PHAGOCYTOSIS BY THE CELLULAR SLIME MOLD *POLYSPHONDYLIUM PALLIDUM* DURING GROWTH AND DEVELOPMENT

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ABSTRACT

The phagocytic ability of amoebae of the cellular slime mold *Polysphondylium pallidum*, grown in shaken suspension, was examined. An established quantitative assay of the uptake of polystyrene (PS) beads was shown to be valid for this organism. The kinetics of phagocytosis were determined, and estimates of the concentration of PS beads necessary to achieve half-maximal phagocytic velocity ($K_p$), as well as the maximal velocity itself ($V_{\text{max}}$), were made. Comparison with previously published data on *Acanthamoeba* and guinea pig leukocytes suggested that the *P. pallidum* amoebae had the lowest $K_p$, while the leukocytes had the highest $V_{\text{max}}$. Beads approximately 1 µm in diameter appeared to be the optimal size for ingestion. Simultaneously with phagocytosis, comparable numbers of beads accumulated at the cell surface; this accumulation did not occur when phagocytosis was inhibited. Phagocytosis was depressed by protein in the medium, by increased osmolarity, and by inhibitors of aerobic metabolism. Starvation-initiated development, leading to encystment, was shown to affect the capacity of the cells to phagocytize, mainly by progressively decreasing the time span over which the cells ingested particles at a constant initial rate.

INTRODUCTION

Phagocytosis may be defined as the process by which certain animal cells internalize solid extracellular particles within vacuoles whose limiting membranes are derived from the surface of the cell. Although phagocytosis has been demonstrated in a variety of cell types, it remains incompletely defined at the biochemical level. The central biochemical question is this: "What, if any, are the special features of a phagocyte's membrane and enzymatic machinery that enable it to phagocytize, and which distinguish it from a nonphagocytic cell?"

We have chosen to approach this problem by attempting to define phagocytic and nonphagocytic states in a single cell type, the amoebae of the cellular slime mold. We reasoned that a comparison of such states from a biochemical point of view might lead to some clues as to the biochemical basis of phagocytosis, at least in this cell type. In addition, we felt that a characterization of phagocytosis in slime mold amoebae would allow some generalizations to be made about phagocytosis in different cell types. Detailed and quantitative studies of this process in guinea pig polymorphonuclear leukocytes (PMN)

1 Abbreviations used in this paper are: BSA, bovine serum albumin; BSS, basic salt solution; PMN, polymorpho-
and in the protozoan Acanthamoeba (29) are available for comparison.

The amoebae of the cellular slime mold are well suited for a study such as this since they are known to phagocytize and grow on a wide variety of bacteria (18) as nutrient. They also possess a well characterized developmental capacity (4) that can be initiated by starvation and that involves, among other events, several changes in the surface properties of the cells (e.g. [10, 23]), which might result in alterations in their phagocytic properties. The rationale of our experiments was that starvation and the initiation of the developmental program of the amoebae would result in changes in their phagocytic ability (as has already been observed in the case of Acanthamoeba, 30), thus allowing us to define in biochemical terms the different phagocytic states alluded to above.

MATERIALS AND METHODS

Organism

The cellular slime mold Polysphondylium pallidum WS-320 was used. A strain was kindly provided by Professor M. Sussman of Brandeis University, Waltham, Mass. It was grown and maintained on an axenic medium containing milk powder, lecithin, proteose peptone, and salts as the principal ingredients, as described by Sussman (25, 26). (See also 11.)

Particles

Polystyrene beads 0.109, 0.357, and 1.1 µm in diameter and a polyvinyltoluene (PVT) bead 1.857 µm in diameter were purchased as 10% (wt/vol) stock suspensions were used without any prior treatment.

Respirometry

The effects of various metabolic inhibitors on respiration were investigated according to conventional methods (e.g. 21) using an A. H. Thomas Warburg apparatus (Arthur H. Thomas Co., Philadelphia, Pa.). Known numbers of washed cells (about 2 × 10^7 cells per flask) were incubated at room temperature in a solution which contained 16 mM phosphate, pH 6.7, 20 mM glucose, and 10 mM EDTA. Inhibitors were placed in the side arms of the Warburg flasks and were added to the cells after their basal rate of respiration had been established.

Assay of Phagocytosis

Phagocytosis was measured by a modification of an assay developed by Roberts and Quatetel (20) and refined by Weisman and Korn (29). This assay involves the uptake of polystyrene (PS) beads by cells in suspension, and the subsequent determination of the ingested PS by its ultraviolet absorption in dioxane extracts of the washed cell pellets.

