Phagocytosis of Latex Beads by *Acanthamoeba*. I. Biochemical Properties*

Robert A. Weisman and Edward D. Korn

ABSTRACT: *Acanthamoeba* sp. take up polystyrene and polyvinyltoluene latex beads. There appear to be no requirements for specific organic molecules for uptake providing the appropriate osmotic conditions are maintained. This requirement can be satisfied by glucose, mannitol, inulin, or proteose–peptone. The proper osmolarity depends on the previous history of the amoebas and they can adapt to an unfavorable osmolarity. Uptake is inhibited by 2×10^{-5} M dinitrophenol, 2×10^{-3} M cyanide, and 2×10^{-4} M azide but not by 4×10^{-2} M fluoride or 2×10^{-3} M iodoacetate. Uptake is optimal at 30–35°. A quantitative measure of bead uptake has been developed by application of the dioxane extraction method of J. Roberts and J. H. Quastel (*Biochem. J. 89*, 150 (1963)). The rate of uptake was found to be a linear function of the time of incubation and the concentration of cells over a broad pH range. Once taken in, beads are retained by amoebae for at least 4.5 hr. The rates of uptake of each of six different size beads (0.126–2.68 μ) were directly proportional to the concentration of beads; the data give straight line in a Lineweaver–Burk plot. When calculated on a weight basis, the values for K_b (concentration of beads resulting in half the maximal velocity) were identical for all six sizes of beads and the values for V_{max} were the same for all sizes of beads within a factor of 2. Therefore, the kinetics of uptake are a function of bead mass (or volume) and not of bead number, surface area, or diameter. The uptake of beads is highly selective, there being essentially no uptake of [^{14}C]glucose or [^{131}I]albumin during the ingestion of 25–30% of the beads present in the same incubation medium. Ingestion of beads of diameter 2.68 μ was inhibited by the presence of beads of diameter 0.126 μ. Taken all together the data indicate that beads are bound to, and accumulate at, the cell surface until an optimum volume, approximately the same for all sizes, is reached, at which time the beads are ingested by a mechanism that excludes the incubation medium. This interpretation has been confirmed by electron microscopic observations which will be published elsewhere.

Phagocytosis, the ingestion of particulate material, can be the sole nutritional mechanism in protozoa and serves a major protective role in metazoa. The morphological details of phagocytosis have not yet been thoroughly studied but, in general, the attachment of a particle to the surface of the cell seems to initiate a complex series of events in which either the plasma membrane, with the attached particulate material, invaginates or the cell flows outward progressively surrounding the particle. Eventually the leading edges of the plasma membrane meet, pinching off a membrane-bound vesicle that contains the particle. The vesicle then moves into the cytoplasm where, normally, digestion of its contents occurs, possibly after fusion with lysosomes (Hirsch and Cohn, 1964).

Pinocytosis is a morphologically similar process that was originally thought to be a mechanism for fluid ingestion (Mast and Doyle, 1934), but which now is believed to be a concentrative mechanism for the uptake of solute molecules (Chapman-Andresen and Holter, 1964). Phagocytosis and pinocytosis may be different manifestations of the same basic process, merely involving materials of different sizes (Brandt and Pappas, 1960). These processes are of fundamental interest because they may be a major mechanism of cellular transport and because they provide a system for the study of membrane function and metabolism and for the study of biochemical mechanisms for the control and integration of a complex series of morphological events.

With a few notable exceptions studies of phagocytosis have involved qualitative microscopic or chemical observations on a very few cells. Karnovsky and his collaborators, however, have obtained quantitative data on metabolic changes associated with the phagocytosis of starch granules and polystyrene latex beads by polymorphonuclear leucocytes and alveolar macrophages (Sbarra and Karnovsky, 1959; Oren *et al.*, 1963). An increased oxygen uptake occurs during phagocytosis that is associated with an activation of a cyanide-sensitive NADPH[1]-oxidase (Cagan and Kar-

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[1] NADPH, reduced nicotinamide–adenine dinucleotide phosphate; PVT, polyvinyltoluene latex beads; PS, polystyrene latex beads.

novsky, 1961). Zatti and Rossi (1965) have suggested that the increased oxygen uptake may be due to increased interaction of NADPH and the oxidase. During phagocytosis there also seems to be a more rapid incorporation of orthophosphate into phosphatidic acid, phosphatidylinositol, and, perhaps, phosphatidylserine (Sbarra and Karnovsky, 1960; Kasnovsky and Wallach, 1961; Berger and Karnovsky, 1966; Sastry and Hokin, 1966).

In this paper we describe some of the general features of the phagocytosis of latex beads by *Acanthamoeba*. A quantitative assay has been developed, based on the method of Roberts and Quastel (1963), that allows the measurement of initial rates of uptake. This system is particularly well suited to a quantitative study of the biochemical parameters of phagocytosis.

Acanthamoeba is available in essentially unlimited quantity as a homogeneous population of cells grown on a soluble medium (Neff *et al.*, 1958) or on a defined medium (Adam, 1959). Latex beads cannot, of course, be metabolized and any metabolic changes occurring during their phagocytosis must, therefore, be related to the uptake process rather than to the subsequent digestion of the ingested material. Also, the latex beads are chemically inert and interactions between them and amoebas should be simpler to analyze than the interactions of cells with more complex particles such as bacteria or proteins. Finally, the availability of a range of latex beads differing in size but chemically identical provides a unique opportunity to study the influence of the size of the ingested particle on the phagocytic process.

Materials and Methods

Monodisperse preparations of polystyrene latex beads of diameters 0.126, 0.264, 0.557, and 1.305 μ, and of polyvinyltoluene latex beads of diameters 1.90 and 2.68 μ, were generously provided by Mr. L. J. Lippie of the Dow Chemical Co. According to the manufacturer the various preparations consist of approximately 99% polymer, 0.5% sulfonate detergent, and 0.5% inorganics of unspecified nature. The detergent is necessary for the emuslion polymerization reaction by which the products are synthesized. The beads in each preparation are uniform in size within standard deviations of about 1–3%.

Fatty acid free bovine serum albumin (Weisman and Korn, 1966) was iodinated with ^{131}I by the Radiopharmaceutical Service of the National Institutes of Health according to the method of Helmkamp *et al.* (1960). After iodination the albumin was dialyzed against distilled water to remove any low molecular weight radioactive impurities that might have been present. Uniformly labeled [^{14}C]glucose was obtained from the New England Nuclear Corp. Proteose–peptone and inulin were obtained from the Difco Co. A molecular weight of 518 was assumed for inulin. Fibrinogen was from the Armour Co., and contained 45% sodium citrate. Thrombin was the gift of Dr. E. Mihalyi. Spectral quality dioxane was from the Matheson Co., Inc. All other chemicals were reagent grade.

Acanthamoeba sp. (Neff, 1957) was grown in proteose–peptone–glucose medium at pH 6.7 as previously described (Weisman and Korn, 1966).

