1987

A STUDY OF PHAGOCYTOSIS IN AMOEBAE OF DICTYOSTELIUM DISCOIDEUM

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A STUDY OF PHAGOCYTOSIS IN AMOEBAE OF *DICTYOSTELIUM DISCOIDEUM*

by

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A thesis submitted to The Council for National Academic Awards in partial fulfilment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

August 1987

Research was conducted at Plymouth Polytechnic in collaboration with The Institute of Marine and Environmental Research, Plymouth, England

A STUDY OF PHAGOCYTOSIS IN AMOEBAE OF *DICTYOSTELIUM DISCOIDEUM*

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Abstract

A study has been made of the effects of various treatments on the phagocytosis of ^{14}C-labelled *E.coli* by amoebae of *Dictyostelium discoideum*. An assay was also developed for the adhesion of amoebae to glass and the effects of a number of inhibitors on this process have been investigated.

The phagocytosis of bacteria was inhibited by chelating agents at millimolar concentrations. The effect of chelators was not apparent in the presence of added divalent cations. However, only a small reduction in cell-glass adhesion was seen with EDTA concentrations that caused large reductions in phagocytosis.

The calcium ionophore, A23187 abolished phagocytosis at 40 ug/ml. Pretreatment of amoebae with lanthanum ions completely inhibited both phagocytosis and cell-glass adhesion at low concentrations. Both phagocytosis and adhesion to glass are also strongly inhibited by calmodulin antagonists. Neither cytochalasin B or colchicine affected phagocytosis.

Concanavalin A strongly inhibited phagocytosis presumably due to a direct interaction with cell surface glycoproteins, since the effect did not occur in the presence of alpha-methyl mannoside. Both phagocytosis and adhesion to glass were greatly reduced on treatment of the amoebae with tunicamycin, again suggesting glycoprotein involvement. Pretreatment of amoebae for 30 min with 1 mg/ml trypsin or pronase had no effect on phagocytosis, although pretreatment with papain at the same concentration caused some reduction. However, phagocytosis became pronase sensitive on exposure to tunicamycin. Beta-glucosidase also caused a small but consistent reduction in phagocytosis and cell-glass adhesion.

Phagosomes were isolated from amoebae by two procedures. In the first, cells were allowed to phagocytose 1 um diameter polystyrene beads. The endocytosed beads were then isolated by flotation on a discontinuous sucrose density gradient. In a second procedure, devised during the course of this work, an attempt was made to isolate phagosomes from ingested glutaraldehyde-fixed *E.coli*. Analysis of these preparations by SDS-polyacrylamide gel electrophoresis showed a number of differences between them. A comparison of these preparations with "bulk" plasma membrane revealed a considerable similarity of the polypeptide profile with that isolated using fixed *E.coli*.

DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted for any other degree.

Signed D. Mealing

Candidate

Date: 25 August 1987

This is to certify that the work here submitted was carried out by the candidate himself.

Signed D. Mealing

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Signed

Supervisor of Studies

Date: 25 August 1987

Acknowledgments

Dr Peter Glynn for encouragement and patience throughout this work and during the writing of this thesis.

The technicians of the Biological Sciences Department, particularly Anne Bell and Dr Annette Wrathmell.

Dr Ann Pulsford for assistance with Electron Microscopy.

Sam Mealing for her unfailing support.

Dr Mike Moore for helpful discussion

Contents

Chapter 1

General Introduction

Phagocytosis

The uptake of particulate material by cells was first observed by Metchnikov (1884), who called the process phagocytosis. As the term is currently used, phagocytosis describes the ingestion of particulate matter 1 um or greater in diameter (Zuckerman & Douglas, 1979).The process can be considered to consist of two phases. The first stage is the attachment of the particle to the phagocytic cell. This is followed by the ingestion phase, during which pseudopodia advance around the particle until they meet at the apex and fuse. The trigger that initiates ingestion in response to particle attachment is poorly understood. However, the overall result of ingestion is that the particle is enclosed in a vacuole whose membrane is derived from the plasmalemma of the phagocyte. This vacuole is also known as a phagosome. The killing and digestion of the particle occurs following the fusion of lysosomes with the phagosomal membrane. This event can happen very quickly and in some cells occurs before the particle is

ingested. Any indigestible remains are either expelled from the cell by regurgitation or, in higher phagocytes, permanently retained within a cytoplasmic vacuole.

Many different types of cells are capable of phagocytosis and the major events of the process are similar in all of them. However, the functional significance of phagocytosis varies. In free living cells such as amoebae, the process is essentially nutritional. This is also true in some multicellular organisms such as sponges and coelenterates where specialised phagocytic cells are responsible for the nutrition of the whole organism (Barrington, 1967; McNeil et al., 1981). In more complex multicellular organisms, such as mammals, phagocytosis serves a variety of functions not concerned with nutrition. One of the most important of these functions is the killing of potentially pathogenic microbes such as bacteria and fungal spores. The neutrophil polymorphonuclear leucocytes are particularly highly specialised in this respect. Similar functions are carried out by macrophages but these cells also have a role to play in antigen presentation to lymphocytes. In addition, macrophages are involved in the clearance of dead cells and other particulate debris.

These "higher" phagocytes have specialised cell surface receptors and have developed biochemical mechanisms (such as free radical production) which enable them to kill bacteria efficiently. Nevertheless,

it is apparent that the mechanisms of ingestion in free living and higher phagocytes are markedly similar. The study of phagocytosis in free living cells may therefore contribute to an understanding of the same process in polymorphs and macrophages. The use of lower eucaryotes as model systems for the study of phagocytosis can result in considerable technical advantages to the experimenter.

Dictyostelium discoideum

The present study is concerned with phagocytosis in amoebae of Dictyostelium discoideum, a member of the class Eumycetozoa. The life cycle of these organisms includes a free living solitary amoebal stage and a multicellular stage leading to spore formation. This multicellular development has been extensively investigated in some members of the group, because it has been seen as a simple model of the differentiation process that is central to the formation of more complex organisms.

D.discoideum is one of about 50 separate Dictyostelid species and was isolated from soil collected from a North Carolina forest (Raper, 1935).Its life cycle has been described in detail in several reviews (Bonner, 1944; Sussman, 1966; Katz & Bourguignon, 1974) and is shown diagrammatically in fig. 1.1. The vegetative stage constitutes free living

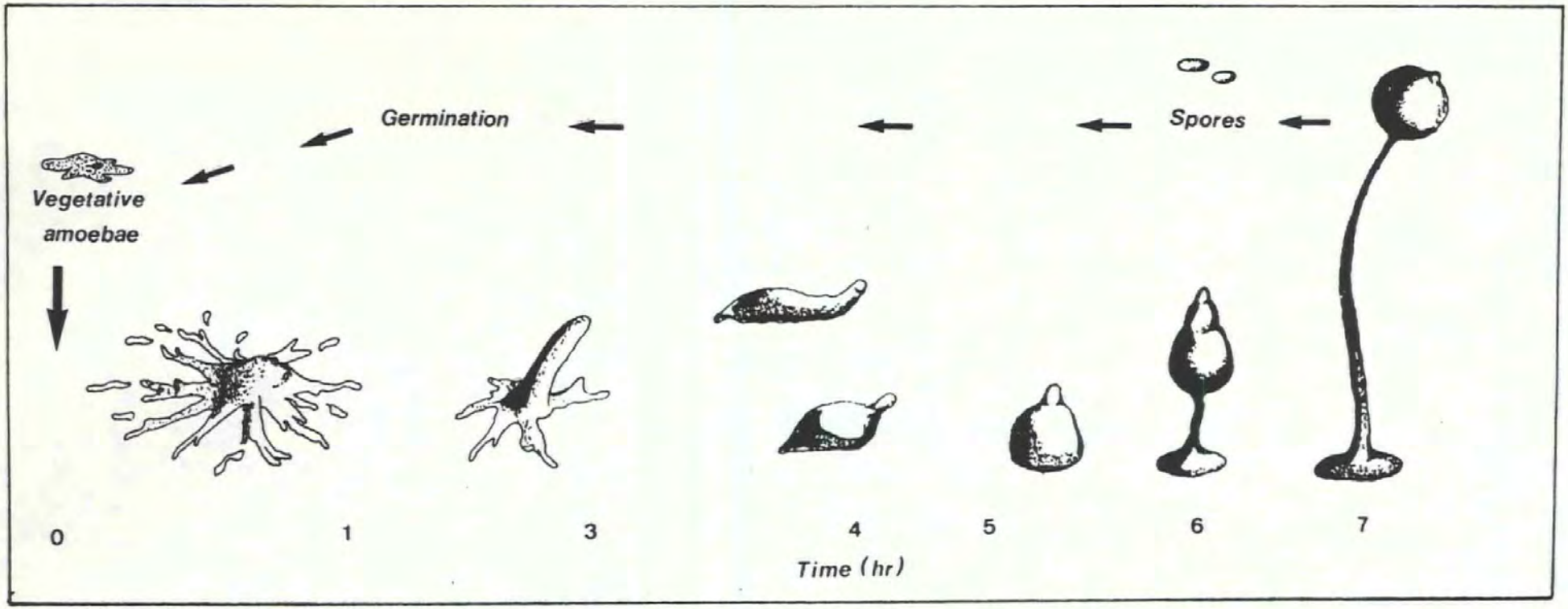


Fig. 1.1 Life cycle of Dictyostelium discoideum with development indicated at approximate time intervals. (not to scale)

solitary amoebae that live in soil and dung, feeding by the phagocytosis of bacteria. This phase of the life cycle may continue indefinitely in the presence of an adequate food supply. The aggregation stage of the life cycle is triggered by the exhaustion of local food supplies. Chemotactic signals emanating from starving amoebae stimulate other amoebae to move into aggregation centers which eventually form a mound of cells. This subsequently develops into a slug-like cylinder, or pseudoplasmodium, which may migrate for some time in response to light or heat. At the end of this period of migration, the cell mass rounds up and differentiates into a fruiting body consisting of a spherical spore mass supported by a stalk about 2 mm in height. Desiccation of the spore mass eventually leads to the release of the spores, which germinate under suitable conditions as single amoebae, so beginning the cycle again.

The wild type strain of D.discoideum (strain NC4) can be grown on bacterial lawns in the laboratory. In addition, mutant strains are now available which feed by pinocytosis (fluid uptake). These axenic strains are able to grow in liquid medium in the absence of bacteria but retain the ability to ingest a wide range of particulate matter (Watts & Ashworth, 1970).

Although it has been less intensively investigated than other aspects of the life cycle,

phagocytosis is the principal mode of nutrition of D.discoideum in nature. A proper understanding of the process is therefore essential if a detailed knowledge of the life cycle is to be gained. In addition, there may be wider implications from such a study. Horn (1971) found that four species of slime moulds could successfully coexist in one small plot. He concluded that this degree of diversity was achieved through the marked preference of each species for different bacteria. This finding bears directly on the process of phagocytosis, implying some degree of selectivity in terms of recognition. Again, a more complete understanding of phagocytic mechanisms may disclose the basis of this selectivity and thus have a contribution to make to an understanding of microbial ecology.

Aims and objectives

In spite of the central role of phagocytosis in the life cycle of D.discoideum, little information is available concerning the general biology of the process in this organism. One of the aims of this project was to use a variety of substances of defined specificity in order to obtain information about the factors affecting phagocytosis. These included chelating agents, ionophores, lectins, proteolytic enzymes and inhibitors of cytoskeletal function. For the most part, the effects of these substances have been studied using

a previously developed assay for phagocytosis which employs ^{14}C-labelled E.coli. However, a simple assay for the measurement of cell adhesion to glass was also developed and the effects of some of these substances on this parameter have also been studied.

A second aim was to investigate the polypeptide composition of phagosome membranes (isolated from around latex beads using density gradient centrifugation) by SDS-polyacrylamide gel electrophoresis. An attempt was also made to isolate phagosomal membranes from around glutaraldehyde-fixed E.coli. This was to investigate the possibility that different binding sites might be responsible for the surface attachment of different types of particle. Finally, an attempt was made to isolate whole plasma membrane to compare its elecrophoretic profile with phagosomal membranes.

Chapter 2

Review

General properties of phagocytosis

Phagocytosis is an energy dependent process in higher phagocytes (Sbarra & Karnovsky, 1959) although evidence suggests that the attachment phase in macrophages can be energy independent (Griffin et al., 1975). These workers reported that antibody or complement coated erythrocytes attached to macrophages at 4°C but were only ingested on warming to 37°C. However, Tadanao et al. (1981), using mouse peritoneal macrophages also separated attachment and ingestion of bovine serum albumin (BSA) coated oil droplets containing spin labelled cholestanone. The addition of a membrane impermeable reducing agent dampened the electron spin resonance signal from particles bound to the outside of the cell. Therefore the two signals could be measured separately. These workers reported that both attachment and ingestion were energy dependent. This difference may be explained by the general observation that attachment of a particle to cell surfaces containing no specific receptor for that particle seems to be energy dependent (Silverstein et al., 1977). The energy required for the phagocytic process of some higher cells appears to be predominantly derived from glycolysis. Stossel et al.

(1972) reported that inhibitors of glycolysis reduced the initial rate of uptake of oil droplets by polymorphs while inhibitors of oxidative phosphorylation and electron transport, such as dinitrophenol and cyanide, were without effect. In contrast, Tadanao et al. (1981) found that inhibition of either the electron transport pathway (by cyanide) or glycolysis (by 2-deoxyglucose) extensively reduced both attachment and ingestion of BSA-coated paraffin oil droplets by macrophages. This suggests that both of these processes contribute towards the energy requirement of both phases of phagocytosis in these cells.

The situation in free living phagocytes is rather different. Bowers (1977) reported that phagocytosis by Acanthamoeba castellanii was highly energy dependent and inhibitors of oxidative phosphorylation completely abolished uptake while glycolytic inhibitors had little effect. The uptake of bacteria by D.discoideum was found to be energy dependent by Glynn, (1981). He found that sodium azide and dinitrophenol abolished phagocytosis while inhibitors of the glycolytic pathway had no effect. Githens & Karnovsky, (1983) reported the inhibition of latex bead uptake by the slime mould Polysphondilium pallidum by inhibitors of aerobic respiration. The general findings seem to be that polymorph phagocytosis is dependent on energy derived from glycolysis while macrophages seem to vary in this

respect depending on species or tissue origin. However, it seems that all free living phagocytes are dependent on aerobic respiration for the phagocytic process.

A requirement for extracellular divalent cations has been reported by several workers. Stossel et al. (1972) observed a 65% drop in the initial rate of phagocytosis by polymorphs if magnesium was absent from the incubation medium and a drop to zero if divalent cations were absent altogether. Tadanao et al. (1981) reported that divalent cations were essential for attachment of oil droplets to macrophages but did not appear to be required for ingestion, as cells allowed to attach oil droplets and then resuspended in cation-free medium ingested the particles normally. The role of external cations in phagocytosis by lower cells is unclear. However, cell-cell adhesion during aggregation of D.discoideum amoebae has been shown to be dependent on the presence of external calcium ions (Europe-Finner et al.,1984). Additionally, Cyr & Bernstein, (1984) reported that non-lethal levels of cadmium ions inhibited phagocytosis in D.discoideum. They suggested that cadmium may be competing with calcium for external binding sites on the plasma membrane.