Exponentially growing amoebae were harvested, washed, and resuspended in the standard medium (SM) (25) which contained Bactopeptone, glucose and salts (but omitted the yeast extract). The cell concentration, determined by from four to six replicate counts of 50–100 cells in a hemocytometer, was adjusted appropriately (usually to 1–2 × 10^5 cells/ml) and the cells were allowed to equilibrate at about 225 rpm on a gyratory shaker at room temperature (23–24°C). The assay was initiated by adding the appropriate amount of PS to the cell suspension (beads 1.1 µm in diameter were used in all experiments, unless otherwise noted). The flask was quickly swirled by hand, triplicate aliquots of 5 ml were withdrawn and each aliquot was mixed rapidly with 5 ml of ice-cold basic salt solution (BSS) containing also 0.2 M sucrose and 0.6 mM 2,4-dinitrophenol, in a 12-ml glass centrifuge tube. This stopped further uptake of PS (see Results and Discussion). The initial aliquots were obtained within 30 s after the addition of PS. After appropriate periods of incubation, further triplicate aliquots were withdrawn in a similar fashion. The cells were sedimented by centrifuging at top speed for 3 min in a table model clinical centrifuge. After the decanting of the supernatant fluids, the sedimented cells were resuspended in 5 ml of ice-cold 0.2 M sucrose in BSS with a plastic pipette, and the tubes were centrifuged for 2 min at top speed in the centrifuge. This washing procedure was repeated once more, and the tubes were inverted and drained. The sedimented cells were extracted overnight at room temperature with 2 ml of p-dioxane (ACS grade, Fisher Scientific Co., Medford, Mass.). After a brief centrifugation to sediment the extracted cells, the optical densities of the dioxane extracts were measured at 259 nm, where PS has an absorption maximum. The amount of PS phagocytized over a given time interval was determined from the difference in the mean optical densities between the zero- time and timed samples by using the known absorbance of PS in dioxane (100 µg PS/ml produced an optical density of 0.230 over a 1-cm light path). This procedure served to correct for incomplete washing and for the optical density due to cellular material which was dioxane-soluble (and which absorbed maximally at about 260 nm). The number of cells
Measurement of Protein

The dioxane-extracted cells were allowed to dry at room temperature and were then dissolved to 0.5 N NaOH. Aliquots of these digests were assayed for protein according to Lowry et al. (14), using bovine serum albumin (BSA), Fraction 5 (Nutritional Biochemical Corporation, Cleveland, Ohio), as a standard. At the same time, known numbers of cells which had not been exposed to PS were carefully washed in ice-cold BSS and assayed for their protein content, both before and after dioxane extraction. Dioxane extraction resulted in an average 7% decrease in cellular protein content. These data allowed us to determine the number of cells recovered in the washed cell pellet after the assay of phagocytosis and thus to express PS uptake on the basis of cells or cellular protein recovered.

RESULTS AND DISCUSSION

Appearance of the Cells after Phagocytosis

A washed suspension of cells which had phagocytized PS for about 15 min was examined by phase microscopy. The assay conditions had little effect on the appearance of the cells, except that PS beads were now visible in the same focal plane as the cellular organelles. Since the motion of the beads (as if they were in a viscous medium) resembled that of the organelles, it was concluded that the beads were inside the cells, and were not simply adsorbed to the cell surface. In addition, a small proportion of the beads could be observed either free in the medium (and undergoing rapid Brownian motion) or immobilized at the cell surface. In a typical experiment over 95% of the cells phagocytized at least one bead during a 15-min incubation at PS and cell concentrations of 265 µg/ml and 1.14 X 10^6 cells/ml, respectively. This PS concentration was about three times the half-saturating PS concentration (see below), and the bead to cell ratio was 330:1. This may be compared with the figures of 70% and 77% obtained with Acanthamoeba (29) and PMN (15), respectively.

Recovery of Cells and PS in the Assay of Phagocytosis

Approximately 95% of the cells present at the beginning of an assay could be recovered in the washed cell pellets after incubations of 0–15 min, as indicated by the measurement of cellular protein recovered, and after correction for the effect of dioxane extraction. The recovery of PS was estimated by determining the amounts of PS present in the washed cells, the wash supernates, and the incubation supernate. The amount of PS in the supernates was determined in two ways: (a) by collecting the PS by high-speed centrifugation and dissolving it in dioxane, or (b) by estimating the PS content in those solutions by their turbidity at 550 nm. The data from five experiments showed that about 84% of the added PS could be accounted for in toto. The major losses of PS were probably incurred in the handling of the rather considerable volumes of supernatant fluids and washings.

Accuracy of the Assay of Phagocytosis

The accuracy of the assay of phagocytosis is a function of the accuracy of the PS and protein determinations in the triplicate zero-time and timed samples. Under a wide variety of assay conditions, the average mean standard deviation of the measurement of PS uptake per cell (expressed as a percentage of the mean in each experiment) was about ±25%, with a range of ±2–42%, 75% of the variability being due to the determination of PS.