Cells were incubated with latex beads in 50-ml ehrlenmeyer flasks on a Dubnoff shaking water bath at 30°, unless otherwise specified. Usually uptake was stopped by adding 2,4-dinitrophenol to a final concentration of 2×10^{-4} M.

In the first experiments uptake of beads was measured by counting the number of beads in each of the cells of a field selected at random. At a magnification of 400×, up to 12 beads/cell could be counted accurately. The exact number of beads in cells with more than about 12 could not be determined. This method has the advantage of providing data on the range of activity of individual cells but suffers certain major disadvantages. Only a limited number of cells can be examined and these might not be representative; the method is only semiquantitative with very active preparations. Nevertheless, useful information can be obtained by this assay (Sbarra and Karnovsky, 1959).

In later experiments a quantitative assay was developed based on the method of Roberts and Quastel (1963). After incubation with beads, amoebas were collected by low-speed centrifugation and washed two or three times with 0.015 M phosphate buffer to remove extracellular beads. The amoebas were then extracted with 2 ml of dioxane for at least 30 min, but usually overnight, and the cell residue was removed by centrifugation. The latex is completely soluble in dioxane and can be quantitated by measuring the absorbance at 259 mμ for polystyrene latex or at 267 mμ for polyvinyltoluene latex. At a concentration of 1 μg/ml the absorbance of polystyrene is 2.3×10^{-3} and the absorbance of polyvinyltoluene is 3.4×10^{-3}.

For the determination of the uptake of [^{14}C]glucose and [^{131}I]albumin the washed cell pads were dissolved in 2 ml of NCS reagent (Nuclear-Chicago Radiochemical Bulletin No. 6) at room temperature. A sample of 1 ml was transferred to a scintillation vial, diluted with 10 ml of 0.4% 2,5-diphenyloxazole in toluene, and counted in a scintillation spectrometer. The radioactivity of aqueous solutions (0.1 ml) was also determined in a one-phase system containing 10 ml of 0.4% 2,5-diphenyloxazole in toluene, and 1 ml of the NCS reagent. Corrections for quenching were made from standard curves prepared for each isotope (Braillie, 1960).

Frozen sections were prepared by the method of Horn *et al.* (1964). Amoebas were washed twice with 0.015 M KH_2PO_4, pH 6.7, suspended in 5% fibrinogen, centrifuged, and the cell pad was clotted by 1 drop of thrombin solution. The cells were fixed for 1 hr in the cold in 10% formaldehyde, rinsed with cold 7.5% sucrose, and sections 2 μ thick were cut in the cryostat.

Results

Microscopic Appearance of Amoebas after Uptake. The ability of amoebas to take up latex beads is

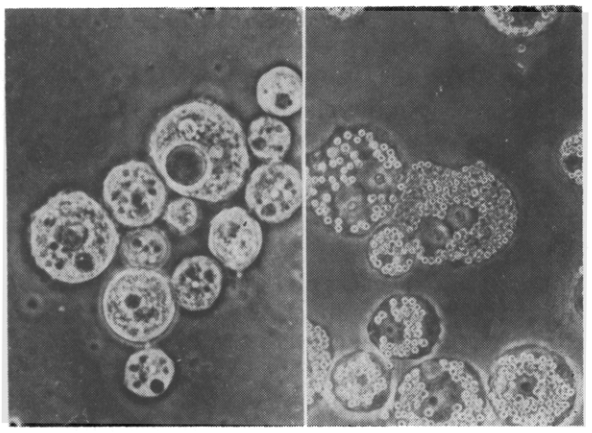

FIGURE 1: Microscopic appearance of *Acanthamoeba* after ingestion of latex beads. (left) Phase micrograph of control cells; (right) phase micrograph of cells after incubation.

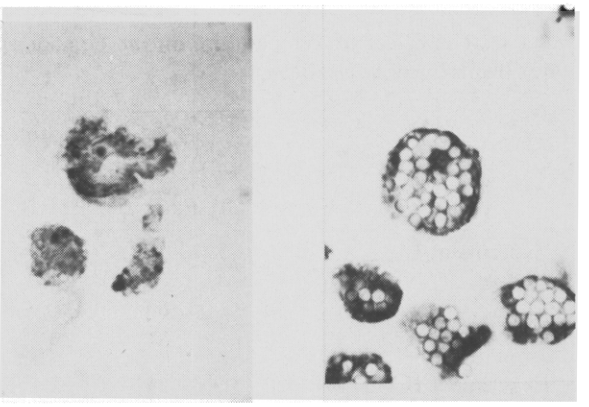

FIGURE 2: Microscopic appearance of frozen sections (2 μ) of *Acanthamoeba* after ingestion of latex beads. (left) Control cells; (right) cells after incubation with polyvinyltoluene latex beads (2.68 μ). A description of the preparation of the sections is given under Materials and Methods.

dramatically shown by micrographs of amoebas after incubation at 30° with and without polyvinyltoluene latex beads of diameter 2.68 μ (Figure 1). To verify that the beads were in fact inside the cells rather than adhering to the surface of the amoebas, frozen sections of cells were examined (Figure 2). The beads were in the same focal plane as the structural elements of the cytoplasm, indicating that the beads were within the cells. Subsequent electron microscopic observations on ultrathin sections have confirmed this conclusion and provided detailed evidence of the morphological basis of the process (E. D. Korn and R. A. Weisman, unpublished data).

Measurement of Uptake by Particle Count. The data shown in Table I illustrate the advantages and limitations of this method of assay. It can be seen that with increased time of incubation more cells contained beads and, among the cells that contained beads, there were more beads per cell. Both effects also occurred as the concentration of beads in the incubation medium was increased. The total number of beads taken up, however, could only be estimated because of the large number of beads that often occur in a single cell. Thus this assay must be considered only semiquantitative. In subsequent tables data obtained by counting beads are presented in a simplified form. The percentage of cells that contained beads is recorded without regard to the number of beads in each cell. The same conclusions could be reached by totaling the number of beads taken up by all the cells in the sample but with less accuracy because more than 12–15 beads in one cell cannot be counted.

Effect of Metabolic Inhibitors and Anaerobiasis. The optimum temperature for uptake (Table II) is between 30 and 35°. The amoeba grows maximally at 29–31° and not at all at 37° (Neff *et al.*, 1958). Inhibitors of oxidative metabolism (dinitrophenol, azide, and cyanide) inhibit phagocytosis, but the inhibitors of glycolysis (fluoride and iodoacetate) do not have a significant effect (Table III). This was true when the inhibitors were added to the cells at the same time as the beads as well as when the inhibitors and cells were incubated together for 25 min before the

TABLE I: Measurement of Phagocytosis of Latex Beads by Bead Count.[a]

Beads/ml of Medium	Incubn (min)	Number of Cells Containing the Indicated Number of Beads/Cell												
		0	1	2	3	4	5	6	7	8	9	10	11	>11
14×10^6	15	64	9	8	6	6	1	2	0	1	1	1	0	1
70×10^6	15	59	7	8	2	3	1	1	4	3	0	2	1	9
14×10^6	30	46	14	8	11	0	3	3	4	4	0	1	0	6
70×10^6	30	20	10	3	3	4	3	3	4	3	0	3	2	42

[a] Amoebas (7.0×10^6) were incubated in 5 ml of 1.5% proteose–peptone in 0.015 M KH_2PO_4, pH 6.7. Uptake was stopped by the addition of dinitrophenol at a final concentration of 2×10^{-4} M. A total of 100 cells was counted for each sample.