The attachment phase

Phagocytosis is a process initiated at the external face of the plasma membrane. One approach taken in an attempt to investigate the factors affecting attachment by phagocytes centres on the study of the surface properties of particles.

The surface charge of a particle has long been considered to be of importance in determining its acceptability for attachment. Stossel *et al*. (1972) prepared oil droplets emulsified with various agents to modify their net surface charge. They reported that particles with a strong net charge (either positive or negative) were ingested more avidly than uncharged or weakly charged particles. Furthermore, they found that some particles required the presence of divalent cations for attachment and ingestion. They concluded that this effect depended on the nature of the substrate particles and did not bear a simple relationship to net surface charge. In apparent contrast, Van Oss (1978) reported that bacteria with a greater surface hydrophobicity than the phagocyte would become attached and ingested whereas bacteria less hydrophobic than the phagocyte would resist attachment. Additionally, Absolom *et al*. (1983) reported that bacteria appeared to adhere better to hydrophilic, rather than hydrophobic surfaces.

Non-specific factors such as surface charge and hydrophobicity are not the only ones governing the interactions between phagocyte and particle. For example, Rottini et al. (1979) have demonstrated a specific interaction between lectin-like substances on the surface of E.coli and mannose residues on the surface of human polymorphs. However, specificity is best exemplified by the phagocytic cells of the vertebrate immune system which have developed a form of recognition involving phagocyte membrane receptors and the coating of particles by "palatable" proteins. Opsonisation, as this coating is called, is one of the primary mechanisms involved in the recognition and subsequent ingestion of foreign particles by phagocytes of the immune system (Kozel & McGraw, 1979). Macrophages possess membrane receptors that recognise the Fc region of the IgG molecule. These receptors mediate the binding of IgG coated particles to the membrane of these phagocytes (Griffin et al., 1975) and participate in the ingestion process (Rottini et al., 1979). It has been reported that a glycoprotein of molecular weight 140 Kd may be identical to (or a component of) the Fc receptor (Howard et al., 1982).

The binding of immunoglobulin to bacteria has been shown to mediate the binding of serum factors that enhance phagocytosis (Johnston et al., 1969). They reported that bacteria coated with IgG were not ingested by human leucocytes unless a small amount of

serum was also present during phagocytosis. They demonstrated that the first four components of complement bound to IgG coated bacteria in the order C1,C4,C2 and C3. The last component C3 is recognised by specific receptors on the phagocyte plasma membrane. Binding of C3 component alone to the particle is mediated by the activation of the alternative complement pathway, and particles triggering this reaction are phagocytosed via cell surface bound C3 receptors (Warr, 1980). Differences in the affinity of these receptors have been reported by Sela et al. (1981) who demonstrated that oral polymorphs bound and ingested Candida albicans via complement receptors far more avidly than peripheral blood polymorphs. These C3 receptors are distinct from the Fc receptors that recognise IgG. Opsonisation can be considered as a means by which higher phagocytes can use a limited range of surface receptors to attach and ingest a wide range of different particles.

Attachment of non-opsonised particles to phagocytes has also been reported. Lung macrophages can (in the absence of serum factors) strongly bind and ingest yeast (Warr, 1980). This author suggested that this binding may be mediated by a lectin-like molecule on the phagocyte plasma membrane that recognises mannose or glucosamine residues on the yeast. Additionally, Benoliel et al. (1980) demonstrated a non specific binding of latex beads that was energy

dependent, and of glutaraldehyde fixed red blood cells that was energy independent, to peritoneal macrophages.

In general, there is less information available concerning the nature of surface attachment in D.discoideum and lower phagocytes. No specific molecule at the surface of nutritive phagocytes has been convincingly shown to mediate attachment. There is, of course, no counterpart of opsonisation in lower phagocytes. However, D.discoideum, along with other phagocytes, can bind and ingest a wide variety of different types of particle, including yeast and latex beads (Hellio & Ryter, 1980), bacteria,tungsten beads and derivatised latex beads (Vogel et al., 1980) as well as being able to bind to glass, plastic and other amoebae. Attachment by D.discoideum to all of these particles or substrata is energy dependent and sensitive to respiratory blockers such as sodium azide. However, the binding of latex beads in buffer to amoebae is energy independent (Glynn & Clarke, 1984). They suggest that this type of binding is non-specific and probably mediated by hydrophobic interactions at the cell surface. The binding of beads coated in axenic growth medium, however, is azide sensitive, presumably because medium makes the beads more hydrophilic.

A detailed investigation of phagocytic attachment in D.discoideum has been carried out by Vogel et al. (1980). Amoebae were mutagenized and subsequently incubated in medium in the presence of tungsten beads

at 27°C (the non-permissive temperature). Cells that did not phagocytose beads could be isolated on the basis of their lower density, and these were screened for temperature sensitive growth at 27°C on nutrient agar plates in association with E.coli. About 5-10% of the cells that did not phagocytose tungsten beads were also found to be temperature sensitive for growth on bacteria and presumably, therefore, phagocytosis. Of these, three strains of mutants were isolated that grew in axenic medium via pinocytosis at both the permissive and non-permissive temperatures with wild type characteristics, but did not phagocytose any substrate particles when incubated in medium at the non-permissive temperature. These strains appeared to carry a mutation specifically affecting phagocytosis without being impaired in other essential cellular functions, including pinocytosis. From the results of experiments measuring the uptake of various types of particles in buffer, or in medium, these workers postulated the existence of two functionally independent binding sites on the cell surface of normal amoebae. One of these was postulated to bind a variety of particles by hydrophobic interactions. It was suggested that this site was altered in mutant cells such that only the most hydrophobic of particles (polystyrene beads suspended in buffer) could attach. However, these mutant cells were able to phagocytose E.coli normally when suspended in buffer but not in

axenic growth medium. It was found that glucose (normally present in the growth medium) completely inhibited the uptake of bacteria in these mutants. To account for this, a second binding site was proposed which was specific for bacteria whose walls contained terminal glucose residues. The inability of these mutants to phagocytose E.coli strain K2754 (whose walls lack terminal glucose residues) was seen as further evidence for this hypothesis. In a later study, Vogel and Duffy (1984) found that phagocytic mutant phenotypes were governed by two loci on different chromosomes. They therefore suggested that the altered non-specific receptor in mutant cells could be a complex of more than one molecule.

Hellio and Ryter (1980) investigated the phagocytosis of polystyrene latex beads, bacteria and yeast in the presence of two lectins known to bind to D.discoideum amoebae plasma membrane (West et al., 1978). They found that concanavalin A (Con A), a lectin that specifically binds glucose or mannose residues, inhibited the attachment and uptake of all three particles for one and a half hours after the addition of the lectin. Addition of wheat germ agglutinin (WGA), which specifically binds glucosamine residues, also inhibited the uptake of all particles for one and a half hours. However, after this time period, cells resumed attachment and ingestion of bacteria and

polystyrene beads but required a further one hour recovery period before the attachment and ingestion of yeast resumed. Importantly, this coincided with the reappearance of WGA binding sites at the plasma membrane. These workers concluded that D.discoideum amoebae may possess specific receptors for the attachment and ingestion of yeast and that these molecules also bind WGA. In contrast binding of Con A was absent in cells that had resumed phagocytosis of bacteria and polystyrene beads. They concluded that some other mechanism was involved in Con A inhibition and was not due to the loss of a specific cell surface molecule that participated in the uptake of bacteria and beads.

Chadwick and Garrod (1983) purified a 126 Kd glycoprotein (gp126) from the plasma membrane of vegetative D.discoideum amoebae. In later work, Chadwick et al. (1984) found that treatment of intact amoebae with a univalent fragment of an antibody to this glycoprotein completely inhibited both phagocytosis and cell-cell cohesion. They proposed that gp126 might be responsible for the EDTA sensitive cohesion of vegetative amoebae (Beug et al., 1970), and also might be directly involved in phagocytosis of bacteria. They suggested that this glycoprotein might be related in some way to the non-specific receptor postulated by Vogel et al. (1980).

Attachment of D.discoideum amoebae to substrata

has been investigated by Bozzaro & Roseman (1983). These investigators reported that vegetative amoebae bound to sugar derivatised gels with varying affinities, depending on the sugar derivative. They concluded that there may be three distinct receptors at the surface of these cells that recognise glucose, mannose and N-acetylglucosamine. They also proposed that binding of amoebae to glucose derivatised gels might be mediated by the glucose receptor postulated by Vogel et al. (1980).

Kayman et al. (1982) isolated mutants of D.discoideum that were defective in migration and in attachment to glass slides. They also noted that these cells were unable to extend pseudopodia or phagocytose bacteria. They suggested that cellular mechanisms involved in these activities may share common elements. These are possibly related to the likely involvement of cytoskeletal components in all of these processes.

The ingestion phase

Subsequent to particle attachment is the extension of pseudopodia around the particle. How attachment of material to the phagocyte membrane 'triggers' the extension of pseudopodia is unclear. However, Griffin et al. (1975), have studied this phenomenon in macrophages and postulate a mechanism for pseudopodial engulfment of attached particles.

These workers treated sheep red blood cells with antibody (IgG) and allowed them to attach to macrophages at 4°C, a temperature which allows attachment but not ingestion. The non attached surfaces of these red blood cells were then treated with anti-IgG to block exposed Fc regions on the erythrocyte surface. On warming to 37°C, no ingestion occurred. On the basis of this result, they suggested that continual apposition of receptors and ligands was essential during pseudopod extension. It was also found that mouse lymphocytes incubated with IgG and allowed to "cap" these antibodies on one hemisphere were ingested only over that hemisphere. These investigators concluded that attachment of particles to specific receptors on the macrophage membrane was not sufficient to trigger ingestion. Rather, ingestion requires the sequential circumferential interaction of particle-bound ligands with plasma membrane receptors not involved in the initial attachment. This hypothesis has been called the "zipper mechanism".

Pseudopodia surrounding particles undergoing ingestion are seen to be free of organelles and composed of diffuse arrays of microfilaments that appear to be predominantly composed of actin (Painter, 1983). Myosin and other contractile network proteins have also been detected at sites of pseudopod extension during phagocytosis by higher cells (Painter, 1983; Stendahl et al., 1980). Additionally, the involvement

of actin in the process of phagocytosis is supported by the inhibitory effect of cytochalasin B on the ingestion of particles by macrophages (Zuckerman & Douglas, 1979) and human polymorphs (Todd-Davies et al., 1971). For any proposed contractile machinery to exert structural influence over the plasma membrane, as during phagocytosis, it would seem likely that interactions between the cytoskeleton and the plasmalemma would take place. In support of this idea, several workers have reported direct attachment of actin to D.discoideum plasma membrane. McMahon and Hoffman (1979) reported that actin and a "spectrin-like" protein are associated with the cytoplasmic face of amoebal plasma membranes. Condeelis & Bennett (1984) reported two different types of actin attachment to D.discoideum plasma membrane. They described an end-on attachment of actin filamemts and also an actin-filament attachment mediated by a rod shaped bridge. Similar bridges have been isolated from intestinal brush border cells and are reported to be associated with calmodulin, a ubiquitous calcium ion binding protein, suggesting regulation by calcium ions (Howe & Mooseker, 1983). Such calcium regulated bridges, if present in phagocytic cells, may modulate filament association with the plasma membrane. An association between actin and the plasma membrane has also been suggested by the work of Luna et al. (1984). They used F-actin derivatised beads to isolate putative

actin binding proteins, from the plasmalemma of D.discoideum and isolated 12 possible membrane actin-binding proteins four of which spanned the plasma membrane. Similar actin-membrane associations have been reported in A.castellanii (Pollard & Korn, 1973).

Although a role for the cytoskeleton in phagocytic ingestion is widely accepted, the details of its involvement remain unknown. Yin and Stossel (1982) have published a rather detailed model of how pseudopodia form and propagate around particles. In brief, attachment of a particle to its receptor causes a local and transient drop in calcium ion concentration in the cytoplasm immediately beneath the attachment site. This reduces the activity of microfilament severing proteins such as gelsolin and thus causes increased gelation of the cortical cytoplasm. Actin filaments on either side of this region are pulled towards it because of the greater concentration of actomyosin complexes in the gelled region. Because some of these filaments are attached to the membrane, the latter is also drawn towards this region and is forced up against the particle. This causes further interactions between the particle and its binding site, so increasing gelation and causing the withdrawal of filaments from the adjacent cortex and the original contact site.

The net result of this process is the propagation of the pseudopodia around the particle. Although this scheme is consistent with many of the known details of cytoskeletal organisation in phagocytes, it nevertheless remains highly speculative.

It has been suggested that during the ingestion of particles, alterations in membrane composition take place so as to selectively include or exclude certain components from the phagosome membrane. The disappearance of a 140 kd protein and appearance of 45 kd and 50 kd proteins during phagocytosis in macrophages (Howard et al., 1982) lends credence to the idea that specific alterations in the plasma membrane may take place as a consequence of ingestion. Parish and Pelli (1974), reported that alkaline phophatase was excluded from phagosome membrane during the uptake of latex beads by D.discoideum amoebae. They also reported that colchicine blocks this normal exclusion process and concluded that colchicine binding proteins were involved in preventing the appearance of alkaline phosphatase in phagocytic vacuoles. Modifications to the plasma membrane during phagocytosis in D.discoideum have also been investigated by Favard-Sereno et al. (1981). They employed a freeze fracture technique to visualise the two faces of the plasma membrane during uptake of yeast cells. They observed 'bumps' and corresponding pits (intramembranous particles) on the two faces of the plasma membrane. They suggest that

some form of membrane modification takes place during formation of the phagosome as they observed a large increase in the number of intramembranous particles in the membrane beneath the attached yeast. Bowers (1980), reported a fourfold increase in the density of intramembrane particles in *A.castellanii* phagosome membranes compared to plasma membrane and showed this to be a consequence of an increased protein:lipid ratio. He also observed that membrane fusion during formation of phagosomes in these cells was achieved by a 'fragmentation' of the neck of the flask-like invagination of the forming phagosome. The fusion of membranes can be considered to be the final event of ingestion, although exactly how this is achieved is not known. However, Korn *et al*. (1974), suggested that modifications in the lipid content may be involved in the fusion event. They reported such modifications in *A.castellanii* and proposed that an increased lysophospholipid content may mediate this final ingestion event.

A natural consequence of phagocytosis is the interiorisation of part of the plasma membrane. This loss of plasma membrane cannot continue indefinitely and there is now evidence for extensive recycling of membrane in both lower and higher phagocytes (Steinman *et al*., 1983). Using macrophages, Muller *et al*. (1980), demonstrated that membrane lost to the inside of the cell during phagocytosis enters some intracellular pool and that a component of this is

subsequently returned to the plasma membrane. They reported that movement between lysosomal membrane and the plasmalemma is rapid and that a bidirectional flow of membrane between these two compartments is present during phagocytosis in these cells.