Time Course of PS Uptake

The uptake of PS beads was a linear function of time for at least 30 min at PS concentrations ranging from 20 to 400 µg PS/ml, as indicated in Fig. 1. No lag in uptake was evident in these experiments where the earliest time point was 5 min. (Another experiment shown in Fig. 9, which included 25-min points, also revealed no lag.) These results established the fact that a two-point assay of phagocytosis of 15 min (the usual incubation period) constituted a measurement of the rate of phagocytosis over the designated range of PS concentrations. Acanthamoeba (29) and PMN (15) were also able to ingest particles linearly with time for 15–30 min at wide ranges of particle concentrations. However, short lag periods were observed in both the latter cell types before the rate of uptake became constant.

The constant rate of bead uptake at low PS concentrations was rather surprising, since it could be calculated in a typical experiment (PS and cell concentrations of 21 µg/ml and 1.5 X 10^4 cells/ml)
FIGURE 1 Time course of PS uptake. Amoebae were assayed for their ability to phagocytize PS beads as a function of time. Uptake of PS was expressed as a percentage of the uptake observed after 15 min of incubation. The curve represents six experiments conducted at PS concentrations ranging from 20 to 400 µg PS/ml. The cell concentrations were 1.5-2.1 X 10^6 cells/ml, and the weights of beads ingested at 15 min reflected the PS concentration used, ranging from 3.5 to 24.6 µg PS/1 X 10^6 cells. The bars represent standard deviations.

that 35% of the PS had been ingested after 15 min of incubation. This phenomenon was apparently independent of the PS concentration, since in another experiment performed at PS and cell concentrations of 277 µg/ml and 6.4 X 10^6 cells/ml uptake was linear with time for 15 min, at which point 56% of the available PS had been ingested. A potential explanation for this phenomenon is offered by the observation (see below) that substantial quantities of PS gradually accumulate at the cell surface during the assay period. Perhaps this accumulation renders the cells less sensitive to the calculated changes in PS concentration occurring as a result of phagocytosis.

Effect of Cell Concentration

At fixed PS concentrations, the rate of uptake of PS was proportional to the number of cells in the standard assay, as shown in Fig. 2. Such data show that the rate of uptake of PS per cell was not changed by varying the number of cells, indicating that uptake was not a function of the "load" (the ratio of PS to cells) under these conditions. The following section indicates that uptake was in fact a function of the PS concentration, within the limits of the PS concentrations examined. Similar conclusions were reached with Acanthamoeba (29) and PMN (15).

Effect of PS Concentration, and Determination of \( K_p \) and \( V_{p,max} \) of Phagocytosis; Effect of Cell Size on \( V_{p,max} \)

The rate of PS uptake into the cells was directly proportional to the PS concentration at low PS concentrations, and reached a maximal value at high PS concentrations. The data from seven experiments were examined graphically according to the method of Eadie and Hofstee (e.g. 12). The method of least squares was used to fit the best line to the experimental points, in order to estimate the maximal velocity of PS uptake \( (V_{p,max}) \), and the PS concentration at which half-maximal uptake occurred \( (K_p) \). The results of these computations are shown in Table I. The variation in the \( K_p \) values appeared to be random, but the variation in \( V_{p,max} \) was closely correlated with the protein content of the cells as indicated in Fig. 3.

Such a correlation would be expected to the extent that the maximal rate of PS uptake per cell is dependent on the surface area (or volume) of the cell. By taking the protein content of the cells as a measure of their size (11) and assuming that the cells are spherical, it is possible to calculate from Table I and Fig. 3 that a 27% increase in the cell surface area (and a 43% increase in volume) was accompanied by a 264% increase in \( V_{p,max} \). Thus...
Each experiment involved five different concentrations of PS beads 1.1 µm in diameter, ranging from 19 to 772 µg/ml. The cell concentrations ranged from 1.2 to 2.1 X 10^6 cells/ml.

Correlation coefficient for the closeness of the fit between the best line plotted according to Eadie and Hofstee (12) and the experimental points. If the fit is perfect, r = 1.0.

| Experiment | Protein content | K_p | V_p^{max} | r |
|------------|----------------|-----|-----------|---|
| I          | 35             | 102 | 17.8      | 0.912 |
| II         | 37             | 107 | 18.3      | 0.609 |
| III        | 37             | 94  | 27.9      | 0.887 |
| IV         | 41             | 64  | 24.0      | 0.929 |
| V          | 43             | 87  | 35.4      | 0.994 |
| VI         | 45             | 51  | 40.4      | 0.969 |
| VII        | 50             | 127 | 55        | 0.961 |
| Mean ±SD  | 90 ± 22        | 31.2 ± 11.6 |

* Each experiment involved five different concentrations of PS beads 1.1 µm in diameter, ranging from 19 to 772 µg/ml. The cell concentrations ranged from 1.2 to 2.1 X 10^6 cells/ml.