TABLE II: The Effect of Temperature on the Uptake of Latex Beads by *Acanthamoeba*.[a]

	Incubn Temp (°C)	% of Cells with Beads	
		Flask A	Flask B
Experiment I	0	5	3
	10	9	13
	20	60	56
	30	84	79
Experiment II	20	34	22
	25	65	57
	30	88	90
	35	92	85
	40	35	44
	45	2	1
	50	0	0

[a] Reaction mixtures contained 2.2×10^7 cells and 6.6×10^8 (6.6 mg) polyvinyltoluene latex beads (2.68 μ) in a total volume of 5.0 ml of 1.5% proteose–peptone, in 0.015 M KH_2PO_4, pH 6.7. The cells and beads were equilibrated at the desired temperature separately before mixing. Incubations were stopped at 15 min by the addition of 2×10^{-4} M 2,4-dinitrophenol. Samples of 100 cells were counted.

TABLE III: Effect of Metabolic Inhibitors on the Uptake of Latex Beads by *Acanthamoeba*.[a]

Inhibitor	Concn (M)	% of Cells with Beads
None		95
Fluoride	4×10^{-2}	83
Iodoacetate	2×10^{-3}	87
Cyanide	2×10^{-2}	3
	2×10^{-3}	24
	2×10^{-4}	54
Azide	2×10^{-3}	0
	2×10^{-4}	28
Dinitrophenol	2×10^{-3}	3
	2×10^{-5}	0

[a] The reaction mixture contained 2.2×10^7 cells, 1.1×10^8 (1.1 mg) polyvinyltoluene latex beads (2.68 μ), and inhibitors as indicated in a total volume of 5 ml of *Acanthamoeba* growth medium, pH 6.5. Incubation was for 30 min at 30°. Samples of 30–40 cells were counted.

addition of the beads. No uptake occurred when air was replaced by nitrogen. The inhibition by dinitrophenol is immediate and, therefore, it has been used to stop the reaction at desired times. Beads that were taken up prior to the addition of dinitrophenol were retained by inhibited cells for at least 3 hr.

Factors Necessary for Uptake and Osmotic Effects. In the first experiments incubations were carried out in complete growth medium which contained 1.5% proteose–peptone, 0.082 M glucose, 2×10^{-4} M methionine, several vitamins, low concentrations of Mg^{2+}, Ca^{2+}, and Fe^{2+}, and 0.003 M KH_2PO_4, pH 6.7 (Korn, 1963). Later, experiments were carried out to determine which components of the growth medium, if any, were required. In these experiments optimal activity was usually found with 1.5% proteose–peptone alone. Glucose, 0.08 M, would, however, inconsistently support as much activity as proteose–peptone. Cells were completely inactive in 0.15 M NaCl–0.015 M KH_2PO_4, pH 6.7.

The variability of buffered 0.08 M glucose to support phagocytosis is explained by the data in Table IV. It was found that when cells were incubated with latex beads as soon after collection as was practicable the optimum molarity of glucose in the incubation medium was about 0.16. (In later experiments in which uptake was measured by the quantitative spectrophotometric assay, 0.12 M glucose was found to be optimum with a fairly sharp drop in activity at lower and higher molarities.) When cells were incubated for 2 hr at 30° in 0.015 M KH_2PO_4 before the addition of beads, however, the optimum molarity of glucose for bead uptake was found to be very much lower.

TABLE IV: The Effect of the Concentration of Glucose on the Uptake of Latex Beads by *Acanthamoeba*.[a]

Concn of Glucose in Incubn Medium (M)	% Cells with Beads	
	No Preincubn	Cells Preincubd for 2 hr in 0.015 M KH_2PO_4, pH 6.7
0	4	18
0.016	3	13
0.032	6	18
0.08	2	6
0.16	31	4
0.32	9	1
0.65	0	0

[a] Cells were collected, washed, and suspended in 0.015 M KH_2PO_4, pH 6.5. One set of flasks was prepared at the concentrations of glucose indicated and immediately incubated with beads for 15 min at 30°. An identical set was preincubated for 2 hr at 30° in 0.015 M KH_2PO_4. Glucose was then added and the cells were incubated with beads for 15 min at 30°. In both sets the final incubation mixture contained 1×10^7 cells and 1.1×10^8 (1.1 mg) polyvinyltoluene latex beads (2.68 μ) in 5 ml. Samples of 100 cells were counted.

TABLE V: The Lack of Specificity of the Incubation Medium for the Uptake of Latex Beads by *Acanthamoeba*.[a]

Pretreatment of Amoebas at 30°	Incubation Condition				
	0.125 M KH_2PO_4	0.125 M Glucose	0.125 M Inulin	0.125 M Mannitol	1.9% Proteose–Peptone
None	12	56	44	52	60
90 min in 0.015 M KH_2PO_4	24	16	16	32	16
90 min in 0.015 M KH_2PO_4; then 60 min in incubation medium	24	72	68	44	72

[a] Amoebas were incubated in the absence of latex beads for varying periods of time in the solutions indicated. The cells were subsequently incubated with latex beads in the media indicated for 15 min at 30°. In the second incubation all vessels contained 1.1×10^8 (1.1 mg) of polyvinyltoluene latex beads (2.68 μ) in 5.1 ml of 0.015 M KH_2PO_4, pH 6.7, and other components as indicated.

In fact the cells were partially active even when incubated with latex beads in 0.015 M phosphate alone. In parallel experiments not shown in Table IV, it was found that when amoebas were incubated in 0.32 M glucose for 2 hr before addition of the beads, the cells adapted and 58% showed uptake when subsequently incubated with beads in 0.32 M glucose. Amoebas were unable to adapt to 0.65 M glucose. These data suggest that the optimum osmolarity of the incubation medium is a function of the internal osmotic pressure of the amoebas and that, given sufficient time, the amoebas can adapt to a wide but not unlimited, range of osmotic conditions. The varying ability of glucose to support uptake originally observed might reflect a variable physiological state of the cells at the time of collection and the length of time they were in dilute buffer before the incubation.

From these data it would appear that the only requirement for organic molecules for phagocytosis of latex beads is to provide an incubation medium of appropriate osmolarity. Probably, neither proteose–peptone nor glucose served as a significant source of energy in these experiments. This conclusion is supported by the fact that amoebas were just as active when incubated in buffered mannitol or inulin as when incubated in glucose or proteose–peptone (Table V). The ability to adapt to varying osmotic conditions could again be demonstrated. Activity was reduced when the amoebas were incubated for 90 min in dilute buffer before incubation with latex beads in media of higher osmolarity, and activity returned following an additional 60-min incubation in glucose, mannitol, inulin, or proteose–peptone before addition of latex beads. In similar experiments not shown here cells remained fully active when incubated for 150 min in 0.125 M glucose or 1.5% proteose–peptone.