Membrane recycling during phagocytosis in D.discoideum has been studied by de Chastellier and Ryter (1977) and de Chastellier et al. (1983). In pinocytosing vegetative cells of D.discoideum, the membrane area of the vacuolar digestive apparatus is approximately equal to that of the plasma membrane. During ingestion of yeast, however, 40% of the plasma membrane was interiorised yet the plasmalemma area remained constant. They showed that this was due to replacement of plasma membrane by membrane of the vacuolar digestive system which decreases in area by a corresponding 40%. De Chastellier and Ryter (1977) proposed a relationship between pinocytic vesicles and newly formed phagosomes which, together with subsequent recycling of phagosome membrane back to the cell surface during exocytosis of undigested components, helps to maintain the plasma membrane area. A further study by de Chastellier et al. (1983), employed the labelling of D.discoideum plasma membrane glycoconjugates with radioactive galactose. This enabled the fate of plasmalemma to be followed during the phagocytosis of latex beads. They found that during phagocytosis, these markers reached an

equilibrium between plasma membrane and internal membranes after one hour, with 57% of label internalised and 43% remaining at the cell surface. They showed that rapid mixing of new and old phagosomes took place and observed that the equivalent of the entire plasma membrane was internalised and recycled every 20 min. Membrane recycling appears to be a mechanism that maintains the area and possibly the composition of the plasma membrane in phagocytic cells.

Chapter 3

Materials and Methods

Reagents

Components of growth media (e.g. bacteriological peptone) were obtained from Oxoid Ltd. (Basingstoke, Hants) or London Analytical and Bacteriological Media (Lab m, Salford, Manchester). Routine reagents (e.g. buffer salts) were obtained from BDH Chemicals Ltd (Poole, Dorset) or the Sigma Chemical Company (Poole, Dorset). More specialised reagents (e.g. enzymes, ionophores, etc.) were also obtained from the Sigma Chemical Company unless otherwise indicated. ^{14}C-lysine was obtained from Amersham International (Amersham, Bucks)

Growth and maintenance of Dictyostelium discoideum

Dictyostelium discoideum, strain Ax2, was obtained from the American Type Culture Collection (Cat. No. 24397). The amoebae were grown in a medium containing (per litre): 14.3 g bacteriological peptone, 7.2 g yeast extract, 15.4 g glucose, 1.3 g $Na_2HPO_4 . 2H_2O$ and 0.5g KH_2PO_4 (Watts & Ashworth, 1970). This medium will henceforth be referred to as

HL5-G. The medium was autoclaved in 250 ml conical flasks (approximately 80 ml per flask) at 15 psi for 15 min. Cultures were initiated by inoculation with spores or subcultured amoebae and grown on a rotary shaker at 22°C and 120 rpm. Under these growing conditions the amoebae doubled in numbers approximately every 10 hr, and reached stationary phase at a cell density of 1-2 x 10^7 cells per ml. The amoebae were subcultured when cell density was between 5-8 x 10^6 per ml and new cultures were initiated from clonally derived spores approximately every 3 months.

Cloning and spore formation

The amoebae used in this study were derived from a clone produced at an early stage of the work. The cloning procedure is straightforward owing to the ability of vegetative amoebae to grow on bacterial lawns and so form clear plaques where the bacteria have been ingested.

A culture of exponentially growing amoebae was diluted with fresh HL5-G medium to a density of approximately 10^3 cells per ml. One volume of this suspension was mixed with an equal volume of an overnight culture of Escherichia coli B. Aliquots of this mixture (0.2 ml per plate) were spread onto HL5-G plus 2% (w/v) bactoagar in petri dishes. These were incubated at 23°C until plaques of cleared bacteria

were visible (usually about 5 or 6 days).

Amoebae from a vigorously growing clone were gently scraped from around the perimeter of the plaque, using a sterile toothpick and as far as possible avoiding contact with the adjacent bacterial lawn. The amoebae were inoculated into 5-10 ml of HL5-G containing 100 ug/ml streptomycin sulphate and incubated on a rotary shaker at 22°C and 120 rpm to a cell density of approximately 5 x 10^8 amoebae/ml. The absence of bacteria was confirmed by subculturing into medium lacking streptomycin.

Spores were formed by transferring amoebae to a non-nutrient solid medium. The cells from approximately 25 ml of a late exponential phase culture were collected by centrifugation at 400 x g for 5 min. The pellet was washed once in an equal volume of sterile Basic Salts Solution (BSS) containing 10 mM KCL, 10 mM NaCl and 5 mM sodium phosphate buffer, pH 6.7. The final pellet was resuspended in 1/10th of the original volume of cell suspension. Aliquots of 0.2 ml were transferred to petri dishes containing 2% (w/v) bactoagar in BSS and spread with a glass spreader. The plates were incubated at 20-23°C for 48 h, by which time spore formation was complete. Fruiting bodies were scraped from the agar surface with a sterile spatula and transferred to 1 ml of HL5-G in sterile screw-capped microcentrifuge tubes (Sarstedt, Leicester). No other cryoprotectant was used. The

spores were frozen at -70°C. After 24 h, a small number of vials were removed and transferred to liquid nitrogen as master stocks. The remaining spores were stored at -70°C.

Growth and maintenance of Escherichia coli B

Escherichia coli B was innoculated into sterile tryptone soy broth (30 g/l) from stocks held in liquid nitrogen. The bacteria were grown overnight on a shaking water bath (60 strokes/min) at 37°C. An innoculum of this culture was then streaked onto tryptone soy agar plates (37 g/l) and incubated at 37°C overnight. The streaked plate was stored at 4°C and replaced every week. Single colonies were taken for innoculation into minimal medium for radiolabelling.

Radiolabelling of Escherichia coli

Escherichia coli B was used as the source of particles in all phagocytosis experiments unless otherwise stated. The bacteria were labelled by overnight growth in a minimal medium containing 1% (w/v) glucose and 0.2 uCi/ml ^{14}C-lysine. (The composition and preparation of the minimal medium are described in appendix I). The bacteria were grown overnight on a shaking water bath at 37°C. The labelled cells were collected by centrifugation at 2000 x g for

5 min, washed once in BSS and resuspended in the same medium. This procedure generally yielded specific radioactivities of the order of 10^4-10^5 cpm per 10^8 viable bacteria. The bacterial suspension was always used within 2 hr of preparation.

Protein determination

The protein content of amoebal suspensions was routinely determined by the method of Sedmak and Grossberg (1977). The technique is based on the formation of a soluble blue complex between protein and coomassie blue. The method is of comparable sensitivity to the Lowry procedure and is much simpler to carry out.

Coomassie blue reagent was prepared by adding 0.6g of Coomassie Blue G-250 to 1 litre of 3% (v/v) perchloric acid. The mixture was stirred overnight at room temperature and filtered. The brownish filtrate was stored in a dark bottle at room temperature and did not deteriorate over several months under these conditions.

Bovine gamma globulin (Cohn Fraction II) was dissolved in 0.05 M NaOH to a final concentration of 200 ug/ml. For calibration purposes a range of concentrations from 0-200 ug/ml was prepared from this stock solution with the final volume of each being 1 ml. Coomassie Blue Reagent (4 ml) was added to the

tubes and mixed. The absorbance at 620 nm was read immediately after mixing, against a blank consisting of 1 ml 0.05 M NaOH and 4 ml Coomassie blue reagent. Readings were plotted against ug of BGG. Amoebal suspensions were diluted with an equal volume of 0.1 M NaOH, causing immediate cell lysis, and treated as described above for standard protein solutions. Further dilutions (with 0.05 M NaOH) of the lysate were made where necessary, to ensure that the absorbances fell within the appropriate calibration range. A typical calibration plot is shown in fig. 3.1. The relationship between protein concentration and absorbance is non-linear but highly reproducible calibration lines were always obtained.

Phagocytosis assay

This assay uses radiolabelled bacteria as particles for phagocytosis by amoebae of D.discoideum (Glynn, 1980). The labelled bacteria are incubated with amoebae under conditions favourable for phagocytosis. Uningested bacteria are removed by washing the amoebae in 5 mM sodium azide which causes the detachment of all surface bound bacteria (Glynn, 1980). Ingested bacteria are subsequently measured by scintillation counting.

Amoebae were harvested during exponential phase by centrifugation at 400 x g for 5 min and washed once in BSS. The washed pellet was resuspended in BSS to the

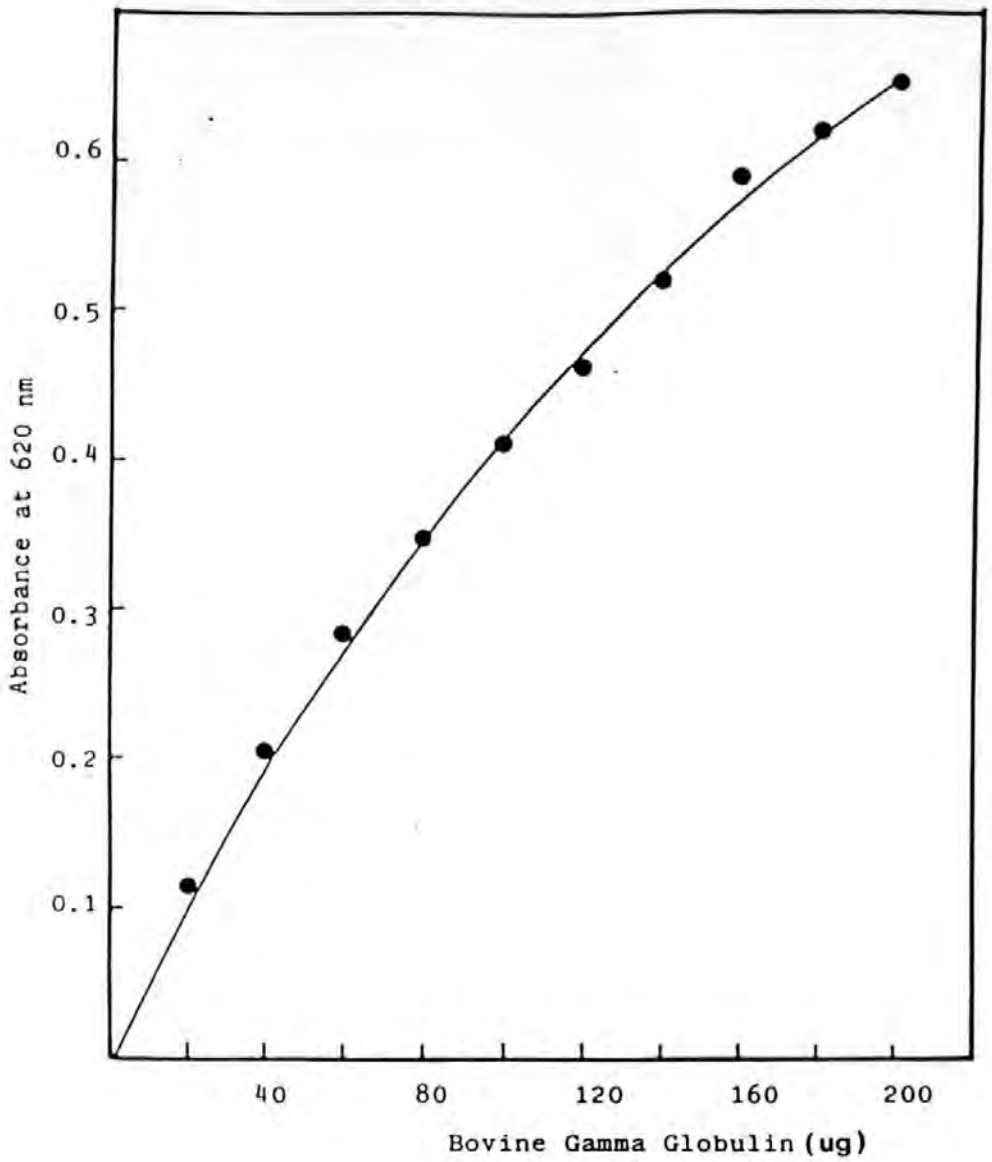


Fig. 3.1. A typical calibration curve relating the amount of bovine gamma globulin to the absorbance at 620 nm of the dye-protein complex.

desired cell density (usually 1-2 x 10^6 cells/ml). Washed amoebae were always used immediately after preparation.

Phagocytosis was carried out in conical flasks in a reciprocal shaking water bath at 23°C and 40 strokes per min. All incubations contained one volume of amoebal suspension, one volume of bacterial suspension and 0.5 volume of test substance. Incubations were started by the addition of bacteria, except where otherwise stated. Aliquots of 1 ml were withdrawn at zero time and subsequent time intervals and transferred to 6 ml of BSS containing 5 mM sodium azide in polypropylene centrifuge tubes. These were centrifuged at 400 x g for 5 min resulting in the separation of the larger amoebae from the uningested bacteria which remained in the supernatant. The pellet was vigorously resuspended in BSS-azide, in order to dislodge any remaining surface attached bacteria, and recentrifuged. The wash was repeated and the final pellet dissolved in Unisolve I scintillation fluid (Koch Light Laboratories, Slough, Bucks) and counted in a Philips PW4700 Liquid Scintillation Counter. Zero time values were subtracted and the results expressed as cpm per 100 ug amoebal protein.

Adhesion assay

A quantitative assay for the adhesion of amoebae to glass was developed, in which the amount of amoebal protein adhering to conical flasks was measured. This method was simple, rapid and inexpensive and provided the opportunity to compare the susceptibilities of phagocytosis and cell adhesion to a variety of test substances.

When amoebae are allowed to settle onto glass they adhere with little apparent lag phase. By arranging cell density so as to minimise mutual cohesion and ensure a monolayer, this assay can reproducibly follow the adhesion of cells to glass. On average 80-85% of cells bind to the flasks and this is routinely achieved within 20-30 min.

Amoebae were harvested during exponential phase, washed twice in BSS and resuspended in BSS or test substance to a density equivalent to 100-150 ug amoebal protein/ml. This is roughly equal to 1-2 x 10^6 cells/ml. Aliquots (20 ml) of this suspension were then pipetted into 250 ml conical flasks slowly shaking (70 rpm) on a rotary shaker. This was to prevent settling out while the amoebae were being dispensed into other flasks. The adhesion assay was started by stopping the shaker. At appropriate time intervals, flasks were

transferred to a second rotary shaker and agitated at 100 rpm for 2 min to remove any non-adherent cells. The supernatant fluid was then poured off and the flasks inverted on absorbent paper for 1 min. The protein remaining in the flask was then measured. This was achieved by solubilising adhered amoebae in 0.1 M NaOH (10 ml). Samples from each flask were then diluted 1:1 with distilled water and protein determined as previously described. Results were expressed as the percentage of added cell protein adhering to the glass (% adhesion).

