximately 1.2×10^8 cells.

In experiment 2 the vesicles were sonicated for six 10-sec intervals, diluted with an equal volume of water, and centrifuged for 15 min at 5000 *g*. The supernatant solution was then centrifuged for 90 min at 38,880 *g*. Further centrifugation of that solution for 6 hr at 38,880 *g* did not sediment any more material. Protein and phospholipid values represent the yield from 2.7×10^9 cells.

1 unit of acid phosphatase is the amount of enzyme that will hydrolyze 1 μmole β-glycerophosphate/minute.

little acid phosphatase and no detectable NADH-dehydrogenase. In several experiments this membrane fraction has been found to have a protein to phospholipid ratio between 1.5 and 1.6. This ratio is lower than that found for plasma membranes from mammalian cells (Korn, 1969) and approaches that found for the yeast protoplast membrane (Mendoza and Villanueva, 1967). DNA was undetectable, and RNA was less than 1%. Total sterols have not been measured by gas-liquid chromatography, but the ratio of Liebermann-Burchard reacting sterols to phospholipid was the same as in the original vesicles.

The electron microscopic appearance of the membrane pellet may depend on the preparative procedures. The membranes have been obtained as closed vesicles of diameter 0.7–500 mμ (Fig. 7) by sonication of the phagocytic vesicles in 10% sucrose. Detectable contamination consisted of glycogen particles, occasional profiles of rough surfaced endoplasmic reticulum, and unidentified fibrillar masses.

A microscopically more homogeneous preparation of membranes as open sheets was obtained when the phagocytic vesicles were pelleted and resuspended in Tris buffer prior to sonication (Fig. 8). Much less glycogen and few microsomes appeared to be present, but amorphous aggregates of unknown composition were seen. The protein, lipid, and nucleic acid analyses of these membranes was the same as those of membranes prepared by the previous method.

DISCUSSION

A useful procedure for the isolation of phagocytic vesicles should be fast (to minimize degradative processes) and provide a high yield of vesicles of high purity. The procedure described in this paper requires only one centrifugation of a whole homogenate to recover about 70% of the phagocytic vesicles. The yield could undoubtedly be improved by using a large capacity swinging bucket rotor (SW 27, for example) since most of the loss is due to the adherence of vesicles to the sides of the tubes in the angle rotor.

Routinely, the amebas ingest 250–300 beads per cell in 30 min. Assuming, as appears to be the case (Korn and Weisman, 1967, and this paper), that each bead is ingested singly, closely surrounded by plasma membrane, this would require internaliza-