The uptake process is sensitive to the ionic strength of the incubation medium; cells were inactive when incubated with beads in KH_2PO_4, pH 6.7, over a concentration range of 1.4×10^{-3}–1.4 M. Although partial activity could be recovered after periods of adaptation to buffers of low ionic strength the amoebas were never observed to adapt to buffers of high ionic strength.

Quantitative Determination of Uptake. The foregoing data demonstrate that the particle-count assay is adequate to define some of the characteristics of phagocytosis in amoebas. But it is obviously inadequate

TABLE VI: Recovery and Reproducibility Data for the Quantitative Spectrophotometric Assay.[a]

	Polyvinyltoluene	
	mg	%
Beads added to incubation	32.4	100
Beads extracted from cells	6.31 ± 0.20[b]	15.9
Beads recovered from medium	24.03 ± 0.99	74.2
Total beads recovered	30.34 ± 1.19	93.7

[a] Four identical reaction mixtures contained 3×10^6 cells/ml and 3.6 mg/ml of polyvinyltoluene latex beads (2.68 μ) in 9 ml of 1.5% proteose–peptone in 0.015 M KH_2PO_4, pH 6.7. The amoebas and beads were equilibrated separately at 30° and then mixed. Samples (1 ml) were taken at zero time and at 30 min and added to 1 ml of 4×10^{-4} M dinitrophenol (DNP). The cells were collected and washed twice with 0.015 M KH_2PO_4, pH 6.7. The cell pads were carefully drained of excess fluid and extracted for 30 min with 2 ml of dioxane. The concentration of polyvinyltoluene in the dioxane extract was calculated from the absorbance at 267 mμ. The beads remaining in the supernatant medium after incubation for 30 min were collected by high-speed centrifugation and dissolved in dioxane. [b] Standard deviation of the mean.

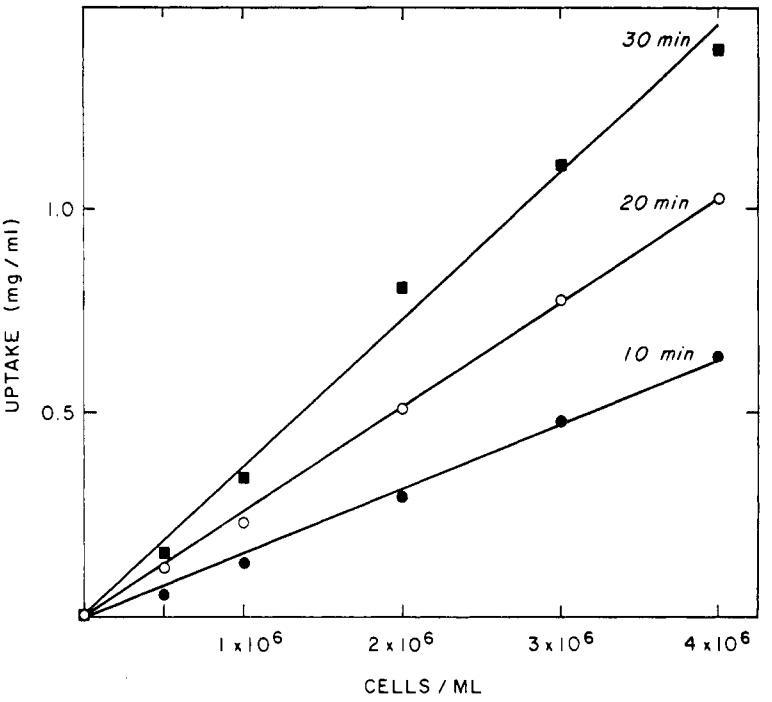

FIGURE 3: The uptake of latex beads as a function of the concentration of amoebas. Three identical reaction mixtures contained 20 mg of polystyrene latex beads (1.305 μ) and cells as indicated in 9 ml of 1.5% proteose-peptone in 0.015 M KH_2PO_4, pH 6.7. Postincubation treatment was as described for Table VI. Each point represents the average of the three values with cell blanks substracted.

for detailed kinetic experiments and for investigations of the initial biochemical events of phagocytosis in which it is necessary to measure accurately the initial rate of reaction. For these purposes we have developed a quantitative assay based on the absorbance of dioxane extracts of cells that have taken up latex beads. Preliminary experiments showed that extraction for 30 min was sufficient to dissolve and extract all the polyvinyltoluene or polystyrene from amoebas that had taken up latex beads. Controls demonstrated that in the presence of dinitrophenol no measurable bead uptake occurs and that extracellular beads are removed completely by the washing procedure. The dioxane extracts of the cells incubated with beads had the ultraviolet absorption spectrum of polyvinyltoluene or polystyrene. Dioxane extracts of cells in which no uptake had occurred showed a low absorbance due to cellular materials.

If this method is to be used to measure the rate of phagocytosis, it must be shown that once beads are taken in they are not lost from the cell. Otherwise the apparent rate of uptake could be the resultant of uptake and output processes. In fact, once taken in latex beads are retained by amoebas for at least 4.5 hr of subsequent incubation in the absence of beads. Finally, the assay has been shown to account for all of the latex beads added to the incubation flask (Table VI). The data in Table VI also show the reproducibility of the assay.

The uptake of latex beads as measured by the spectrophotometric assay was a linear function of cell concentration and was proportional to the length of the incubation period (Figure 3). The rate of uptake was maximal from pH 7 to 9, but appreciable uptake occurred over the range of pH 5-10 (Figure 4).

The Kinetics of Bead Uptake. The time courses of the uptake of latex beads of six different sizes at several concentrations of each bead are shown in Figure 5. The concentrations of beads are plotted on a weight basis rather than as a function of the number of beads. Each point is the mean of three samples each of which contained 3×10^6 cells. Each of the experiments has been repeated twice with identical results. Several of the experiments have been repeated after an interval of 9 months with the same results. Two types of control were included in each experiment. One control contained cells but no beads, and the other control flask contained cells and beads, but uptake was inhibited by 2,4-dinitrophenol. Both controls gave the same blank value. These blanks have been subtracted from the experimental values. The coincidence of the two different types of controls show that extracellular latex beads did not contaminate the washed amoebas.