The relationship between cell density, (measured as protein added to the flasks), and protein adhering to the flasks is seen in fig. 3.2a, and the kinetics of adhesion in fig. 3.2b.

The isolation of phagosomes using polystyrene beads

The isolation of phagosomes using polystyrene beads depends on the low density of the beads (1.05 g/ml). Because of this property the ingested beads plus their surrounding membranes can be separated from the rest of a cell homogenate by flotation on a discontinuous sucrose density gradient (Korn, 1974).

Ten 250 ml conical flasks containing approximately 80 ml of HL5-G medium were innoculated with amoebae and grown to a cell density of 5-7 x 10^6/ml. The amoebae were collected by centrifugation, washed once in BSS

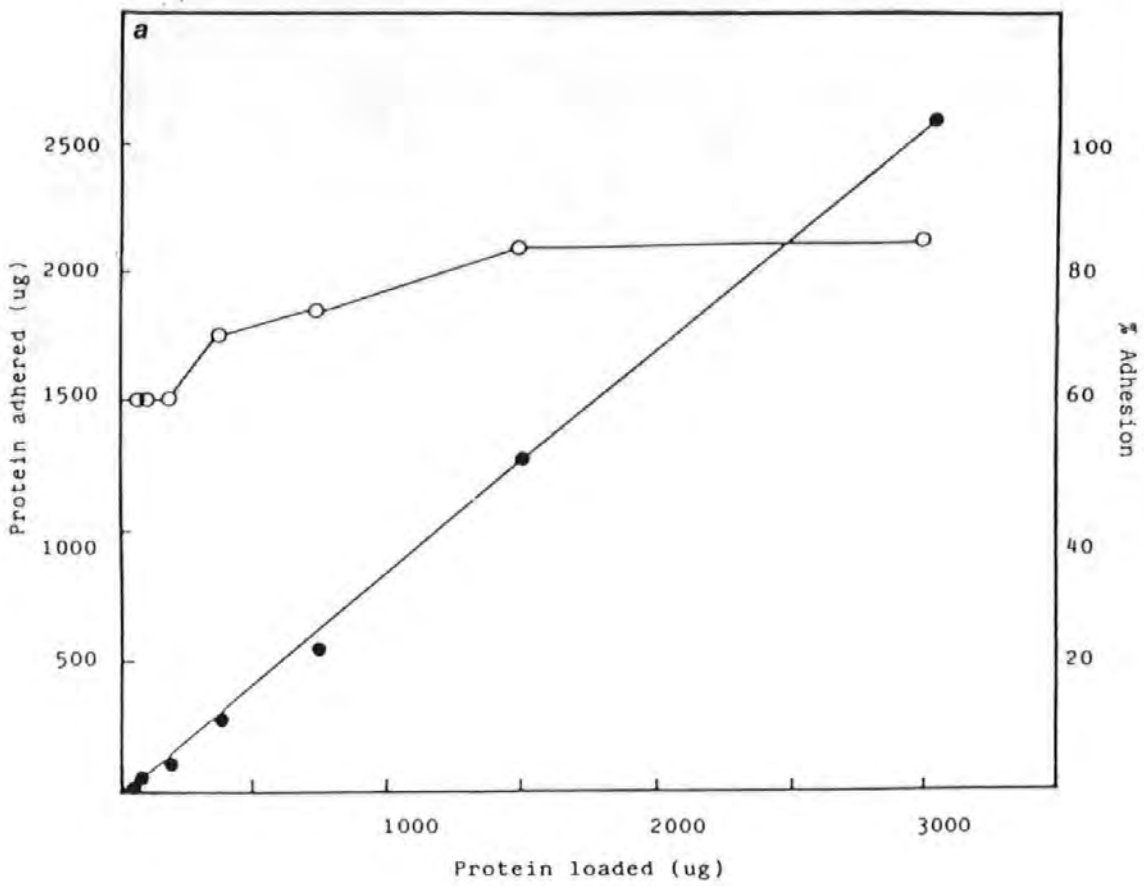
Fig. 3.2. Adhesion of D.discoideum amoebae to glass.

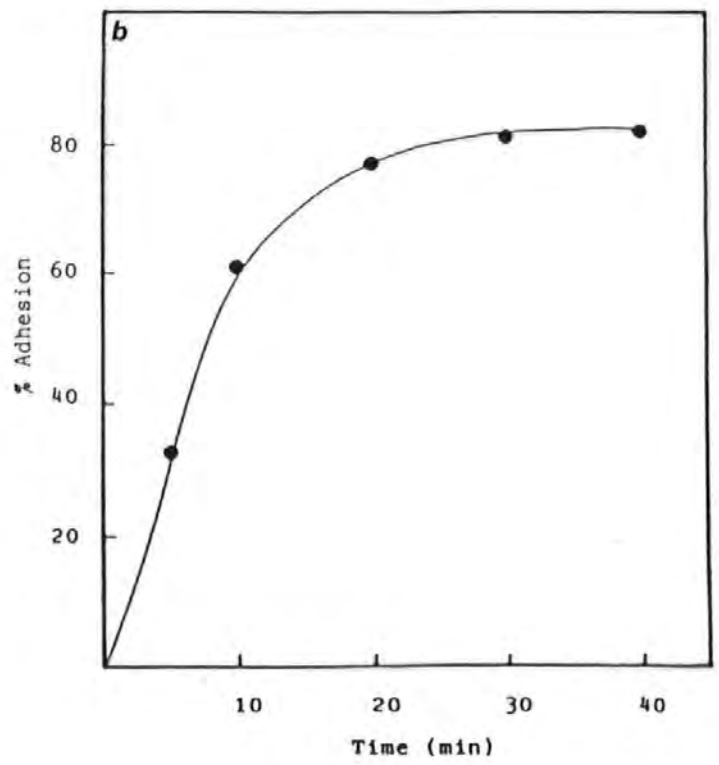
a) Adhesion of cells to 250 ml conical flasks was measured after 40 min incubation at 22°C.

(O) Adhesion expressed as a percentage of total amoebal protein added to flasks, (●) Relationship between adherence of cell protein and cell protein added to flasks.

b) Time course of adhesion of amoebae to glass.

Adhesion is expressed as a percentage of amoebal protein added (2400 ug).


a
2500
2000
1500
1000
500
Protein adhered (ug)
100
80
60
40
20
% Adhesion
1000
2000
3000
Protein loaded (ug)


b
80
60
40
20
% Adhesion
10
20
30
40
Time (min)

and resuspended in 400 ml of BSS distributed between 2 x 500 ml conical flasks. Polystyrene beads of diameter 1 um (Polysciences Inc., Northampton) were added to a final concentration of 2.5 x 10^9 /ml. Phagocytosis was allowed to proceed for 1 h on a rotary shaker at approximately 120 rpm and 22°C. After phagocytosis all procedures were performed at 4°C or on ice. The cells were harvested by centrifugation at 400 x g for 5 min and repeatedly washed in cold BSS until the supernatant was clear of beads. The washed pellet was resuspended in 20 ml of ice cold 30% (w/v) sucrose in BSS. The cells were broken in a motor-driven Potter homogeniser (900 rpm) to approximately 85% breakage (20-30 strokes). Every 10 strokes the homogenate was placed on ice for 5 min to avoid overheating.

Six ml of homogenate was distributed into 3 x 25 ml MSE polypropylene ultracentrifuge tubes and successively overlayered with 7 ml each of 20% (w/v) and 10% (w/v) sucrose in BSS. The tubes were then centrifuged in a 3 x 25 ml swing out rotor at 100,000 x g for 2 h in an MSE superspeed 50 centrifuge. The appearance of the tubes before and after the run is diagrammed in appendix II. The beads with their associated membranes, were collected just below the 10-20% interface. This suspension was diluted fourfold with BSS and collected by centrifugation at 4000 x g for 30 min at 4°C. The pelleted beads were then suspended in a small (200 ul) volume of SDS

electrophoresis sample buffer and incubated at 37°C for 2 hr. The beads were removed by centrifugation in an Eppendorf 5414 microcentrifuge for 2 min and the supernatant retained for electrophoresis. For electron microscopy, the beads were resuspended in glutaraldehyde after the 4000 x g centrifugation step.

Isolation of phagosomes using fixed bacteria

Unlike polystyrene beads, fixed bacteria cannot be separated from the cellular components of the homogenate by flotation. However, it was reasoned that glutaraldehyde fixation of the bacteria would leave them rigid and indigestible (and therefore intact) following phagocytosis and homogenisation. They would then constitute one of the larger types of particle in the homogenate and perhaps might be isolated by a suitable centrifugation protocol.

E.coli B were grown in a single 250 ml conical flask containing approximately 80 ml HL5-G. This was innoculated from a single colony and grown up overnight at 37°C on a shaking water bath. The bacteria were harvested by centrifugation at 2000 x g in a bench centrifuge and washed twice in 15 mM phosphate buffer, pH 6.7. Fixation was carried out by resuspending the bacteria in 2.5% glutaraldehyde in 15 mM phosphate buffer (10 ml), pH 6.7 at 4°C for the desired time (usually 1 h). After fixation, the bacteria were washed

four times in 15 mM phosphate buffer, pH 6.7 and stored as a suspension in BSS at 4°C for not more than 8 h.

The phagocytosis of fixed bacteria was carried out in a 500 ml conical flask on a rotary shaker at 22°C and 120 rpm. The amoebae (approximately 5 x 10^6/ml) were incubated with fixed bacteria (5-10 x 10^8/ml)in a total volume of 100 ml. Phagocytosis was allowed to proceed for 1 h. After incubation, the internalised bacteria were visible under phase contrast microscopy as black rods moving inside the amoebae.

All procedures from this point onwards were performed at 4°C or on ice. The amoebae were harvested by centrifugation at 400 x g for 5 min. The pellet was washed repeatedly in BSS (on average 5 times) until the supernatant was visibly clear. The cell pellet was resuspended in approximately 10 ml of 10% (w/v) sucrose in 20 mM Tris-HCl, pH 7.5. Homogenisation was carried out using a Potter homogeniser with a tight fitting teflon pestle attached to an electric drill operating at 900 rpm. On average 10-20 strokes was sufficient to produce a cell breakage of >85%, as determined by light microscopy. The homogenate was placed in ice every 10 strokes to avoid overheating.

Whole cells and debris were removed by centrifugation at 400 x g for 5 min at 4°C. The supernatant was retained and recentrifuged twice under the same conditions. This was to ensure the elimination of all whole cells and large debris from the

homogenate. Contamination by such coarse debris was checked at each stage by phase contrast microscopy. Ingested bacteria were recovered by centrifugation at 1000 x g for 10 min. The pellet was resuspended in 20 mM Tris-HCl, pH 7.5 and recentrifuged until clear of visible contaminants under the light microscope. The final bacterial pellet was suspended in electrophoresis sample buffer and held at 37°C for 2 h to solubilise phagosomal membrane. Bacteria were cleared from the sample by centrifugation for 2 min in an Eppendorf microcentrifuge. For electron microscopy, the final bacterial pellet was resuspended in glutaraldehyde.

Preparation of plasma membrane

Plasma membrane preparation was by the method of Rossamando and Cutler (1975) and was based on cell lysis by amphotericin B followed by fractionation of the lysate by density gradient centrifugation. All procedures were carried out at 0-4°C except where otherwise stated.

Amoebae were suspended in 0.1 M Tris-HCl pH 7.5 to a cell density of approximately 2.5 x 10^7/ml. A solution of amphotericin B in dimethyl sulphoxide was added to a final concentration of 360 ug/ml and the suspension was incubated at 22°C on a rotary shaker for 1 h at 120 rpm. Cell lysis after this time was

typically 80-90%, as determined microscopically. The lysate was centrifuged at 400 x g for 5 min to remove unlysed cells and the supernatant retained on ice. The remaining pellet was washed once in buffer to remove any entrapped membranes. The two supernatants were combined and centrifuged at 30,000 x g in an MSE High Speed 18 for 30 min to recover membranous material. The supernatant was then removed with a pasteur pipette and the pellet resuspended in fresh buffer and recentrifuged as before. The final pellet was resuspended in 1 ml of buffer and kept on ice.

Sucrose concentrations of 39.9, 41.6 and 42.6% (w/v) were made up in 0.1 M Tris-HCl, pH 7.5 and kept on ice. The sample was prepared by the addition of 1 ml of 39.9% sucrose to the 1 ml derived from the previous step in the preparation. The sucrose gradient was prepared by pipetting 6 ml of each of the three sucrose solutions in order from high to low concentration into a 25 ml polypropylene centrifuge tube. The sample was then layered onto the gradient and centrifuged at 100,000 x g for 18 h in an MSE superspeed 50. The appearance of the tubes before and after the run is diagrammed in appendix II. Plasma membrane was recovered from just below the sample/39.9% interface. This was diluted four fold with buffer and the membranes recovered by centrifugation at 30,000 x g for 30 min in an MSE High Speed 18 centrifuge.

Determination of alkaline and acid phosphatase activity

Alkaline and acid phosphatase activity in phagosome and plasma membrane preparations were assayed by following the production of p-nitrophenol from the hydrolysis of p-nitrophenyl phosphate. The reaction was followed on a Pye-Unicam SP8-400 spectrophotometer at 405 nm.

The reaction mixture for alkaline phosphatase activity contained: 670 ul of 0.1 M tris-HCl (pH 8.5) containing 5 mM $MgSO_4$, 170 ul of p-nitrophenyl phosphate (1 mM) and 160 ul of sample solubilised in 0.5 % (v/v) triton X-100 in buffer. The reaction was carried out in a 1 ml cuvette and started by the addition of sample. The production of p-nitrophenol was followed on a chart recorder and the activity determined as the initial change in absorbance per minute. One unit of activity was defined as that amount which will liberate 1 nmole of p-nitrophenol per minute under these assay conditions. The reaction mixture for acid phosphatase contained: 4 ml of 0.1 M Glycine-HCl (pH 3.5), 1 ml of p-nitrophenyl phosphate (1 mM) and 1 ml of sample solubilised in 0.5 % (v/v) Triton X-100 in buffer. The reaction was carried out in 10 ml glass test tubes and started by the addition of sample. At appropriate time intervals 500 ul aliquots were removed and placed in a 1 ml cuvette containing an equal volume of 0.1 M NaOH, thus stopping the reaction.