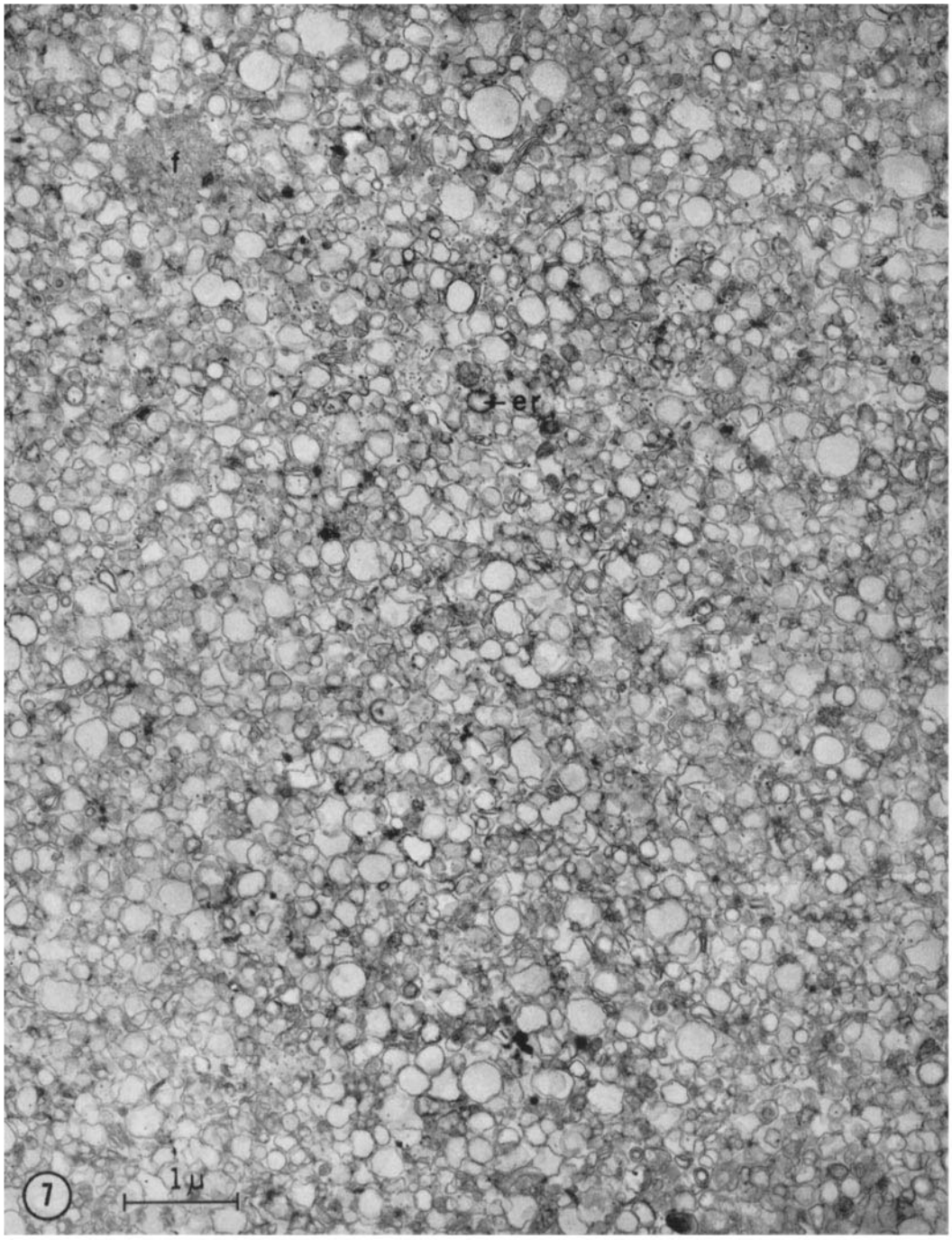


FIGURE 7 Membrane fraction prepared from isolated phagocytic vesicles following ultrasonication in 10% sucrose. The membranes formed closed vesicles when ultrasonication was performed in 10% sucrose. Numerous small glycogen particles may be seen (presumably released from the bead-containing vesicles), as well as occasional microsomes (*er*) and aggregates of fibrillar material (*f*). × 16,900.