Uptake was not always linear from zero time. The significance of the lag phase is not known with certainty. Its length varied with cell batch and bead size. The lag period might reflect the time necessary for the cells to recover from shock incurred while collecting them from the culture flasks or the lag may represent the time

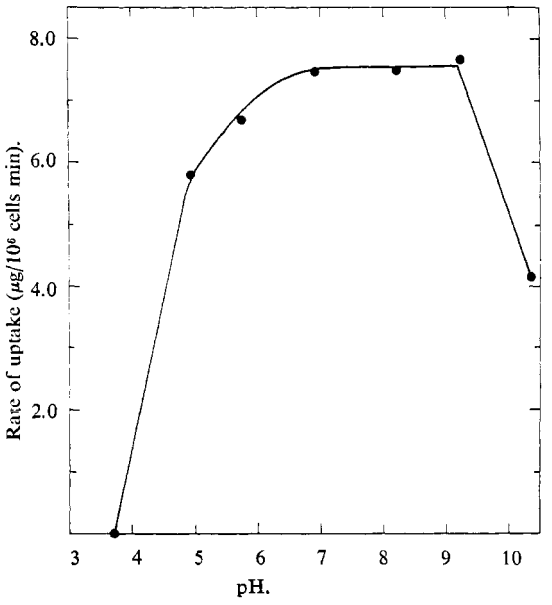

FIGURE 4: The effect of pH on the rate of uptake of latex beads by *Acanthamoeba*. Three parallel reaction mixtures contained 20 mg of polystyrene beads (1.305 μ) and 2.7 $\times$ 10^7 cells in a total volume of 9 ml of 1.5% proteose–peptone in 0.015 M KH_2PO_4, pH 6.7. Samples were taken at 10, 20, and 30 min and treated as described in Table VI. Each point represents the average of the three values with cell blanks substracted.

required to reach a steady state at the cell surface.

After the lag period there was a long period in all of the experiments during which the rate of uptake was constant. Moreover this rate was proportional to the concentration of latex beads in the incubation medium. This becomes more apparent when the data are presented as Lineweaver–Burk plots (Figure 6) using rates calculated from the linear portions of the curves in Figure 5. When this is done, straight lines are obtained for each bead size which give finite values for the terms analogous to the V_{max} and K_m of enzyme kinetics, *i.e.*, the maximal velocity of bead uptake (V_{max}) and the concentration of beads at which uptake proceeds at half the maximal velocity (K_b).

The fact that bead uptake shows saturation kinetics suggests that an amoeba contains a finite number of binding "sites" that can be saturated. The most striking aspect of these data, however, is that, when calculated on a weight basis, the values for K_b for the beads of all sizes are identical, and the apparent values for V_{max} are the same at least within a factor of 2 (Table VII).

An amoeba can ingest 4000 beads of diameter 0.126 μ in the same time it takes to ingest one bead of diameter 2.68 μ. The 2.68-μ beads can be ingested at a mean maximal rate of 1.5 beads/cell per min. The time actually required for the uptake of one bead may, of course, be much less or much more than the mean

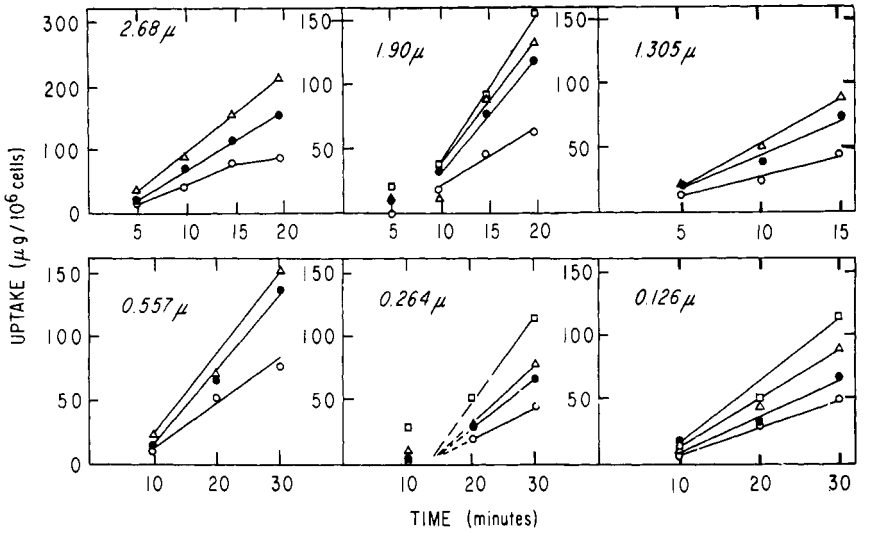

FIGURE 5: The uptake of latex beads of different sizes by *Acanthamoeba*. Triplicate reaction mixtures contained 2.7 $\times$ 10^7 cells and polyvinyltoluene or polystyrene latex beads as indicated in a total volume of 9 ml of 1.5% proteose–peptone in 0.015 M KH_2PO_4, pH 6.7. The cells and beads were equilibrated separately at 30° before being mixed to start the experiment. At the times indicated 1.0-ml samples were withdrawn and diluted with 1 ml of 4 $\times$ 10^{-4} M DNP in a 12-ml centrifuge tube. The cells were collected and washed with 0.015 M_2 $KHPO_4$, pH 6.7, until the supernatant solution was clear (usually three times but sometimes more for the smaller beads). The cell pads were extracted with 2 ml of dioxane for 30 min and the absorbance of the dioxane extract was measured at 267 mμ for polyvinyltoluene and 259 mμ for polystyrene. Each point represents the average of the values from the three flasks. The curves in each graph are for different concentration of beads. Beads of 2.68 μ: 368 (—○—), 736 (—●—), and 1840 μg/ml (—▲—); 1.9-μ beads: 368 (—○—), 1227 (—△—), and 2454 μg/ml (—□—); all other beads: 333 (—○—), 666 (—●—), 1120 (—△—), and 2240 μg/ml (—□—).