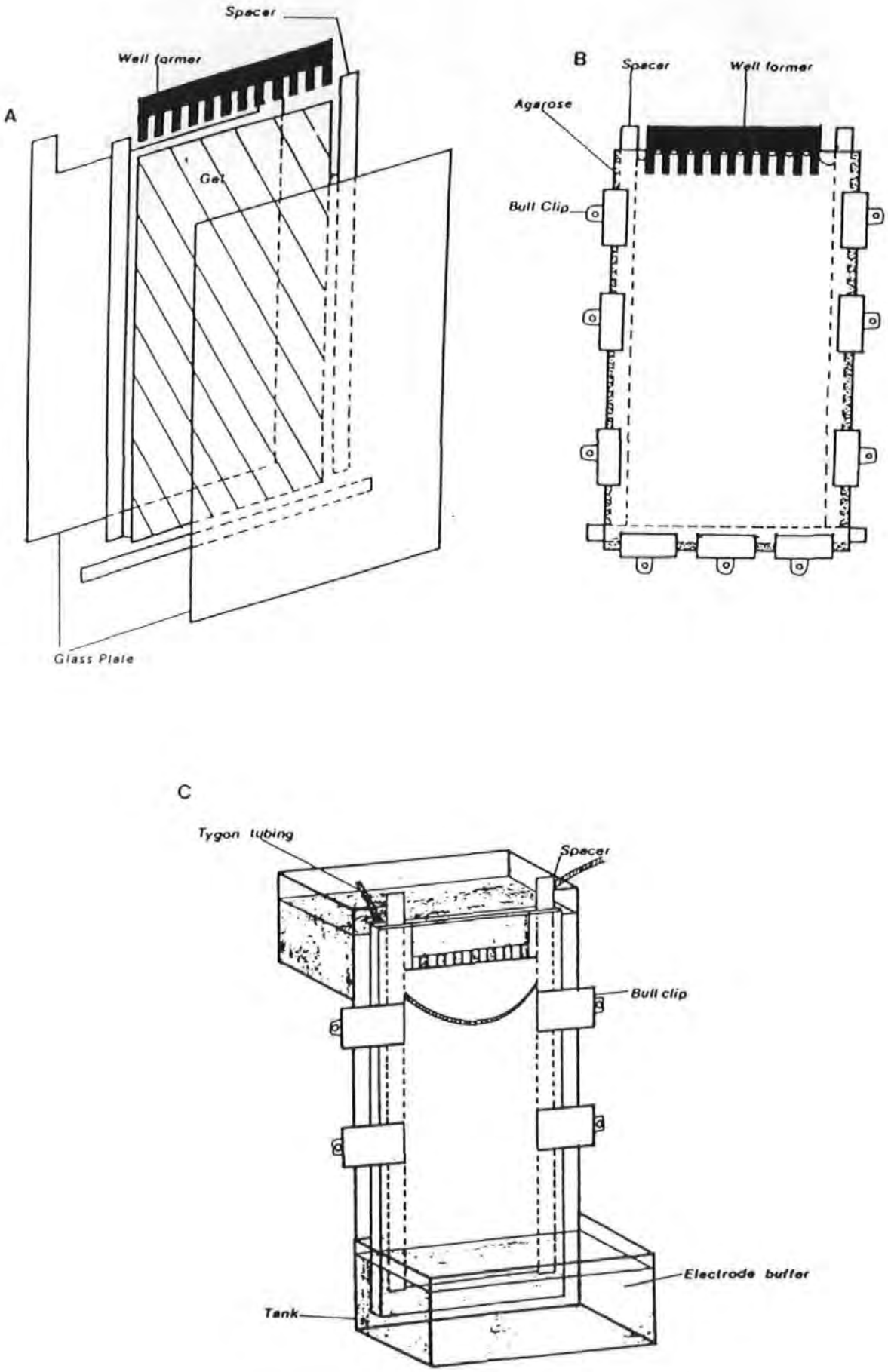
Absorbances were read immediately at 405 nm and the activity determined as the initial change in absorbance per minute. One unit of activity was defined as that amount which will liberate 1 nmole of p-nitrophenol per minute under these assay conditions.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli (1970) using a 13% separation gel and a 4.5% stacking gel. The compositions of the stock solutions and the volumes of each required to produce a gel of given concentration are listed in appendix III. One millimetre thick slab gels were prepared using the apparatus illustrated in fig. 3.3 (a-c). The sides and bottom of the apparatus were sealed with 1% (w/v) agarose in water.

The separation gel solution was poured into the gel former to a height of 10-12 cm, overlayered with water-saturated n-butanol and allowed to polymerise in the dark for approximately 45 min. After polymerisation the n-butanol was washed off with successive volumes of distilled water. The stacking gel solution was then pipetted onto the separation gel and a well former was placed in position. Polymerisation was in the dark for approximately 1 h. Polymerised gels were sometimes stored overnight at 4°C, enclosed in cling film to

Fig. 3.3. Apparatus used in polyacrylamide gel electrophoresis of membrane proteins from D.discoideum. a) Exploded view of gel former, b) Gel former during acrylamide polymerisation, c) Electrophresis tank with gel in position


A
Spacer
Well former
Gel
Glass Plate
B
Spacer
Well former
Agarose
Bull Clip
C
Tygon tubing
Spacer
Bull clip
Electrode buffer
Tank

prevent evaporation and shrinkage.

The gel apparatus was set up as shown in fig. 3.3c. The gel was secured into the electrophoresis tank using metal bullclips after removal of the bottom spacer. Tygon tubing was used to isolate the reservoirs preventing leakage of buffer from top to bottom. Electrode buffer was poured into top and bottom reservoirs so that both the top and bottom of the gel were in contact with buffer, (approximately 500 ml per reservoir). Between 10-40 ul of sample were loaded into each well using a glass microsyringe. Electrophoresis was carried out using a constant current supplied from a Pharmacia power pack ECPS 3000/150. A current of 10 mA was applied for the first hour and then 20 mA until the dye front was approximately 1 cm from the bottom of the gel. The relationship between relative mobility and polypeptide molecular weight for the standard polypeptides used is detailed in fig. 3.4.

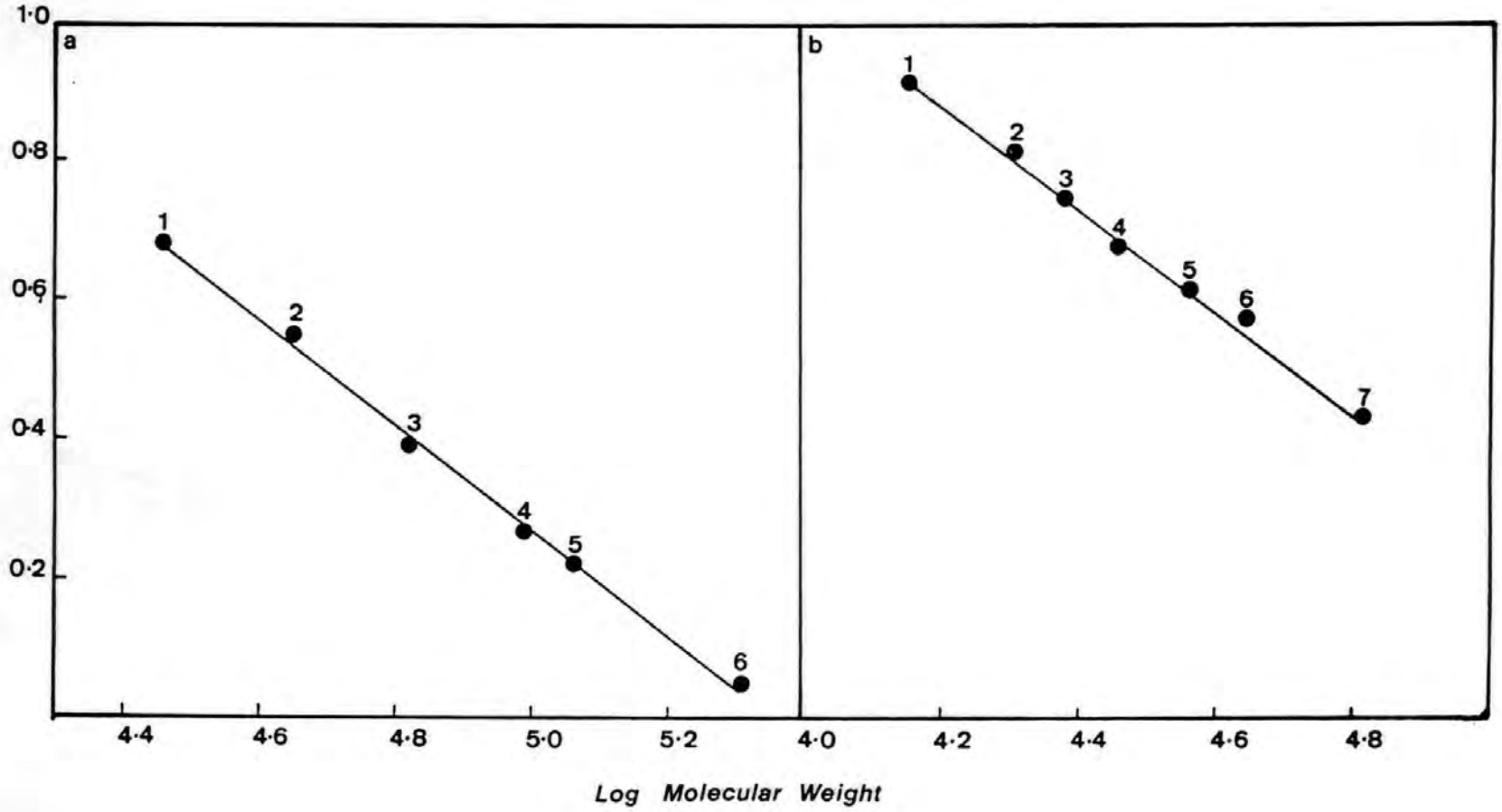
Protein samples were solubilised in a small volume of sample buffer and incubated at 37°C for 2 h. Samples were concentrated, when necessary, using the 10 Kd exclusion range Amicon microconcentrator system (Amicon Ltd, Stonehouse, Glos). Protein samples dissolved in approximately 2 ml of sample buffer were loaded into the top reservoir. The microconcentrators were then centrifuged for 30 min at 6,000 rpm in a High Speed 18 centrifuge using an 8 x 50 ml fixed angle rotor with rubber inserts. The concentrated sample (approximately

Fig. 3.4 a) Calibration curve typically obtained from the MW-SDS-200 Kit (Sigma) on 13% gels.

Proteins	Approx.Mol.Wt.
1. Carbonic Anhydrase	29,000
2. Albumin (Egg)	45,000
3. Albumin (Bovine)	66,000
4. Phosphorylase B	97,400
5. Beta-Galactosidase	116,000
6. Myosin	205,000

b) Calibration curve typically obtained with proteins from the MW-SDS-70L Kit (Sigma) on 13% gels.

Proteins	Approx.Mol.Wt.
1. Alpha-Lacalbumin	14,200
2. Trypsin Inhibitor	20,000
3. Trypsinogen	24,000
4. Carbonic Anhydrase	29,000
5. Glyceraldehyde-3-phosphate Dehyrogenase	36,000
6. Albumin (Egg)	45,000
7. Albumin (Bovine)	66,000


1·0
0·8
0·6
0·4
0·2
Relative Mobility (Rm)
a
b
1
2
3
4
5
6
7
4·4
4·6
4·8
5·0
5·2
4·0
4·2
4·4
4·6
4·8
Log Molecular Weight

100-200 ul) was recovered from above the filter using a pasteur pipette. Proteins in sample buffer were stored at -20°C in multiple aliquots in order to minimise freeze/thawing effects.

Polyacrylamide gel staining

Coomassie brilliant blue R-250 (2 g) was dissolved in 1 litre of 50% (v/v) methanol containing 10% (v/v) acetic acid. Gels were stained in this solution overnight. Staining solution was freshly prepared for each gel or batch of gels. Destaining was carried out at 55°C in 10% (v/v) methanol containing 10% (v/v) acetic acid for approximately 24 hr with 2 changes of solution. Bands were visualised on a light table and the distance moved relative to the bromophenol blue tracking dye (Rm value) calculated. Stained gels were photographed through an orange filter (Wratten no. 22).

Glycoprotein staining was carried out using the concanavalin A / peroxidase technique described by Parish et al. (1977). Con A possesses four equivalent sites which can react with sugars or glycoproteins. If the sugar residues are in an insoluble form, Con A is unable to engage all four active sites, probably because of steric hindrance. This is the case when Con A binds to glycoproteins fixed in gels. The remaining active sites can then operate as acceptors for other sugars added to the system, such as the carbohydrate

groups of horse radish peroxidase. The catalytic activity of this enzyme allows visualisation of Con A binding sites by the reduction of diaminobenzidine to a visible brown dye.

On completion of electrophoresis the SDS was removed from the gel by washing in approximately 200 ml of 25% (v/v) isopropanol containing 10% (v/v) acetic acid for 20 h, the solution being changed once. The gel was then washed in approximately 200 ml of 15 mM phosphate buffer (pH 6.8) for 2 h with one change of solution. This was followed by incubation in 100 ml of a solution containing 0.5 mg/ml concanavalin A in the same buffer, for 2 h after which the gel was washed for a further 20 h in 15 mM phosphate buffer with one change of solution. The gel was then incubated with horseradish peroxidase (Sigma grade B) at a concentration of 0.1 mg/ml for 30 min, after which it was washed for a further 20 h in phosphate buffer with one change of solution.

Staining was achieved by immersion in 100 ml phosphate buffer containing 50 mg of diaminobenzidine and 20 ul of 30% (v/v) hydrogen peroxide at room temperature until a suitable density of staining had developed. Diaminobenzidine was dissolved by heating to 60-80°C for 5 min on a hot plate while stirring and cooled before adding to the gel. Gels were stored in distilled water containing 5 mM sodium azide.

Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde in 15 mM phosphate buffer at pH 7.5 at 4°C overnight. After washing by resuspension in phosphate buffer and centrifugation, samples were treated with 1% osmium tetroxide in phosphate buffer, dehydrated through a graded alcohol series and treated with acetone before impregnation with Spur resin. The pellet was embedded in Spur resin, sectioned on a Port. Blum microtome and viewed with a Philips EM 300 transmission microscope.

Chapter 4

The effect of divalent cation chelators on phagocytosis and adhesion

Introduction

Ethylenediaminetetraacetic acid (EDTA) and ethylene glycol bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), are organic cation chelators with the ability to sequester calcium and magnesium ions from solution. However, EGTA has a greater affinity for calcium than magnesium while EDTA has a roughly equal affinity for both cations (Thomas, 1982). A previous observation suggested that EDTA depressed phagocytosis in amoebae of D.discoideum (Glynn, unpublished observation). A series of experiments was therefore carried out to investigate the effects of chelating agents, both on phagocytosis and on the adhesion of vegetative amoebae to glass.