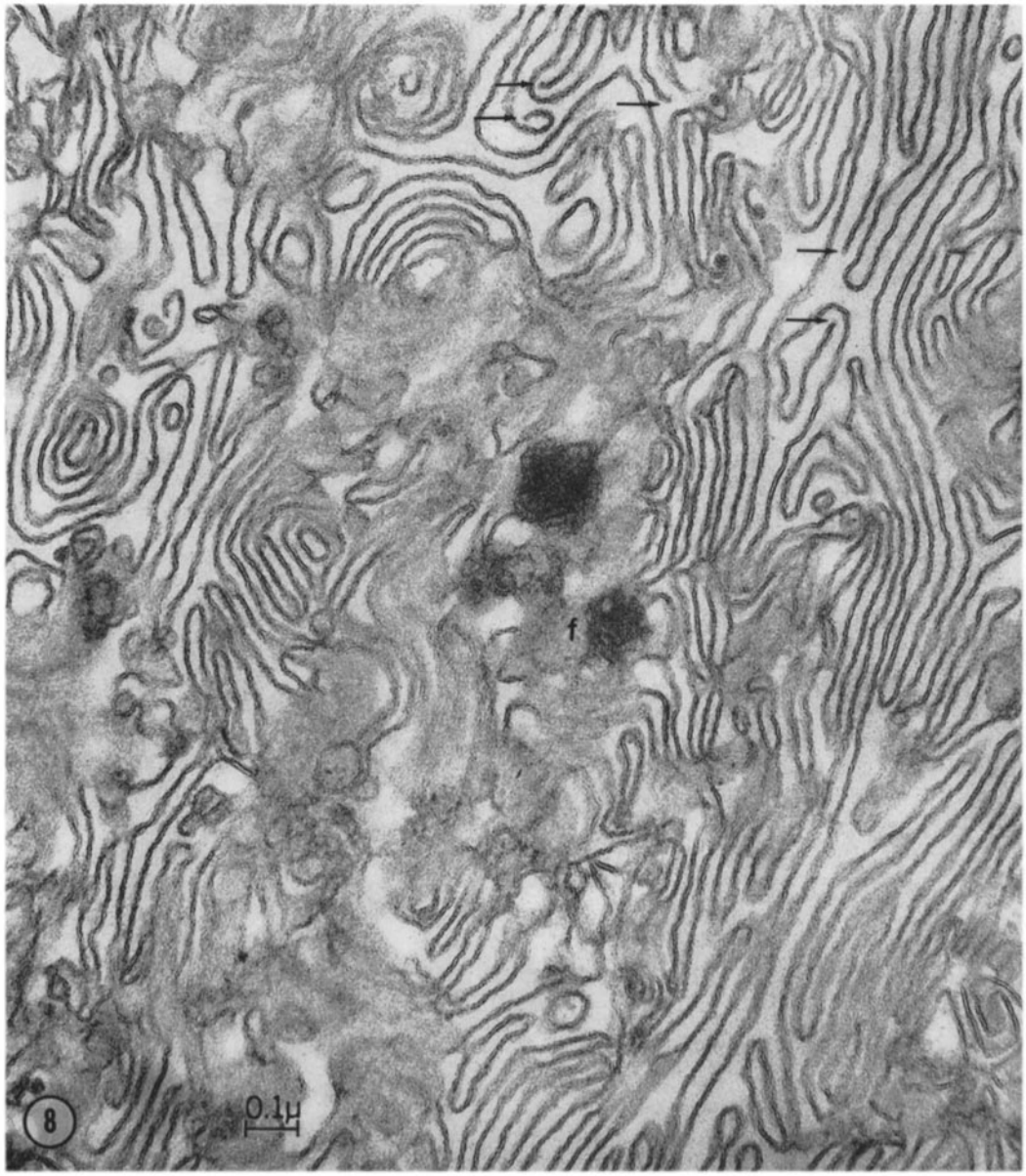


FIGURE 8 Membrane fraction prepared from isolated vesicles following ultrasonication in 0.02 M Tris buffer. Open sheets of membrane were formed when the phagocytic vesicles were resuspended and sonicated in Tris buffer. Arrows indicate edges of the membrane sheets seen in cross-section. Poorly defined grey areas represent obliquely cut membrane. Ultrastructurally, these phagocytic membranes have dimensions similar to those of plasma membranes of the amebas. Occasional glycogen particles and dense fibrous masses (*f*) represent the only identifiable nonmembrane components of this purified fraction. × 75,000.

tion of at least 950–1150 μ^2 of membrane (the surface area of 250–300 beads of diameter 1.099 μ) per cell. An average ameba, when rounded by exposure to dinitrophenol, has a diameter of 26 μ and a calculated surface area of about 2100 μ^2 (Bowers and Korn, 1969). Thus, the ingested membrane is a rather high percentage of the total surface membrane. The fact that this membrane sur-

rounds inert plastic is also advantageous for biochemical experiments.

The electron microscopic, chemical, and enzymatic analyses indicate that the phagocytic vesicles are of sufficient purity for most experimental purposes, many of which should not require the further isolation of the phagocytic membrane. For example, the phospholipids and sterols of the vesicle are almost certainly contained in the surrounding membrane. It will be of great interest to compare the detailed composition and metabolic turnover of the lipids of this membrane to that of the lipids of the plasma membrane isolated directly. Changes in composition of the lipids of the phagocytic vesicle may also occur following the internalization of these derivatives of the plasma membrane. Some of these studies are currently in progress in this laboratory.