§ µg protein/1 X 10^6 cells.

‖ µg PS/ml.

¶ Correlation coefficient for the closeness of the fit between the best line plotted according to Eadie and Hofstee (12) and the experimental points. If the fit is perfect, r = 1.0.

Figure 3: Dependence of V_p^{max} on cell protein content. The V_p^{max} values of Table I were plotted as a function of cell protein content. Protein content and V_p^{max} are expressed as microgram protein per 1 X 10^6 cells and microgram PS per 1 X 10^6 cells and 15 min, respectively.

An increase in cell size caused a quite disproportionate increase in the ability of the cells to phagocytize. This is an interesting finding, since it suggests that larger cells possess greater phagocytic capabilities per unit of surface area (or volume) than do smaller cells. It may also suggest, as one possible explanation, that if phagocytic regions of the cell surface are in “patches”, more of these occur per unit area of larger cells than of smaller cells.

The data for the K_p and V_p^{max} of PS uptake in P. pallidum can be compared with similar data available from studies on Acanthamoeba (29) and PMN (15), as summarized in Table II. These data suggest that the P. pallidum amoebae are more avid phagocytes than the other two cell types from the point of view of “affinity” for particles, while in terms of the maximal uptake velocity per unit surface area (considering all three cell types as spheres) the leukocytes are the most effective. The K_p and V_p^{max} terms must be regarded very tentatively. They are not rigorously consistent with terms used in enzyme reactions, but might be useful in considering the phagocytic activities of different cells, or of given cells under different conditions. Comparisons, for example, between amoebae and PMN, must also be viewed conservatively when there are differences in the type of particle used and in the assay conditions employed, as summarized in Table II.

Attachment of PS to Cell Surface During Phagocytosis

A characteristic feature of the assay of phagocytosis was that a substantial portion of the PS which sedimented with the cells in the first centrifugation could be removed from the cells by the subsequent washing procedures. This fraction of PS, referred to here as “bound PS”, accumulated as a function of time during the incubation, rising from insignificant levels at zero time to substantial quantities (similar to the amount of PS which had
Based on measurements of the average cell diameters (11, 16, 29) and the assumption that cells are spherical. Expressed as square microns per cell.

Micrograms PS per milliliter.

§ Micrograms PS per $1 \times 10^6$ cells and 15 min.

¶ Starch granules with an average diameter of 2.58 µm were used rather than PS, the cells were assayed as monolayers, and the kinetic parameters were estimated by the present authors. (The reported rates of uptake of PS beads by rat PMN in suspension (20) are roughly consistent with the more detailed data reported for guinea pig cells in monolayer by Michell et al. (15).) It should also be noted that the maximal velocity actually observed was only 30% of the calculated $V_{p}^{\text{max}}$, since particle uptake was inhibited at high particle concentration (15).

It is possible to estimate the proportion of the cell surface area which was occupied by bound PS after 30 min of incubation in the experiment shown in Fig. 4. Approximately 70 µg of PS beads 1.1 µm in diameter were bound at that time. Assuming that the cross-sectional area of the bead corresponds to that part of the surface area of the cell which is occupied by the bead, and postulating an average cell surface area of 230 (µm)$^2$ (from an average diameter of 8.55 µm, 11), approximately 100% of the cell surface was occupied by beads.

Thus the observed accumulation appears to be feasible.

Since the first event in the phagocytosis of a particle by a cell must involve an initial contact of sufficient duration to allow the internalization of the particle into the cell, it was not surprising to find that cells exposed to PS accumulated some beads which could subsequently be washed free of the cells. However, the rather slow rate and the very large extent (relative to the amount actually ingested) of this accumulation, and its absence under conditions which blocked bead uptake, indicate that the accumulation of bound PS was not due to a simple adsorption process. This accumulation appears to be due to a slow surface accumulation of beads in a form that does not permit immediate phagocytosis and is mediated by a process which appears to occur only under those condi-

**Table II**

$K_p$ and $V_{p}^{\text{max}}$ of Phagocytosis in Several Cell Types

| Cell type       | Surface area* | $K_p$‡ | $V_{p}^{\text{max}}$ (cells)§ | $V_{p}^{\text{max}}$ (area)¶ |
|-----------------|---------------|--------|-----------------------------|-------------------------------|
| $P$. pallidum   | 230           | 90     | 31.2                        | 0.136                         |
| Acanthamoeba    (29) | 1250       | 748    | 180                         | 0.144                         |
| PMN¶ (15)      | 316           | 910    | 443                         | 1.41                          |

* Based on measurements of the average cell diameters (11, 16, 29) and the assumption that cells are spherical. Expressed as square microns per cell.