Results

The effect of EDTA on the phagocytosis of E.coli can be seen in fig. 4.1. It is clear that EDTA significantly reduced phagocytosis at 1 and 2 mM and that this reduction was almost equal for both

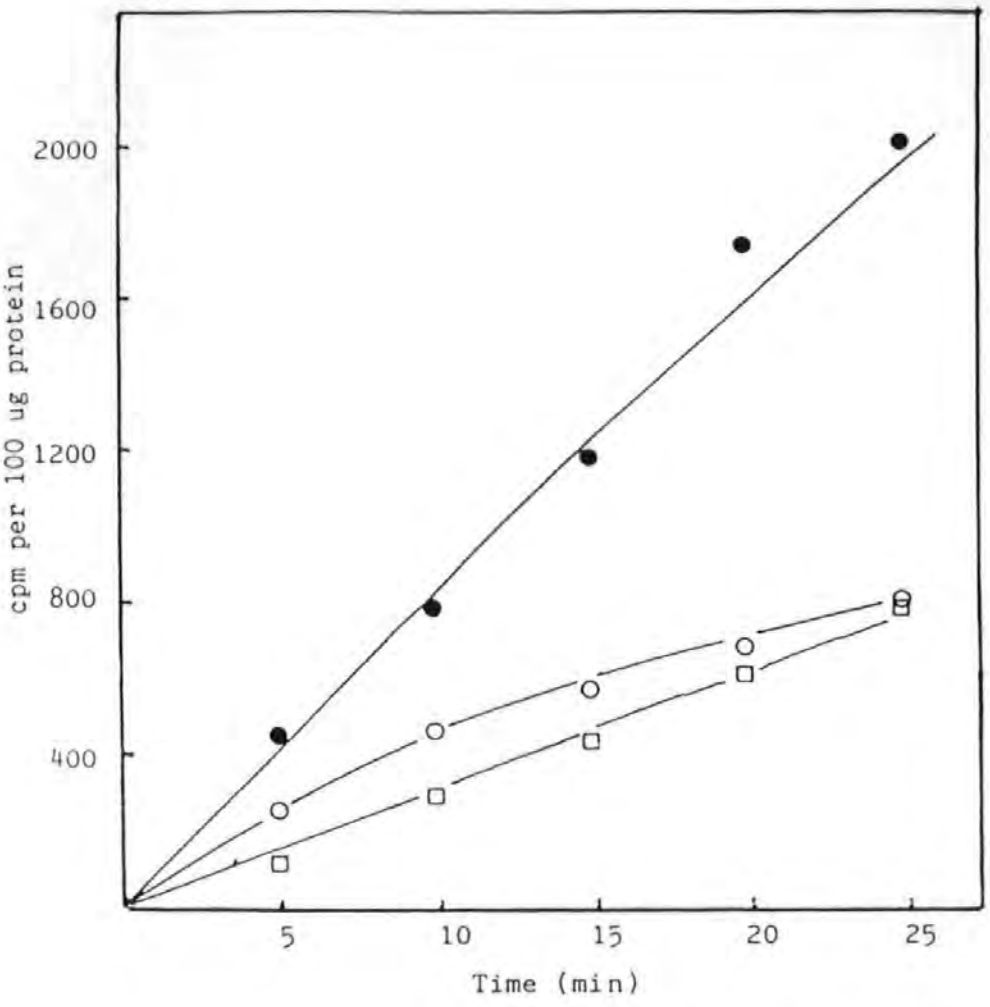


Fig. 4.1. Comparison of the rates of uptake of E.Coli at 23°C by amoebae in the absence (●) or presence of 1 mM (○) or 2 mM (□) EDTA.

concentrations. Phagocytosis was only about 40% of control levels after 25 min incubation. The relationship between chelator concentration and phagocytosis is illustrated in fig. 4.2a. Chelator concentrations were varied over the range 0.05-10 mM and incubations carried out for 30 min. EDTA elicited a sharp decrease in phagocytosis over the range 0-1 mM. Further increases in concentration upto 10 mM produced little additional inhibition. This correlates well with the previous observation that 1 and 2 mM EDTA gave rise to almost similar levels of inhibition.

This effect of EDTA is unlikely to be due to some effect on the surface properties of bacteria. Bacteria preincubated in 10 mM EDTA for upto 40 min and then resuspended in BSS were phagocytosed as readily as control bacteria (table 4.1)

It is noteworthy that phagocytosis was not completely inhibited at any EDTA concentration tested. The reason for this effect is not clear but even at the highest EDTA concentration tested (10mM), a residual uptake of 30-40% of control was observed. Doubling the incubation time and therefore the exposure time to EDTA resulted in similarly shaped curves (fig. 4.2b). However, the extent of inhibition levelled off at lower concentrations of EDTA (0.1-0.5 mM as opposed to 1-5 mM for the 30 min incubation). This may reflect some time dependent component in the inhibition of phagocytosis by this chelator.

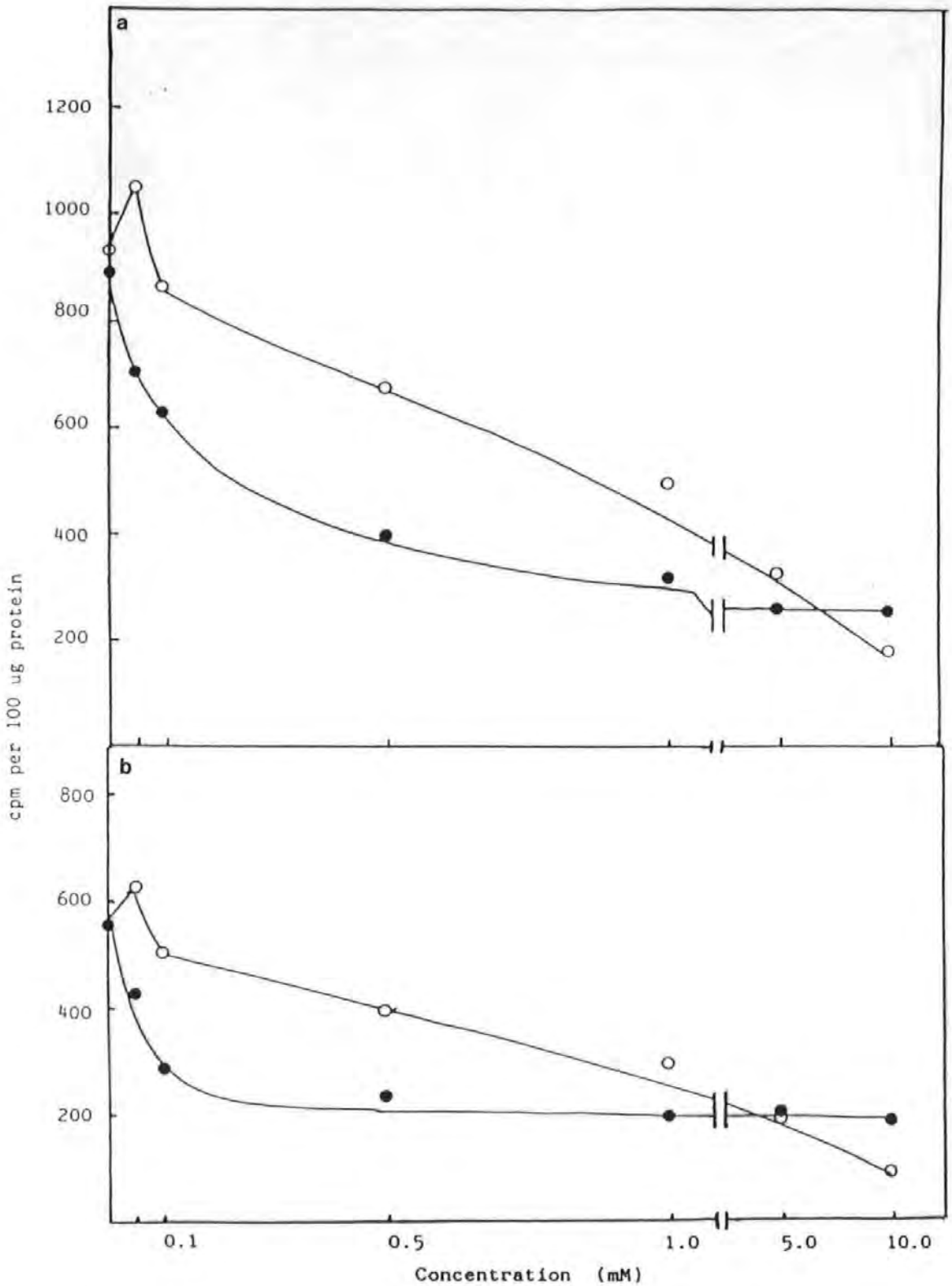


Fig. 4.2. The effects of increasing EDTA (●) or EGTA (○) concentration during the uptake of E.coli by amoebae. a) Phagocytosis for 30 min, b) Phagocytosis for 60 min

Table 4.1 Phagocytosis of EDTA pretreated E.coli

Exposure time (min)	Cpm/ 100ug amoebal protein		% Control
	Control	EDTA	
0	1448	1380	95
10	1415	1342	95
20	1386	1482	107
30	1484	1503	101
40	1662	1520	91

E.coli were incubated for upto 40 min in 10 mM EDTA or BSS. Bacteria were washed twice in BSS prior to phagocytosis.

Although EGTA also inhibited phagocytosis, it appears to be different from EDTA mediated inhibition in terms of concentration dependence. There was a consistent rise in phagocytosis at concentrations of 0.05 mM but over most of the remaining concentration range, inhibition of phagocytosis was less than that seen at equivalent EDTA concentrations (fig. 4.2a). The same pattern of concentration dependence was also seen in 60 min incubations (fig.4.2b).

Although EDTA and EGTA normally exert their biological effects by the sequestration of divalent cations, it was important to show that this was the case here. In order to demonstrate the specificity of these chelators, magnesium and calcium ions were added to phagocytosis incubation mixtures together with chelators at identical concentrations. Both calcium and magnesium ions overcame the inhibitory effect of EDTA, (fig. 4.3).

The restoration of phagocytosis by cations in the presence of EGTA was also investigated (fig. 4.4). It was found that calcium restored phagocytosis to a greater extent than did magnesium.

The foregoing results clearly indicate a requirement for divalent cations during phagocytosis. However, it does not appear that this requirement is for ions in free solution in the suspending medium. This is suggested by the results shown in fig. 4.5. In this experiment, BSS was prepared in plastic containers

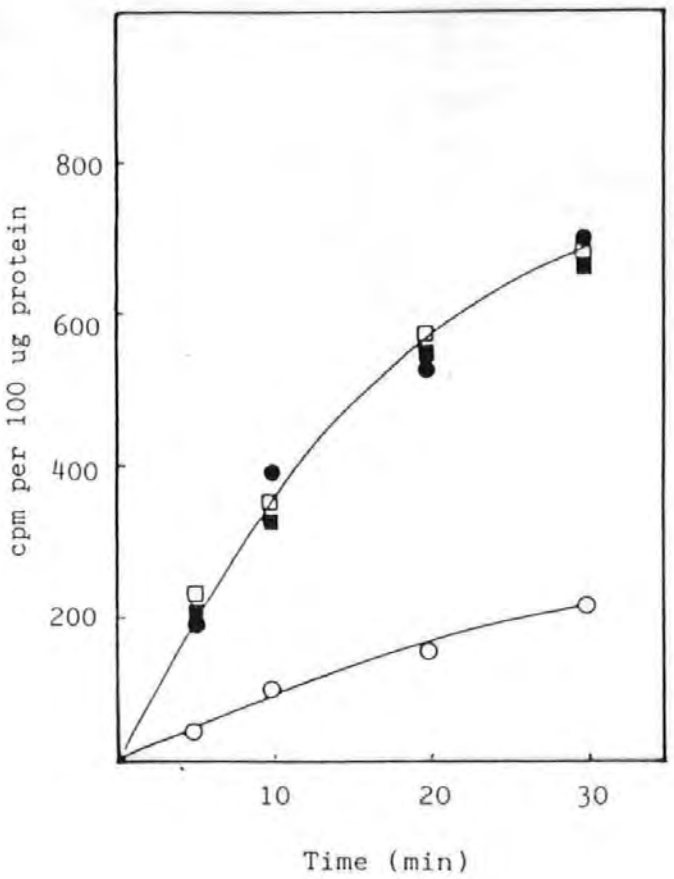


Fig. 4.3. Comparison of the rates of uptake of E.Coli by amoebae at 23°C exposed to 10 mM EDTA in the presence or absence of added divalent cations.
(●) BSS (control), (○) 10 mM EDTA, (□) 10 mM EDTA + 10 mM Ca^{++}, (■) 10 mM EDTA + 10 mM Mg^{++}

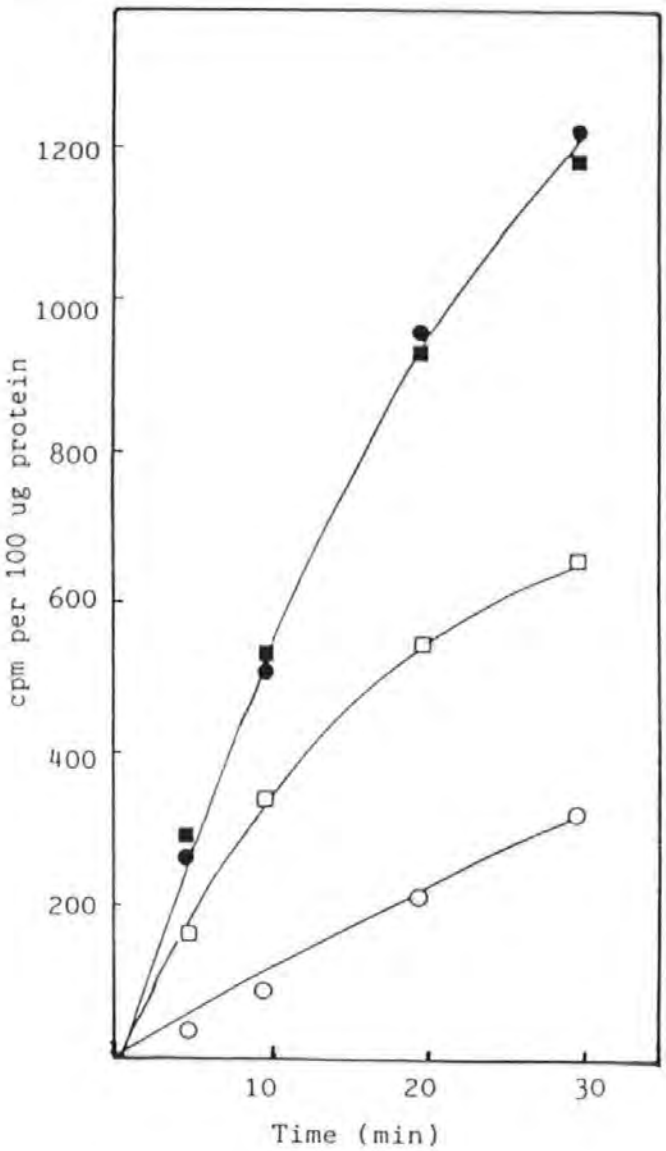


Fig. 4.4. Comparison of the rates of uptake of E.Coli by amoebae at 23°C exposed to 10 mM EGTA in the presence or absence of added divalent cations. (●) BSS (control), (○) 10 mM EGTA, (■) 10 mM EGTA + 10 mM Ca^{++}, (□) 10 mM EGTA + 10 mM Mg^{++}

to avoid the possibility of ions being leached from glassware (Europe-Finner, personal communication). The concentrations of magnesium and calcium ions in this medium were $3x10^{-10}$ M and $8x10^{-7}$ M respectively, as determined by atomic absorption spectroscopy. As can be seen from fig. 4.5, the addition of 2 mM calcium and/or magnesium ions to this medium had no effect on phagocytosis over a 40 min time course.