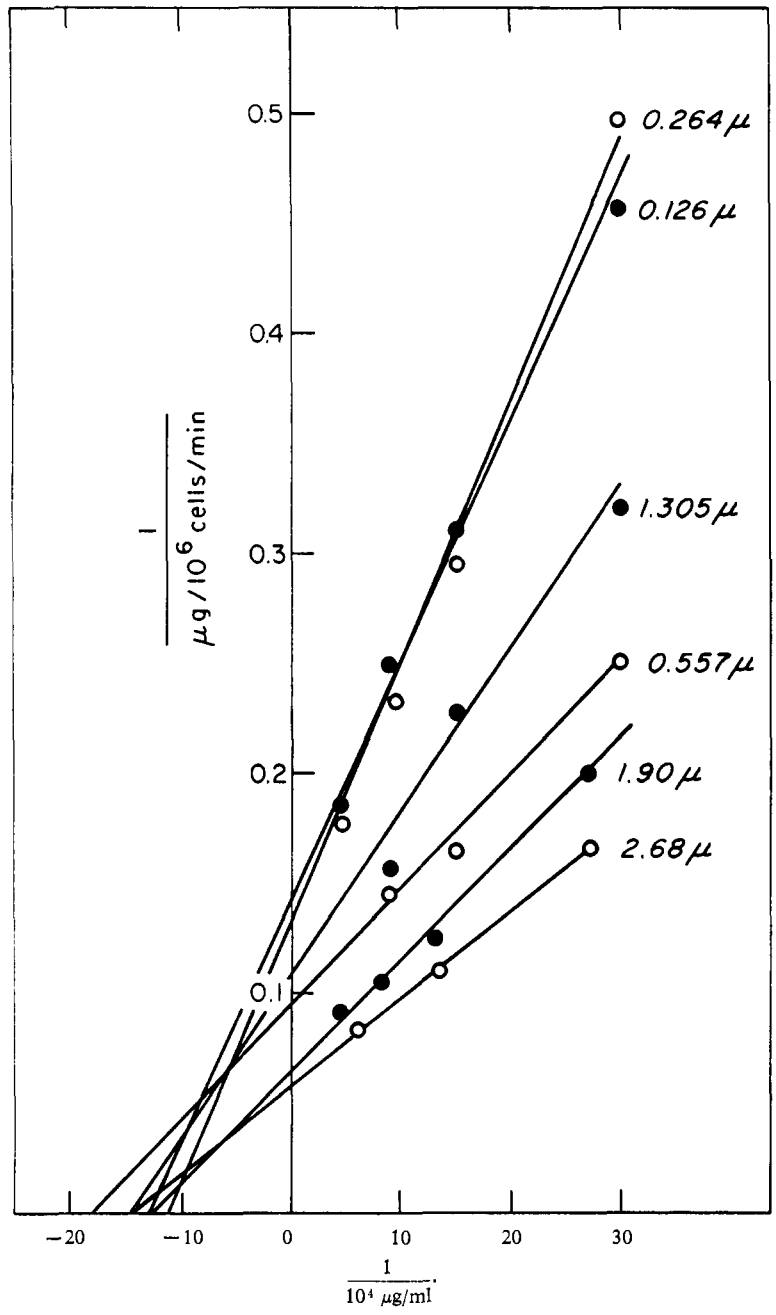

FIGURE 6: Reciprocal plots of the rate of uptake of latex beads as a function of the concentration of beads. The data are taken from the experiment described in Figure 5, using the linear portions of the curves to determine the rate of uptake.

rate. In another experiment it was found that at saturation one amoeba contained 63 beads of diameter 2.68 μ which occupied 15% of the volume of the amoeba.

Simultaneous Uptake of Beads of Different Sizes. The uptake of large and small beads from the same reaction mixture was examined to test for possible competitive effects. This experiment was facilitated by the fact that the 2.68-μ beads consist of polyvinyltoluene while the 0.126-μ beads consist of polystyrene. The absorption spectra of the two polymers differ permitting the simultaneous determination of the concentration of each in the presence of the other. From the extinction coefficients of each polymer at the two wavelengths, 274 and 259 μ, the following equations can be written: $A_{274} = (2.4 \times 10^{-3}V) + (0.31 \times 10^{-3}S)$ and $A_{259} = (2.41 \times 10^{-3}V) + (2.36 \times 10^{-3}S)$ in which V and S represent the concentration of polyvinyltoluene and polystyrene, respectively, in micrograms per milliliter, and the coefficients are the

TABLE VII: The Kinetic Constants of the Uptake of Latex Beads by *Acanthamoeba*.[a]

Diameter of Bead (μ)	Vol. of 1 Bead (μ^3)	K_b (μg/ml)	K_b (beads/ml)	V_{max} (μg/10^6 cells min)	V_{max} (beads/cell min)
2.68	10.1	670	6.4×10^7	16	1.5
1.90	3.6	840	2.2×10^8	17	4.6
1.305	1.2	720	5.7×10^8	10	7.9
0.557	9.2×10^{-2}	550	5.7×10^9	12	1.2×10^2
0.264	9.7×10^{-3}	910	8.9×10^{10}	8	7.9×10^2
0.126	1.1×10^{-3}	800	7.7×10^{11}	8	6.9×10^3

[a] The values in terms of micrograms were calculated from the data of Figure 6. The values in terms of beads were then calculated from the densities at 20° (1.05 g/ml for polystyrene and 1.03 g/ml for PVT). K_b is the concentration of beads at which uptake proceeds at half the maximal velocity.

TABLE VIII: The Simultaneous Uptake by *Acanthamoeba* of Latex Beads of Different Sizes.[a]

Latex Beads Added to Flask	Bead Uptake (μg/10^6 cells) 10 minutes PS	10 minutes PVT	25 minutes PS	25 minutes PVT	35 minutes PS	35 minutes PVT
0.126-μ PS	5	—	57	—	101	—
2.68-μ PVT	—	17	—	266	—	223
0.126-μ PS + 2.68-μ PVT	0	6	9	0	80	0

[a] Reactions were carried out as described in the legend to Figure 5 with the exception that cells were washed six times after incubation. PS were added at concentrations of 3.3 mg/ml and PVT were added at a concentration of 3.6 mg/ml. Each value is the average of three analyses of different flasks.

absorbances of each at a concentration of 1 μg/ml at the indicated wavelength. When A_{274} and A_{259} are measured V and S can be calculated.

From the data shown in Table VIII it can be seen that the presence of 2.68-μ beads may inhibit slightly the uptake of 0.126-μ beads. But there is a very dramatic inhibition of uptake of the larger beads by the smaller beads. This is more clearly seen in the data in Table IX where the rate of uptake of 2.68-μ beads is shown in the presence of increasing concentrations of 0.126-μ beads.

Uptake of [^{14}C]*Glucose.* One possible mechanism for the uptake of latex beads might be simply the uptake of the incubation medium with the beads being carried in passively. The fact that bead uptake exhibits saturation kinetics is one argument against this mechanism. The hypothesis was tested experimentally by measuring the uptake of [^{14}C]glucose from the incubation medium in the presence and absence of latex beads (Table X). In the absence of latex beads the amoebas take up very little glucose from the medium. Essentially all of the [^{14}C]glucose not recovered from the cells was recovered from the medium at the end of the incubations. Less than 0.01% of the added radioactivity appeared as CO_2. When latex beads were present in the medium and actively ingested by the amoebas there was no detectable increase in the uptake of [^{14}C]glucose (Table X). The very small uptake of medium as indicated by the low uptake of glucose, cannot account for the uptake of beads. In fact the phagocytic process is highly selective and beads are ingested but not the medium in which they are suspended.

The total volume of cells in each reaction mixture in the experiments described in Table X was $36 \times 10^9\ \mu^3$ = 0.036 ml. In the experiment with the 2.68-μ beads the amoebas ingested the beads originally present in 0.72 ml of incubation medium. At the same time the amoebas took up an amount of glucose present in only 0.003 ml of medium. Thus, while removing the beads from a volume of medium 10–20 times their own volume the amoebas removed glucose from a volume of medium only one-tenth their own volume.