‡ Micrograms PS per milliliter.

§ Micrograms PS per $1 \times 10^6$ cells and 15 min.

¶ Starch granules with an average diameter of 2.58 µm were used rather than PS, the cells were assayed as monolayers, and the kinetic parameters were estimated by the present authors. (The reported rates of uptake of PS beads by rat PMN in suspension (20) are roughly consistent with the more detailed data reported for guinea pig cells in monolayer by Michell et al. (15).) It should also be noted that the maximal velocity actually observed was only 30% of the calculated $V_{p}^{\text{max}}$, since particle uptake was inhibited at high particle concentration (15).
conducted at PS and cell concentrations ranging from signify standard deviations. These experiments were

A similar finding has been reported in the case of *Acanthamoeba* (13). Essentially no beads accumulated at the surface of these amoebae under conditions that inhibit bead uptake, while substantial accumulations occurred during normal uptake.

**Effect of PS Bead Size**

When amoebae were incubated with equal concentrations, by weight, of beads ranging in diameter from 0.109 to 1.857 µm, the results shown in Fig. 5 were obtained. The weight of beads ingested by the cells was found to depend on the average bead size. Control experiments showed that uptake of the beads 0.109 and 0.357 µm in diameter was linear with time for 15 min (samples taken at 5, 10, and 15 min), indicating that the normal assay period of 15 min constituted a measurement of the rate of uptake of those beads. The uptake of the largest bead was, however, linear with time for no more than 5–10 min. Thus the uptake of the smaller beads was limited by their decreased rate of uptake, while the uptake of the large bead was limited by the relatively low maximal extent of its uptake. It should be pointed out that variations in the cell size had no apparent effect on this phenomenon (the cells used ranged from 35 to 53 µg protein per 10⁶ cells) suggesting that the ratio of PS size to cell size was not important, at least over the range studied.

The possibility that the reduced rate of uptake of the smaller beads was due to increases in the *K*ₚ values for uptake was not tested. At the PS concentrations used in several of the experiments, a 17-fold increase in the *K*ₚ (based on the weight of beads) would have been required to account for the difference in the rate of uptake of beads 0.109 and 1.1 µm in diameter.

The different rates of uptake of the three smaller beads cannot be explained by suggesting that the number of phagocytic events was independent of the bead size and that only one bead was phagocytized at a time. If that were true, then the relative rates of uptake, in terms of weight per unit time, would have been in the ratio of 0.1:0.35:100 for the three bead sizes, rather than the ratio of 21:55:100 that was found. These figures also indicate either that the number of phagocytic events was drastically increased when the cells were presented with the smaller beads, or that the smaller beads were ingested in large clusters (from one to three beads per vacuole was typical with the 1.1 µm beads, as revealed by phase-contrast microscopy). A relative increase in the number of phagocytic events (vacuoles formed) might in fact account for the decreased weight uptake of small beads, since the amount of surface membrane internalized per unit weight of beads ingested would thereby be increased. If the amount of surface membrane were limiting, this would result in a decrease in uptake of smaller beads, as was noted.

Studies on the uptake of beads 0.126–2.68 µm in diameter by *Acanthamoeba* also revealed a correspondence between bead size and the rate of bulk uptake (29). *K*ₚ and *V*ₚmax were determined for each bead size; *K*ₚ showed no systematic variation, while *V*ₚmax increased only by a factor of two from the smallest to the largest bead. These data suggested that the average phagocytic vacuole contained equal weights of beads, irrespective of the bead size (29), and further electron microscopic data showed that small beads were in fact ingested as large clusters (13).

**Effect of Composition and Osmolarity of the Incubation Medium**

The choice of SM as the incubation medium for assaying bead uptake was dictated by the fact that the use of media containing no polypeptide compo-
The rate of uptake was found also to be a function of the osmolarity of the incubation medium; higher osmolarities inhibited uptake. This was indicated by experiments in which ingestion of beads was measured either in media containing varying concentrations of Bactopeptone (the principal ingredient of SM) or in SM containing variable concentrations of sucrose. Uptake of beads by cells preincubated for 15–40 min in 0.5% Bactopeptone (about 30 mosM) was about three times that seen in 4.5% Bactopeptone (about 150 mosM). The effect on phagocytosis of adding various concentrations of sucrose (50–400 mosM) to SM (about 150 mosM) is shown in Fig. 6.