In order to ascertain if the inhibition of phagocytosis persisted after the removal of the chelating agent, amoebae were preincubated for 5 min and 30 min in 10 mM EDTA and then washed free of the chelator with BSS. The amoebae were then tested for phagocytosis over a 60 min time course in the presence and absence of 10 mM EDTA. The results shown in fig. 4.6a, indicate that the inhibition was fully reversible after 5 min pretreatment but only partially so after 30 min (fig. 4.6b), the final level attained being only about 45% of the control value. A similar level of partial reversibility was attained with 60 min preincubation in EDTA. Interestingly, however, washing 60 min preincubated amoebae in BSS containing 2 mM calcium ion resulted in a far greater recovery of phagocytosis, although only after a 20 min lag phase (fig 4.7). Thus the partial irreversibility of the effect of 60 min treatment with EDTA can be overcome by exogenously supplied divalent cations.

The attachment of particles to phagocyte surfaces

Fig. 4.5. Comparison of the rates of uptake of *E.coli* by amoebae at 23°C in the presence or absence of externally added divalent cations.

a) Calcium free BSS ($[Mg^{++}] = 3 \times 10^{-10}$ M, $[Ca^{++}] = 8 \times 10^{-7}$ M.)

b) plus 2 mM Ca^{++}

c) plus 2 mM Mg^{++}

d) plus 2 mM Ca^{++} and 2 mM Mg^{++}

Calcium free BSS was prepared from Analar reagents in plastic containers and used the same day.

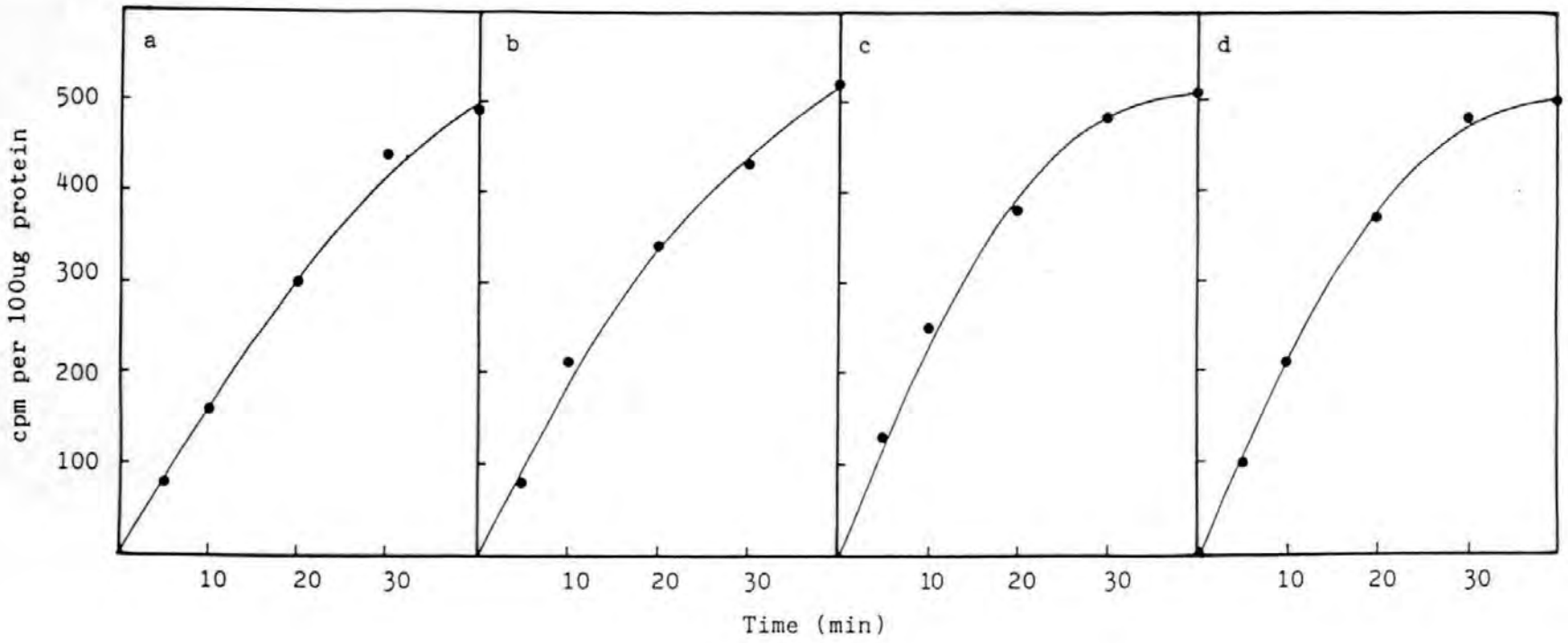
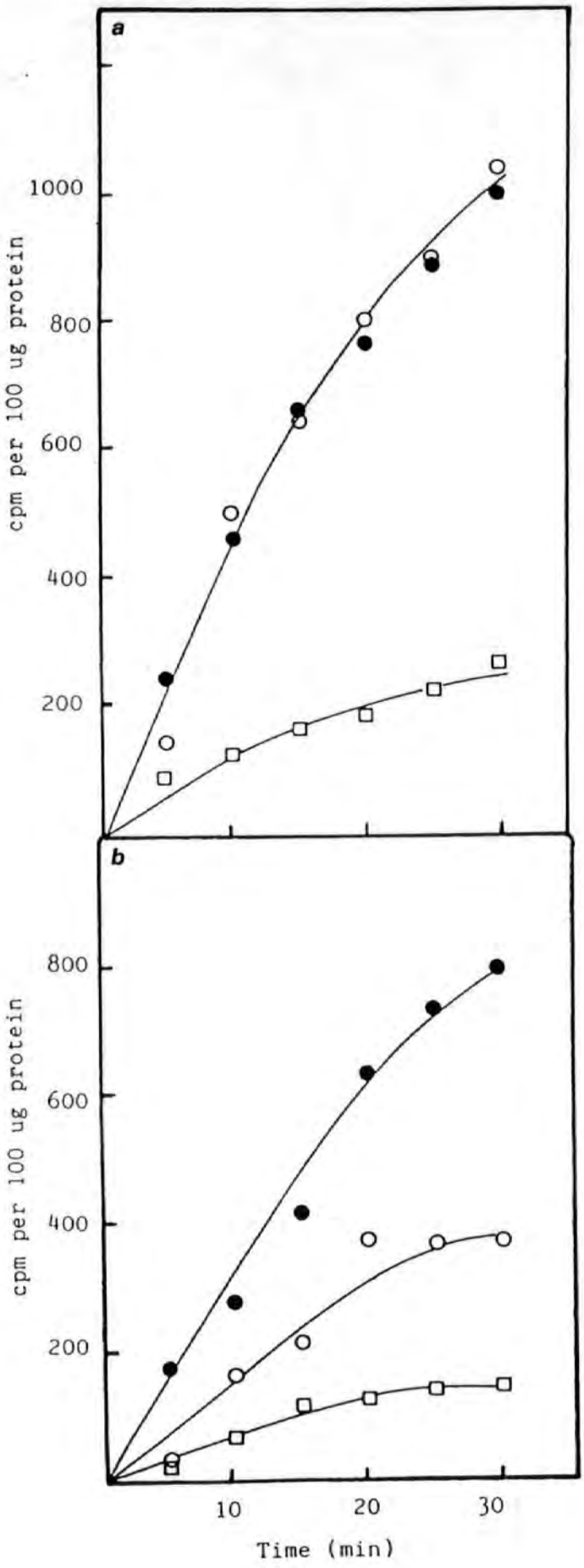

a
b
c
d
cpm per 100ug protein
500
400
300
200
100
10
20
30
Time (min)

Fig. 4.6. The effect of preincubation of amoebae in 10 mM EDTA on the phagocytosis of E.coli.

a) Cells were preincubated for 5 min in 10 mM EDTA or BSS. Removal of chelator was achieved by washing the cells three times in fresh buffer. (●) Preincubation and phagocytosis in buffer, (○) Preincubation in 10 mM EDTA with phagocytosis in buffer, (□) Preincubation in 10 mM EDTA with phagocytosis in 10 mM EDTA

b) As above except preincubation was for 30 min.


a
b
1000
800
600
400
200
cpm per 100 ug protein
10
20
30
Time (min)

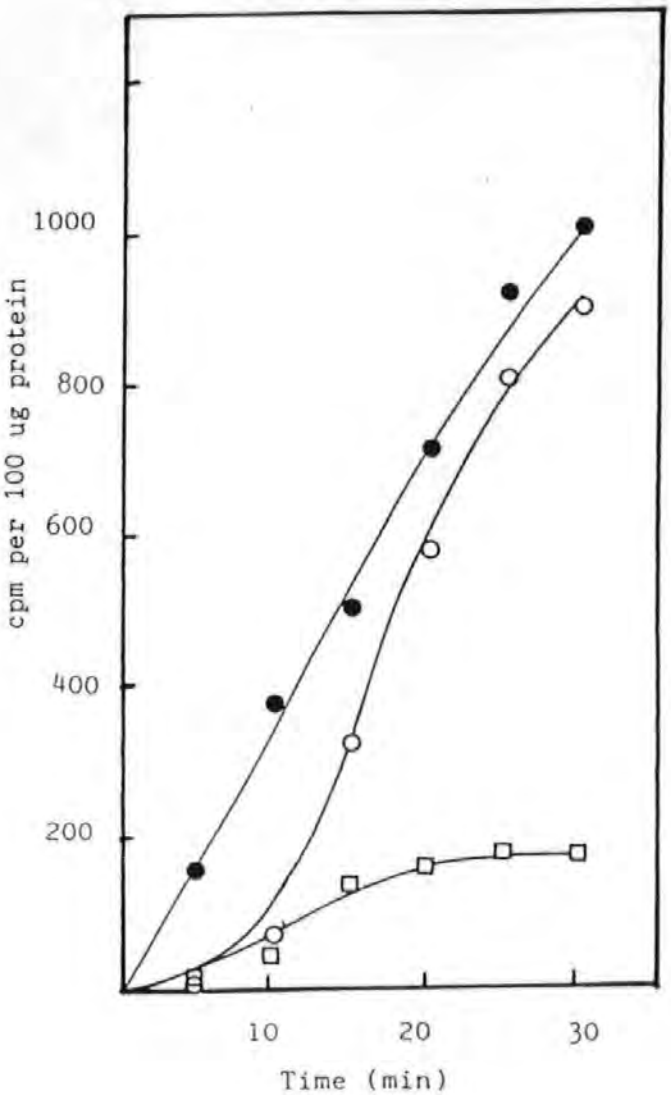


Fig. 4.7. Comparison of the rates of uptake of E.coli by amoebae at 23°C after preincubation in 10 mM EDTA with subsequent removal of chelator and addition of external Ca^{++}. Cells were preincubated for 60 min in 10 mM EDTA. Removal of chelator was achieved by washing the cells three times in buffer containing 2 mM Ca^{++} prior to phagocytosis. (●) Preincubation and phagocytosis in buffer, (O) Preincubation in 10 mM EDTA with phagocytosis in buffer, (□) Preincubation and phagocytosis in 10 mM EDTA.

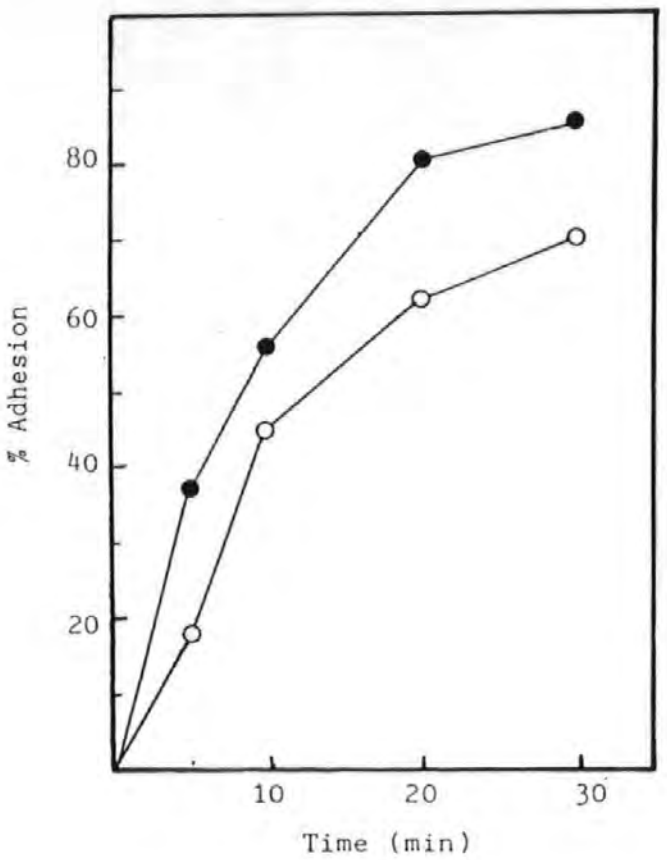


Fig. 4.8. Comparison of adhesion of amoebae to glass in the absence (●) or presence (○) of 10 mM EDTA. Each time value is the mean of three separate experiments.

has been suggested to possess common functional components with cell adhesion of phagocytes to substrata (Bozzaro & Roseman, 1983). In view of this suggestion the adhesion of D.discoideum amoebae to glass in the presence of EDTA was investigated. Fig. 4.8 illustrates the time course of adhesion of amoebae to glass in the presence of 10 mM EDTA. It can be seen that only a small reduction in adhesion to glass was observed. There was also a slight reduction in the rate of attachment. However, the extent of this effect was far less than that caused by the same concentration of EDTA on phagocytosis.

Discussion

The results presented here indicate a requirement for divalent cations for full phagocytic activity in amoebae of D.discoideum. However, the concentration dependence of the inhibition was not the same for EDTA and EGTA and the significance of this is not clear. It does seem, however, that both substances are genuinely acting as chelating agents in view of their lack of effect in the presence of added cations.

On the other hand, it seems unlikely that the required cations are in free solution in the suspending medium. Phagocytosis is not stimulated by the addition of calcium or magnesium ions to BSS which itself contained only 3×10^{-10} M magnesium and 8×10^{-7} M

calcium. If concentrations such as these represented the divalent cation requirement for phagocytosis, the process might be expected to be sensitive to much lower concentrations of EDTA and EGTA.