Identical results have been obtained in experiments in which amoebas were incubated with and without beads in the presence of radioactive valine, aspartic acid, glutamic acid, phenylalanine, and lysine. In each case the cells took up that amount of radioactive

TABLE IX: The Inhibitory Effect of 0.126-μ Latex Beads on the Rate of Uptake of 2.68-μ Beads.[a]

Concn of 0.126-μ Beads (mg/ml)	Rate of Uptake of 2.68-μ Beads (μg/10^6 cells min)
0	18
0.33	6.4
0.67	5.3
1.33	4.3
3.33	3.2

[a] Reaction mixtures contained 33 mg of 2.68-μ polyvinyltoluene beads, 0.126-μ polystyrene beads as indicated, and 2.7×10^7 cells in a total volume of 9 ml of 1.5% proteose–peptone in 0.015 M KH_2PO_4, pH 6.7. Samples were taken after 10, 16, and 24 min of incubation at 30°. Rates of uptake were calculated from the linear portions of the plot of uptake *vs.* time. Average values from triplicate flasks were used after substraction of the cell blanks.

compound present in a volume of medium equivalent to about 0.1 their own volume. This is presumably a measure of fluid uptake which is not significantly altered by the phagocytosis of latex beads.

Acanthamoeba does not seem to have a concentrative mechanism for glucose or amino acid uptake. The very low rate of uptake of solute molecules may explain the long division time (70 hr) of *Acanthamoeba* when grown on media containing glucose and amino acids as the sources of energy (Adam, 1959). On more complex media the mean generation time is 12–15 hr.

TABLE X: The Uptake of [^{14}C]Glucose during the Uptake of Latex Beads by *Acanthamoeba*.[a]

Addition	% Uptake	
	Beads	[^{14}C]Glucose
None	–	0.14
2.68-μ beads	12	0.09
2.68-μ beads + DNP	–	0.04
0.126-μ beads	24	0.12
0.126-μ beads + DNP	–	0.07

[a] Reaction mixtures contained 9×10^6 cells and 0.01 M uniformly labeled [^{14}C]glucose (1.1×10^6 cpm) in a total volume of 3.0 ml of 1.5% proteose–peptone in 0.005 M KH_2PO_4, pH 6.7. Where indicated, 55 mg of polyvinyltoluene latex beads (2.68 μ) or polystyrene latex beads (0.126 μ) and 3.3×10^{-4} M DNP were added. Incubations were for 1 hr at 30° followed by seven washes of the entire sample after which the cell pads were analyzed for radioactivity or bead uptake. Each value is the average of three samples and cell blanks have been subtracted from bead uptake values.

Uptake of [^{131}*I*]*Albumin.* Experiments were carried out to compare the uptake of protein and latex beads and the influence of one on the other. Chapman-Andresen and Holter (1964) have reported that *Amoeba proteus* will take up serum albumin at pH 4.3, where it carries a positive charge, but not at pH 7.4. *Acanthamoeba* were found to take up very little albumin at any pH between 4 and 9 and there was no dependence on the concentration of albumin. When the uptake of latex beads and [^{131}I]albumin was compared in the same experiment (Table XI), the results were very similar to those obtained with [^{14}C]glucose. About 30% of the beads were ingested but only about 0.1% of the albumin. Again the phagocytosis of latex beads was found to be highly selective with a concentrating factor of about 300.

TABLE XI: Uptake of [^{131}I]Bovine Serum Albumin during the Uptake of Latex Beads by *Acanthamoeba*.[a]

Addition	% Uptake	
	Beads	[^{131}I]Albumin
[^{131}I]Albumin	–	0.13
Latex beads	26	–
[^{131}I]Albumin + latex beads	27	0.11

[a] All reaction mixtures contained 2.1×10^7 cells in 9.0 ml of 1.5% proteose–peptone in 0.015 M KH_2PO_4, pH 6.7. Where indicated, 45 mg of [^{131}I] bovine serum albumin (4.6×10^4 cpm/mg) and 6.6 mg of polyvinyltoluene latex beads (2.68 μ) were added. Incubation was at 30° for 15 min. Duplicate 1-ml samples were removed from each flask and added to 1 ml of 4×10^{-4} M DNP. The cells were washed three times with 0.015 M KH_2PO_4, pH 6.7, and the cell pads were analyzed for their content of ^{131}I or latex beads. Each value is the average of three separate incubations.

Effect of Bead Uptake on Amoeba Volume. From the previous experiments it is apparent that at saturation each amoeba can contain a very large number of latex beads. This might be expected to result in a volume change; either an increase because of the volume occupied by the beads, or a decrease if the plasma membrane used to form the phagocytic vesicles were not replaced by new plasma membrane.

In the experiment reported in Table XII, amoebas were incubated either alone, with beads, or with beads plus dinitrophenol. Each incubation was carried out in quadruplicate and one of the flasks from each set was used to measure bead uptake in the usual way. The contents of the other three flasks of each set were pooled and the cells were collected in a calibrated

TABLE XII: The Volume of Amoebas before and after Uptake of Latex Beads.[a]

Incubn Condn	Bead Uptake (mg)	No. of Cells	Total Cell Vol. (ml)	Vol./Cell (μ^3)
No beads	0	1.6×10^8	0.65	4.1×10^3
Beads	44.0	1.6×10^8	0.60	3.7×10^3
Beads + DNP	0	1.7×10^8	0.78	4.6×10^3

[a] All flasks contained 5×10^7 amoebas in 9 ml of 1.5% proteose-peptone in 0.015 M KH_2PO_4, pH 6.7. Two sets of flasks contained 200 mg of 1.305-μ polystyrene latex beads. One set of flasks contained 3.3×10^{-4} dinitrophenol. Incubation was for 90 min at 30°. Each set contained four flasks. For other experimental details, see text.

centrifuge tube. The cells were washed eight times to remove all the extracellular beads. To each tube 0.1 ml of [^{14}C]glucose (3.5×10^5 cpm) was added and the volumes were carefully adjusted to 2 ml. An equal amount of [^{14}C]glucose was diluted to 2 ml in a control tube. The samples were thoroughly mixed and the cells were sedimented by centrifugation. From the clear supernatant solutions, three 0.1-ml aliquots were taken to measure radioactivity. Since glucose is not significantly taken up by the amoebas its concentration is inversely proportional to the volume of the extracellular space. The cells were resuspended and aliquots were taken for cell counts (Weisman and Korn, 1966). From these data the total cell volume and the volume per cell can be directly calculated (Table XII).

The amoebas incubated with latex beads of diameter 1.3 μ ingested an average of 270 beads/cell which was equivalent to 7% of the cell volume. The volume of beads ingested was too small to cause measurable changes in cell volume. But the amount of plasma membrane used to form vesicles was very great. Electron microscopic observations have shown that each bead of diameter 1.3 μ is surrounded individually by plasma membrane (E. D. Korn and R. A. Weisman, unpublished data). Therefore, the total area of plasma membrane used to form phagocytic vesicles in one amoeba was approximately 1430 μ^2. If an amoeba of volume 4000 μ^3 is assumed to be a sphere its mean diameter is 20 μ and its surface area 1250 μ^2 or about the same as the amount of membrane used to form vesicles.