It will also be of interest to examine changes in the enzymatic content of the phagocytic vesicles with time. We do not know the source of the acid hydrolases in *Acanthamoeba* nor the ultimate fate of the vesicle and its contents. Other investigators have studied the intracellular localization of hydrolytic enzymes in *Chaos chaos* (Holter and Lowy, 1959; Müller et al., 1962; Lagunoff, 1965; Chapman-Andresen and Lagunoff, 1966; Rothschild, 1967), and several have noted a redistribution of acid phosphatase from small, scattered cytoplasmic granules into the newly formed phagocytic vesicles (Müller et al., 1962; Lagunoff, 1965; Chapman-Andresen and Lagunoff, 1966). *Acanthamoeba* is obviously rich in acid hydrolyses but, except for their presence in the autolysosomes of encysting cells (Bowers and Korn, 1969), we know nothing of their cytoplasmic distribution. The density distributions of several enzymes usually associated with lysosomes have been measured by Müller (1969), after sucrose gradient centrifugation of homogenates of *Ancathamoeba*. It seems likely from his data that particles similar to lysosomes must be present in the ameba, but they have not yet been identified with morphological elements in the trophozoite.

In mammalian systems it is generally believed (see Novikoff et al., 1964; Cohn et al., 1966; de Duve and Wattiaux, 1966; Dingle, 1968) that hydrolytic enzymes such as acid phosphatase are synthesized in the rough endoplasmic reticulum from which they move to the Golgi region to be enclosed in small vesicles that are pinched off as primary lysosomes. These can fuse with pinocytic vesicles or phagocytic vesicles (Cohn and Wiener, 1963) to form secondary lysosomes (heterolysosomes). Pinocytic vesicles may also fuse with existing secondary lysosomes. Such organelles may be formally equivalent to the digestive vacuoles of amebas. We do not know if phagocytic vesicles in the ameba acquire their content of hydrolytic enzymes by fusion with digestive vacuoles, with "primary" lysosomes, or with both.

Mammalian lysosomes have a density that is very similar to the densities of mitochondria and peroxisomes (de Duve and Baudhuin, 1966). When rats are injected intraperitoneally with Triton WR-1339, the detergent accumulates in hepatic lysosomes making them significantly less dense than the mitochondria and peroxisomes from which the lysosomes can then be separated by isopycnic centrifugation (Wattiaux et al., 1963, Leighton et al., 1968). Conversely, dextran has been used to increase the density of the lysosomes (Baudhuin et al., 1965). These precedures are conceptually similar to the one described in the present paper but differ significantly in that they introduce serum proteins into the lysosomes (Trouet, 1964) and require a waiting period of 3 days between the injection of the detergent and the excision of the liver.

For many experimental purposes it will be necessary to study the isolated membrane of the phagocytic vesicle. From the data presented in this paper, it appears likely that the membrane preparation described is of suitable purity, but more extensive evidence is needed to supplement this preliminary characterization. The low protein to phospholipid ratio, the absence of contaminating enzymatic activity, and the electron microscopic appearance all attest to the relative purity and homogeneity of the isolated membranes. Details of the sterol and phospholipid composition of these membranes will be of interest as will, of course, studies on their protein and possible enzymatic content.

It appears likely that the membrane surrounding vesicles isolated after incubation of the cells with beads for 30 min, is largely composed of newly ingested plasma membrane. In this preparation, most of the beads are still individually surrounded by a tightly applied membrane. With time, fusion of vesicles with each other and probably with digestive vacuoles occurs; this may lead to changes in the membranes. Such fusion processes may be the source of the glycogen particles

that contaminate the vesicle and membrane preparations since glycogen is present in some digestive vacuoles (Fig. 1) and autolysosomes (Bowers and Korn, 1969). The procedures described in this paper provide a useful method for studying these possible changes in membrane composition and properties.

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