Inhibition of phagocytosis by a medium of high osmolarity is not surprising in a cell which does not rapidly adapt to the change. The higher osmolarities appeared to dehydrate the cells and round them up, presumably making the deformations of the membrane required for phagocytosis more difficult and decreasing the available space for ingested particles inside the cell. It was not determined whether the cells could adapt to increased osmolarity.

**Figure 6** Effect of osmolarity of medium. Amoebae were exposed to variable osmolarities by preincubation for 15–20 min at room temperature in SM (approximately 150 mosM) containing varying concentrations of sucrose as indicated. Phagocytosis was then assayed in the usual way and was expressed as a percentage of the uptake in the absence of sucrose. The curve represents the results of two experiments, in which the PS and cell concentrations were 400 µg/ml and 1.5–1.7 × 10^6 cells/ml, and control PS uptake was 17–54 µg/1 × 10^6 cells and 15 min. A third experiment gave similar results.

Previous studies of PS bead uptake by Acanthamoeba revealed that these cells were also sensitive to the osmolarity of the incubation medium (29). Either an increase or a decrease of the osmolarity away from the optimum was inhibitory. The optimum osmolarity could, within limits, be shifted by preincubation of the cells at different osmolarities.

Bead uptake was also sensitive to the presence of proteins in the incubation medium. When the PS beads were first mixed with varying concentrations of BSA, and these mixtures were added to the cells, uptake was inhibited to an extent dependent on the BSA concentration, as indicated in Fig. 7.

One possible explanation for this inhibition of PS bead uptake by BSA is competition for uptake, if the BSA is pinocytized by these cells, as it is by several types of amoebae (6, 7). Bowers and Olszewski (6) calculate that the cell surface of Acanthamoeba is turned over 2–10 times per hour as a consequence of pinocytosis. An alternative explanation involves a coating by BSA of the cell or PS surface, thus reducing the efficiency of the contact between the PS and the cell surface. Such a phenomenon has been observed with human PMN, whose capacity to phagocytize PS was inhibited by a prior incubation of the PS with a protein, glucose oxidase (2). Protein factors were, however, necessary for the optimal ingestion of starch granules by PMN (21), indicating that the interactions among different proteins, particles, and cells are rather complex.

**Figure 7** Effect of protein in the medium on ingestion. Amoebae were assayed for their ability to phagocytize PS in the presence of various concentrations of BSA. The PS and the protein were mixed and added to the cells simultaneously. Uptake was expressed as a percentage of the uptake in the flask containing no BSA. The final BSA concentration is given as g/100 ml. The curve represents three experiments which involved PS and cell concentrations of 400 µg/ml and 1.2–1.6 × 10^6 cells/ml. Control PS uptake ranged from 27 to 35 µg PS/1 × 10^6 cells and 15 min.
Effects of Metabolic Inhibitors on Uptake

Uptake of beads was dependent on metabolic energy, as shown by the data in Table III. The dependence appeared to be primarily on energy generated by aerobic pathways of metabolism, as indicated by the effects of azide, cyanide, and dinitrophenol, while the characteristic inhibitors of glycolysis, fluoride and iodoacetamide, had lesser effects. The lack of effects by fluoroacetate, malonate, and iodoacetate was very likely due to the inability of these charged molecules to penetrate the cells adequately. The amoebae of the slime mold D. discoideum have been shown to be relatively impermeable to molecules such as glucose and methionine (33, 34), as have other amoebae (8, 29).

In order to show that the effective substances did actually inhibit the energy metabolism of these cells, their influence on cellular oxygen consumption was also determined. The representative data shown in Table IV are consistent with the supposition that these substances affected phagocytosis by interfering with metabolic energy generation.

This pattern of the metabolic dependence of phagocytosis resembles that seen in Acanthamoeba (29), except that the latter were inhibited only slightly by 40 mM fluoride. (Cyanide, azide, dinitrophenol, and iodoacetate were also employed.) Although the authors concluded that phagocytosis did not depend on glycolysis, it would have been more conclusive to have used iodoacetamide to avoid the possibility that the lack of effect by iodoacetate was due to the inability of that substance to enter the cells adequately. The PMN, on the other hand, are primarily dependent on glycolysis, and relatively independent of pathways of aerobic metabolism (e.g. 21).