Despite this large utilization of plasma membrane the decrease in volume of the amoebas was minimal. The figures in Table XII indicate a decrease in volume of about 10–20% depending upon whether the comparison is made to the controls incubated without beads or to the controls incubated with beads but inhibited by dinitrophenol. This decrease in volume is equivalent to a decrease in surface area of 7–14% (if the amoeba is assumed to be a sphere) and is only 6–14% of the area of membrane necessary to enclose the phagocytic vesicles. The data suggest, therefore, that the plasma membrane is rapidly replaced during phagocytosis either by synthesis *de novo* or by translocation of existing intracellular membranes (Bennett, 1956). Of course, if the actual surface area of an amoeba is very much larger than that calculated for a sphere, because of numerous surface invaginations, it might be possible to lose a high percentage of plasma membrane with little change in volume.

Discussion

The fact that the uptake of latex beads is completely inhibited by anaerobiosis, and by inhibitors of oxidative phosphorylation (azide, cyanide, and dinitrophenol) but not appreciably by inhibitors of glycolysis (fluoride and iodoacetate) does not necessarily indicate an intimate relationship between phagocytosis and oxidative phosphorylation. More probably it reflects the fact that the amoebas are strict aerobes whose general viability is dependent on oxidative pathways. Phagocytosis in other cells is blocked by either or both classes of inhibitors depending on whether oxidative or glycolytic pathways are the major energy source. Thus the uptake of starch and polystyrene particles by guinea pig polymorphonuclear leucocytes which occurs under both aerobic and anaerobic conditions is not inhibited by cyanide or dinitrophenol, but is prevented by iodoacetate and fluoride (Sbarra and Karnovsky, 1959). Phagocytosis by guinea pig alveolar macrophages is sensitive, however, to either type of inhibitor (Oren *et al.*, 1963). On the other hand, to block phagocytosis by Ehrlich ascites cells both oxidative and glycolytic metabolism must be inhibited simultaneously (Roberts and Quastel, 1963).

Our results suggest that phagocytosis in amoeba can take place only when the cells are incubated in a medium of appropriate osmolarity. This could be provided by any of the compounds tested provided the ionic strength was not too high. A similar effect has been observed for polymorphonuclear leucocytes (Sbarra *et al.*, 1963). If given sufficient time the amoebas can adapt to a medium of unfavorable osmotic pressure. It is not known whether the cells are inherently unable to take up particles when the internal and external osmotic pressures are different, or whether the difference in osomotic pressures forces the cell to move water in a process that may be competitive with phagocytosis. Studies with $H_2{}^{18}O$ may answer this question.

Proteose–peptone has been reported to contain a factor that stimulates phagocytosis in *Tetrahymena* (Seaman and Mancilla, 1963). This does not seem to be true for *Acanthamoeba*. Leucocytes require the presence of a serum protein for maximal ability to engulf starch particles but not for the uptake of latex beads (Sbarra and Karnovsky, 1959). We have never observed any requirement for added protein for the uptake of latex beads.

In other amoebas, the uptake of protein appears to occur only at a pH below its isoelectric point (Marshall *et al.*, 1959; Rusted, 1959; Chapman-Andersen and Holter, 1960). This apparent requirement for a positively charged species may be related to the presumed presence of a sulfated mucopolysaccharide coat on the external surface of *Amoeba proteus* and *Pelomyxa carolinensis*. Polystyrene and polyvinyltoluene latex beads do not carry a positive charge or any other functional group that can be imagined to interact with a component of the cell surface unless the anionic detergent is irreversibly bound to the beads. It must be assumed that mechanical contact is sufficient to initiate the phagocytoic process.

The kinetic data provide considerable insight into the phagocytic process. The simplest mechanism for the ingestion of particulate material would be for the cell to engulf medium and take in particles passively. This mechanism would not show saturation kinetics, *i.e.*, the K_b would be infinite. The fact that the identical finite K_b was found for latex beads of six different sizes is a strong argument against this mechanism. It is unlikely that the saturation kinetics are an artifact due to the presence of an inhibitor in the preparations of latex beads because such an inhibitor would have to be present in the same concentration in each of the six different preparations. Identical results have also been obtained with latex beads that were washed free of soluble contaminants by repeated centrifugation from distilled water. A passive mechanism for bead uptake is also incompatible with the fact that soluble components of the medium are not taken in together with the latex beads.

If each bead were ingested individually the kinetics of bead uptake would be expected to be a function of the number of beads or of the surface area of the beads present in the incubation medium. Similarly a mechanism of phagocytosis that involved simply the binding of latex beads to the cell surface and ingestion of the plasma membrane would be expected to exhibit kinetics that were a function of the diameter of the beads or something approaching the surface area depending on the extent of interaction between the cell surface and the bead. The data clearly show that the uptake of latex beads is independent of the diameter, surface, area, and number of beads but is instead a function of the volume of beads.

The kinetic data, therefore, suggest that latex beads are ingested in phagocytic vesicles containing approximately equal volumes of beads irrespective of the volume of the individual beads that are being taken up. Furthermore, the vesicles would have to be almost completely occupied by beads and contain little of the incubation medium in order to explain the almost total exclusion of glucose, protein, and amino acids during the uptake of beads. According to this hypothesis many small beads would accumulate in a closely packed aggregate on the surface of the amoeba until their total volume approximated that of a large bead at which time they would be internalized within one phagocytic vesicle. This mechanism can also explain the inhibition of the uptake of beads of diameter 2.68 μ by beads of diameter 0.126 μ. When present in the medium at equal concentrations there would be 8000 small beads for every large bead. The small beads would, therefore, preferentially accumulate on the cell surface and by the time a large bead made contact the resultant volume might be too large for the cell to ingest. Or the cell might be unable to bind a large bead when already binding so many small beads. The time required for the accumulation of beads at the surface of the amoeba could also explain the lag phase in the uptake curves that is especially apparent and reproducible with the smaller beads.

This interpretation of the kinetic data is confirmed and extended by electron microscopic studies to be reported elsewhere (E. D. Korn and R. A. Weisman, unpublished data). The micrographs show that latex beads of diameter 1.3 μ and greater are ingested individually, each bead closely surrounded by membrane derived from the plasma membrane. Latex beads of diameter 0.557 μ and smaller, on the other hand, are accumulated at the surface of the amoeba and are then ingested with many beads (often more than 1000) tightly packed within one membrane-bound vesicle. The accumulation of beads, often at a convex surface, implies the secretion of some substance by the amoeba that binds the beads to each other and to the cell.

The initial events of phagocytosis are complex. Contact between a chemically inert latex bead and an amoeba is apparently sufficient to cause the secretion of a substance by the amoeba which binds the beads to the cell, the envelopment of the beads by the plasma membrane, the internalization of the phagocytic vesicle, and the maintenance of the continuity of the plasma membrane. Throughout this process a high degree of specificity is maintained so that soluble molecules, and presumably water also, are almost entirely excluded. Finally, the fact that there appears to be a highly favored size for the phagocytic vesicle implies an underlying structural basis in the organization of the plasma membrane.

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