"Consumption" of Surface Membrane During Phagocytosis

The quantity of cell surface membrane ingested by P. pallidum amoebae during a 30 min incubation at saturating particle concentrations was calculated to exceed the available surface area of the cells, as follows. The cells were assumed to be spherical and to have an average surface area of 230 (pm)², based on an average cell diameter of 8.55 μm (11). The amount of surface area consumed was assumed to be equal to the surface area of the particles ingested. Given an average \( V_p \) of 31.2 μg PS/1 \( \times 10^6 \) cells and 15 min, a bead diameter of 1.1 μm, that 1 μg of PS represented 1.4 \( \times 10^6 \) beads, and the assumption that each vacuole contained only one bead (two or three beads were in fact occasionally observed in a single vacuole), the average surface area consumed per cell in 30 min was 332 (μm)², or about 1.5 times the available surface area. Similar calculations may be made for Acanthamoeba and PMN.

Several explanations for this paradox may be

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**Table III**

| Conditions | Inhibitor concentration (mM) | PS uptake ‡ |
|------------|-----------------------------|-------------|
| 0°C        | —                           | 13          |
| Sodium azide | 2                       | 6           |
| Potassium cyanide | 2                   | 14          |
| 2,4-dinitrophenol | 0.15                 | 30          |
| Fluoroacetate | 8.6                    | 88          |
| Malonate   | 1.4                        | 100         |
| Iodoacetate | 2                       | 83          |
| Iodoacetamide | 1-2                   | 28-46       |
| Sodium fluoride | 20                    | 87          |
|            | 40                        | 2           |

* The data were compiled from experiments involving PS and cell concentrations of 66-408 μg/ml and 0.91-2.2 \( \times 10^6 \) cells/ml. The cells were preincubated 15-40 min with inhibitor before the addition of PS.

† Expressed as a percentage of the control value.

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**Table IV**

| Inhibitor          | Inhibitor concentration (mM) | Oxygen consumption † |
|-------------------|------------------------------|----------------------|
| Sodium azide      | 0.2                          | 55                   |
|                   | 3.0                          | 30                   |
| 2,4-dinitrophenol | 0.01                         | 130                  |
| Sodium fluoride   | 20                           | 100                  |
|                   | 40                           | 44                   |
| Iodoacetate       | 0.1                          | 100                  |
|                   | 2.0                          | 100                  |

* Expressed as a percentage of the control values.
offered: (a) “New” surface membrane may have been synthesized to replace that consumed by phagocytosis, but this explanation has been explicitly ruled out in the cases of Acanthamoeba (27) and PMN (22). (b) The calculation of the amount of available surface membrane may be in error, to the extent that the cells are not spherical. Electron micrographs of phagocytizing Acanthamoeba (10), for instance, indicate that this is the case, but the deviations from the spherical (evident in electron micrographs of thin sections) appear to be insufficient to account for the large amount of cell surface putatively internalized during phagocytosis. (c) The surface membrane may be capable of stretching and thus provide in effect a reserve of membrane. (d) The cells may contain in their cytoplasm membrane “subunits” or components which can replenish the surface membrane as it is consumed by phagocytosis. (e) The “consumption” of surface membrane may be minimized by the merging of lysosomes with the incipient food vacuoles, allowing the particle to be enclosed within a membrane which is derived in part from lysosomes, thus conserving the surface membrane. In the PMN, lysosomes have been observed to merge with an incipient vacuole before it has been pinched off from the cell surface (35). (f) Finally, the cell may simply return endocytized cell membrane by exocytosis, as Acanthamoeba, at least, is suggested to be capable of doing (6).

Effect of Starvation and Subsequent Development on Phagocytosis

The developmental sequence of the amoebae was initiated by harvesting exponentially growing cells, washing them several times, and resuspending them in SM at about 24°C. The cells responded by encysting, as described elsewhere (11).

The effect of these conditions on the phagocytic ability of the amoebae was determined by periodically removing aliquots of the cell suspension and assaying as usual for the ability of the cells to ingest PS beads. In several experiments, the amoebae were starved at cell concentrations convenient for the subsequent assay of phagocytosis. In other experiments the amoebae were starved as concentrated suspensions and were diluted about fivefold with fresh SM before measuring phagocytosis, in order to diminish the possibility that substances accumulating in the medium might have an effect on phagocytosis. These two sets of experiments gave similar results, and further control experiments, in which the medium obtained from starving cell suspensions was added to freshly harvested cells, showed that no phagocytosis-inhibiting substances accumulated in the medium during starvation.

The results of the experiments, as shown in Fig. 8, indicate that starvation caused an immediate decrease in the ability of the cells to ingest PS beads, when uptake was measured over a 10–15 min assay period. The apparent first-order kinetics of the change indicated a half-time of about 10 h for the decay in phagocytic ability. Essentially all of the cells remained viable during these experiments, as evidenced by the fact that a constant

![Figure 8](image-url)

**Figure 8** Effect of starvation on phagocytosis.

Starvation of the amoebae was initiated as described in the text, and aliquots of starving cells were tested periodically for their ability to phagocytize PS. Uptake was expressed as a percentage of the rate of PS uptake in the unstarved samples, based on cell number. The data points represent five separate experiments, and the best line was fitted to the points by the method of least squares. The PS and cell concentrations ranged from 125 to 286 µg/ml and 1.2 to 3.3 × 10⁶ cells/ml, and the zero time PS uptake was 13–41 µg PS/1 × 10⁶ cells and 15 min.
95% of the cells ingested at least one particle when assayed every 7 h over a 20-h starvation period (approximately 100 cells were examined by phase contrast microscopy at each time point). This result also shows that the diminution of phagocytic ability was a consequence of a reduction in the amount of PS ingested per cell.

The loss of phagocytic capacity during starvation was found to be due primarily to a progressive decrease in the period over which PS bead uptake was linear with time, as shown in Fig. 9, even though there seems to be a modest increase in activity after about 1 h. That this overall change was due to a decrease in the maximum number of beads which a single cell could phagocytize was suggested by the data shown in Fig. 9 E. When these cells (which had been starved for 10.5 h) were incubated at a range of concentrations of PS, we noted (a) that at the highest PS concentration the uptake of PS per cell reached a plateau value after 5–10 min of incubation, unlike the case with growing cells, while (b) at the lower PS concentrations, uptake was still continuing after 15 min of incubation.

A similar study has been made previously of the effect of starvation (leading to encystment) on the phagocytic capacity of Acanthamoeba (30). Weisman and Moore (30) observed a rapid loss (about 50% in 4 h) of the ability of the amoebae to phagocytize PS beads under conditions which produced the first cysts in about 8 h of starvation. The kinetics of phagocytic loss thus correspond roughly to those reported for P. pallidum above, when the slower rate of encystment of the slime mold amoebae is taken into account.

There are three major explanations for the observed reduction in phagocytic capacity of these amoebae as they respond to a starvation medium: (a) a depletion of the cells' energy stores; (b) a decrease in the supply of components (structural or catalytic) essential to the formation of phagocytic vacuoles, or (c) a generalized change in the cellular membrane leading to decreased phagocytic activity. The first explanation is a major possibility in the case of Acanthamoeba, since the starvation medium contained only salts and buffer (30). It is less likely in the case of P. pallidum, since the starvation medium contained glucose and Bactopeptone, and the cellular glucose levels did not decrease during the first 20 h of starvation (11). Both cell types did, in any case, possess sufficient energy stores for the extensive cellular alterations involved in encystment.

The second possibility, that the change in phagocytic ability was due to the loss of a cell component essential for the formation of the phagocytic vacuole, is more interesting, since such a substance might be shown to be essential to (and perhaps unique to) the phagocytic process. A likely candidate for a component essential to phagocytosis that might be depleted during starvation is a contractile apparatus required for the membrane deformations undergone by the cell during phagocytosis. The microfilament system, as reviewed by Wessells et al. (31), is a possibility. Microfilaments, which have been shown to contain actin (e.g., Pollard et al. 17), are present in Acanthamoeba (17, 28), and D. discoideum (closely related to P. pallidum) has been
shown to contain an actomyosin-like component (32). The involvement of microfilaments in phagocytosis has been implied by experiments demonstrating that cytochalasin B, which disrupts microfilaments, also inhibits phagocytosis (1, 9). However, as discussed by Spudich (24), cytochalasin may inhibit phagocytosis by some other mechanism, such as interaction with the cell surface more generally.

The third explanation (not unrelated to the second above) may hinge on such observations as those of Weisman and Moore (30) who in their study of Acanthamoeba regarded the rapid loss of phagocytic ability as a sensitive indicator of early cell surface changes associated with encystment. Further, Bowers and Korn (5) noted surface changes during the early phases of encystment of Acanthamoeba. Theirs was an electron microscopic study and they documented the gradual secretion to the cell surface of a noncellulose material, which might well reduce the area of the cell surface available for phagocytosis. Cell surface changes have also been reported to occur during the starvation-induced development of D. discoideum amoebae (10, 23). Alternatively, there might simply be a decrease in phagocytosis-competent cell membrane: the amoebae of P. pallidum (11) and Acanthamoeba (5) decrease in size during the encystment process. Rational answers to the question posed initially as to the specific nature of certain cells, and particularly their membranes, that permit phagocytosis could (it appears from the information presented here and in papers cited) come from the isolation and characterization of pure membrane preparations of such a cell as P. pallidum during starvation. In starving cells phagocytic activity decreases quite rapidly, paradoxically in a way, since one might have believed that starving cells would even more actively seek nutrients, and especially particulate nutrients (11). The data obtained after a very short period of starvation tend to indicate that this might possibly occur.

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