It is a reasonable assumption that both the chelating agents used are acting extracellularly. Their size (molecular weights around 400 daltons) and strongly polar nature make it extremely unlikely that these substances enter the cell. One possibility is that these agents act by simply leaching cations out of the cell by effectively creating a suitable gradient across the membrane. Such a gradient might deplete the levels of cations within the cell, thus inhibiting phagocytosis. However, this seems unlikely because of the rapidity of the effect. Inhibition of phagocytosis is detectable after just 5 min exposure to EDTA and this short exposure is completely reversible, suggesting no serious depletion of internal stores. Another possibility is that there is a cation binding site on the external surface of the plasma membrane which is involved in phagocytosis. Cations would presumably be quite tightly bound to such a site because EDTA concentrations of around 0.05 mM are necessary to inhibit phagocytosis in solutions containing only very low concentrations of free calcium and magnesium ions. The existence of such a site would also explain how washing EDTA pretreated cells in solutions containing calcium ions results in full

restoration of phagocytosis. Gingell and Vince (1982) have reported that both calcium and magnesium ions bind to the surface of D.discoideum amoebae and Sussman and Boschwitz (1975) report the presence of two classes of cation binding sites. They have shown the presence of a calcium/manganese site and a second calcium binding site present on the surface of vegetative amoebae. Roblee & Shepro (1976) have shown that blood platelets have calcium ions bound to their surfaces and that on removal of these ions by chelating agents, the platelets are no longer able to aggregate. The idea of functionally important cation binding sites at the cell surface therefore has a clear precedent. However, some of the present observations are difficult to explain in terms of these ideas. The complete reversibility of 5 min exposure to EDTA might perhaps be explained by the binding of residual calcium ions in BSS on resuspension in this medium, but the partial reversibility of 30 and 60 min pretreatments is not consistent with this possibility. In addition, although 60 min EDTA pretreated amoebae can be restored to almost full phagocytic activity by washing in solutions containing calcium ions, this restoration shows a 20 min lag phase which is also difficult to explain in terms of a simple cation binding site. The existence of such a site must therefore remain only a tentative proposal.

A third possibility is that external chelators may affect the cell cytoskeleton either by removing calcium

from the surface of amoebae or leaching out internal stores. In support of this idea Maeda and Kawamoto (1986) showed that D.discoideum vegetative amoebae, shaken in 10mM EGTA for 30 min exhibit low actin concentrations in cytoskeletal preparations. Additionally, ultrastructural studies revealed that these cells have greatly reduced attachment of microfilaments to the internal face of the plasma membrane. Such effects of chelators could well affect phagocytosis. Indeed, it is possible that a combination of the suggested effects of chelators may play a part in inhibiting phagocytosis.

It is not clear whether chelating agents inhibit the attachment or the ingestion phase of phagocytosis. This is because only ingested bacteria are measured by the assay used here, any surface attached bacteria being removed by the washing procedure. However, attachment to glass is clearly less sensitive to chelating agents than phagocytosis, an observation supported by the work of Yabuno (1971) who found, with an entirely different assay, that EDTA did not reduce the adhesion of D.discoideum amoebae to glass. In addition, Glynn and Clarke (1984) found that amoebae of D.discoideum adhered to columns of polystyrene beads coated in growth medium even in the presence of 20 mM EDTA. These results suggest that the attachment of amoebae to substrata is relatively insensitive to chelating agents. It is possible that the same is true

of phagocytic attachment of bacteria. On the other hand, the mutual adhesion of vegetative amoebae is completely inhibited by EDTA and this suggests that there may be more than one attachment mechanism in these cells.

Chapter 5

The effect of inhibitors of calcium ion and cytoskeletal functions

Introduction

Intracellular calcium ions are known to play regulatory roles in a variety of cellular processes. For example, these ions are known to influence the activity of key cytoskeletal proteins and because of this have been postulated to be involved in regulating phagocytic ingestion (Yin & Stossel, 1982). Extracellular calcium has also been implicated in the mechanism of cell cohesion in amoebae of D.discoideum (Collodi et al.,1986) and other cell types (Gogstaf et al., 1983). The inhibitory effects of EDTA and EGTA on phagocytosis by D.discoideum amoebae also indicate the involvement of divalent cations in this process and calcium ion is the most likely candidate for a regulatory role. In view of these findings, investigations were carried out into the effects on phagocytosis and cell adhesion of a variety of substances which interfere with calcium ion function.

The lipophilic carboxylic acid ionophore A23187, isolated from Streptomyces chartreusensis, is thought to be a mobile carrier of calcium ions across

biological membranes, partly as a result of its ability to dissolve in the phospholipid bilayer. Each molecule possesses a ring structure and two such molecules are required to translocate one calcium ion across the membrane (Houslay & Stanley, 1982). It has a threefold greater affinity for calcium over magnesium ions (Campbell, 1983), and has been shown to alter cell permeability to calcium (Reed & Lardy, 1972; Shroeder & Strickland, 1974).

The effects of lanthanum ions on phagocytosis and adhesion were also studied. Lanthanum ions have long been known to interfere with calcium mediated functions. The antagonistic effects generally occur at concentrations in the 1-10 mM range and inhibition of a process by lanthanum ions is widely taken as an indication of the involvement of calcium ions (Campbell, 1983).

Many of the functions of intracellular calcium ions are modulated by a small protein called calmodulin. This protein, which is ubiquitous among eucaryotic cells, has the capacity to bind calcium ions, resulting in a conformational change to a form which modifies the activity of other proteins (Klee et al., 1980). Phenothiazines inhibit the formation of calcium-calmodulin complexes and have been widely used as indicators of calmodulin involvement in specific biochemical and cellular functions (Weiss & Levin, 1978). One of this family of drugs, trifluoperazine

(TFP), was used to treat amoebae of D.discoideum in order to ascertain its effects on phagocytosis.

The effect of compound 48/80 was also studied. Compound 48/80, has been used extensively as a histamine releaser in mammalian mast cells (Woldemussie & Moran, 1984). It is a cationic amphiphile that has been shown to specifically inhibit calmodulin dependent stimulation of the Ca^{2+}-ATPase of red blood cell membranes and of brain phosphodiesterase (Gietzen, 1983).

Finally, the involvement of microfilaments and microtubules during phagocytosis was investigated using the compounds cytochalasin B and colchicine. The involvement of the cytoskeleton in phagocytosis and cell motility has been well documented (Taylor & Fecheimer, 1982; Yin & Stossel, 1982). Compounds that disrupt cytoskeletal function might therefore be expected to affect these processes. One such compound, cytochalasin B, a metabolite from the mould Helminthosporium dermatoideum is known to disrupt microfilaments in the cell by binding to the growing end of actin microfilaments (Bray,1979). It has been shown to inhibit several cellular processes that involve the cytoskeleton, including phagocytosis (Todd-Davies, 1971; Malawista et al., 1971; Axline & Reaven, 1974).

Colchicine is known to prevent microtubule polymerisation by binding to tubulin dimers (Alberts et

al., 1983) and to disrupt processes that involve microtubules.

Results

The effects of A23187

As is shown in fig. 5.1, phagocytosis is sharply reduced between 5 and 10 ug/ml A23187 and abolished at a concentration of 38 ug/ml. A control containing equivalent amounts of the solvent, dimethyl sulphoxide, is also shown. At concentrations of ionophore that completely inhibit phagocytosis, the solvent alone reduces uptake by only 25%. It is therefore clear that the large additional inhibition of phagocytosis is due to the ionophore.

The effects of lanthanum ions

In preliminary experiments on the effect of lanthanum ions, it was found that the concentrations used caused extensive clumping of bacteria if included in the incubation medium during phagocytosis. This led to difficulty in separating uningested bacteria from amoebae during the assay washing procedure. To avoid this problem, the amoebae were treated with lanthanum chloride and washed by centrifugation prior to assaying their phagocytic capacity. Europe-Finner et al. (1985)

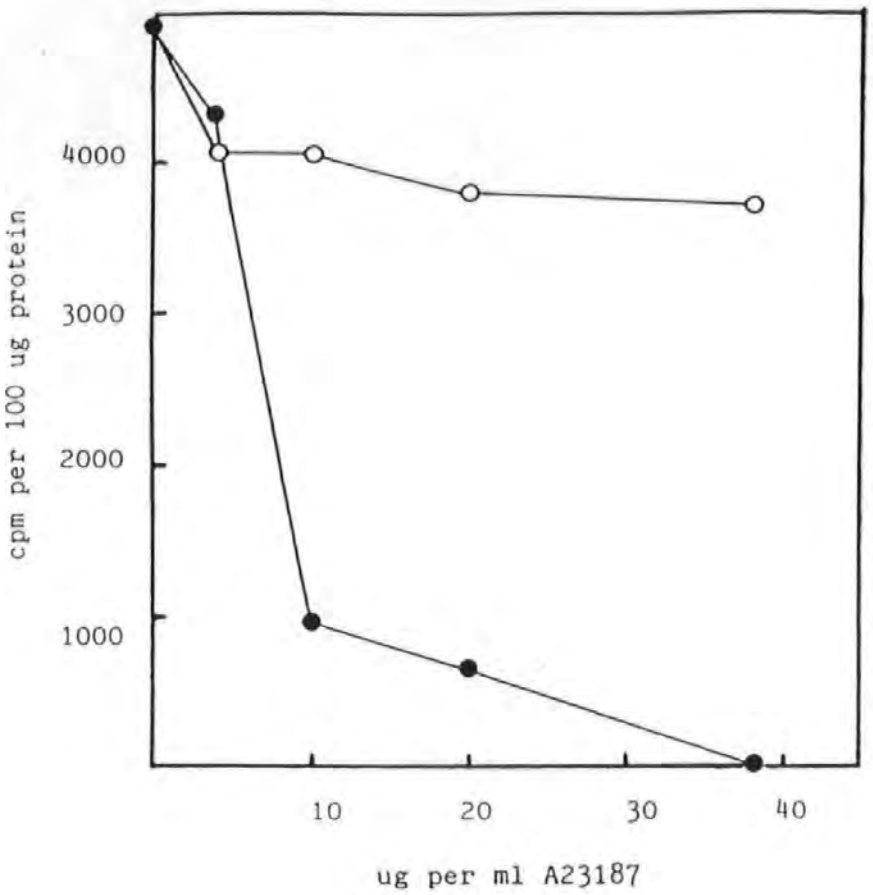


Fig. 5.1. The effect of ionophore A23187 on the phagocytosis of E.coli by amoebae. (●) DMSO+ 10 ug/ml A23187, (○) DMSO. Phagocytosis was for 60 min. Values are the means of duplicate samples.

reported that lanthanum ions caused some lysis of D.discoideum amoebae. However, no significant cell lysis was recorded when amoebae were incubated in 0.5 mM lanthanum chloride for 60 min as determined by cell protein in the supernatant after centrifugation of treated amoebae. The effect of pretreating amoebae for 30 min with various concentrations of lanthanum chloride is shown in fig. 5.2. There is a sharp drop in phagocytic capacity upto a concentration of about 0.1 mM. Phagocytosis was almost completely abolished at higher concentrations. This result clearly illustrates the very high sensitivity of phagocytosis to pretreatment with lanthanum ions.

Lanthanum ions also inhibited adhesion to glass, with concentrations of 0.05 mM causing virtually complete inhibition (fig. 5.3). At lower pretreatment concentrations (0.02 mM), some adhesion to glass occurred but as shown in fig. 5.4, there was a lag phase of some 10 min before any adhesion was detectable and both the rate and final level of adhesion were reduced. Microscopic examination showed that addition of 0.5 mM lanthanum to cells already attached to glass caused rapid detachment (approximately 30 sec) from the substratum with subsequent rounding of the cells.

The effect of lanthanum pretreatment on adhesion to glass is largely resistant to subsequent washing of the amoebae in solutions containing EDTA. In the experiment shown in fig. 5.5a, amoebae were pretreated

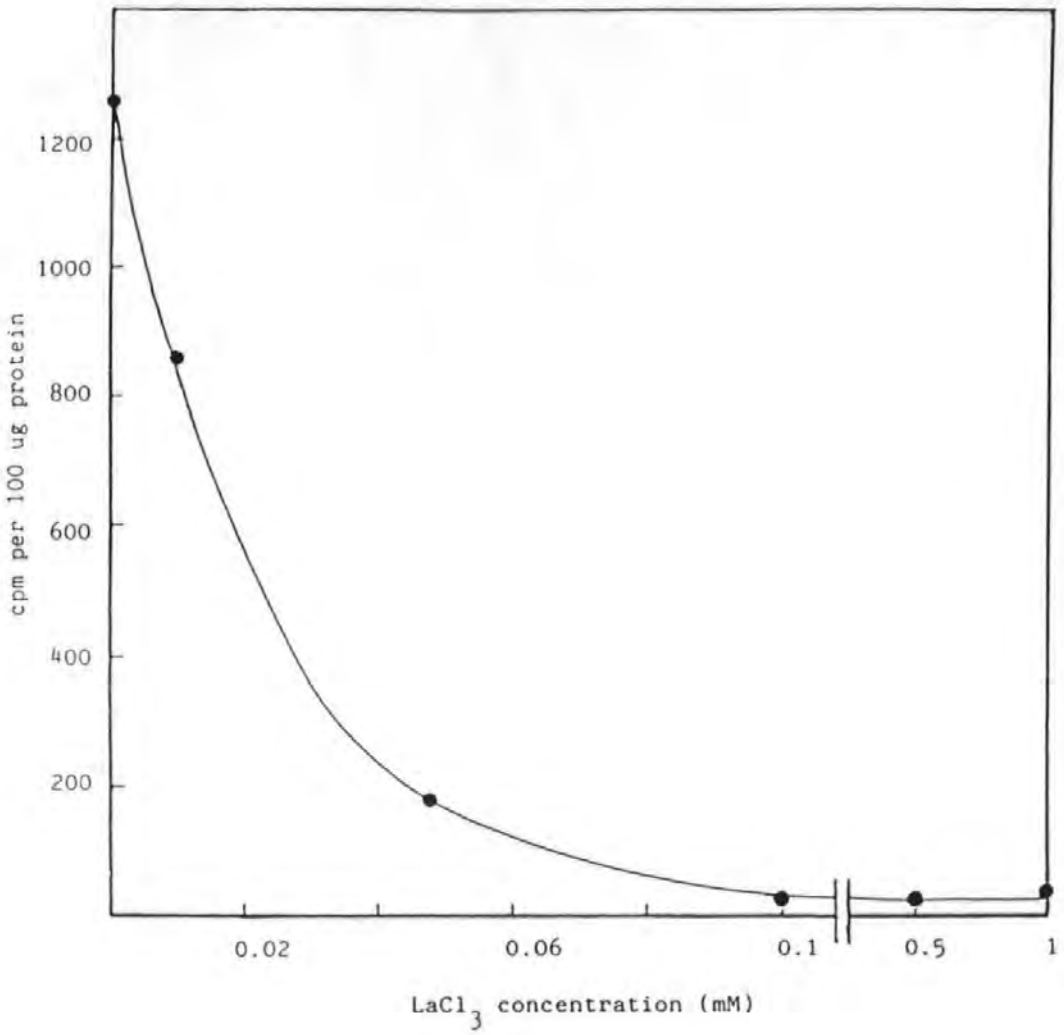


Fig. 5.2. The effect of lanthanum ion concentration on phagocytosis of E.coli by D.discoideum. Cells were pretreated for 30 min and LaCl3 washed free with BSS. Phagocytosis was for 60 min. Values are the mean of duplicate samples.

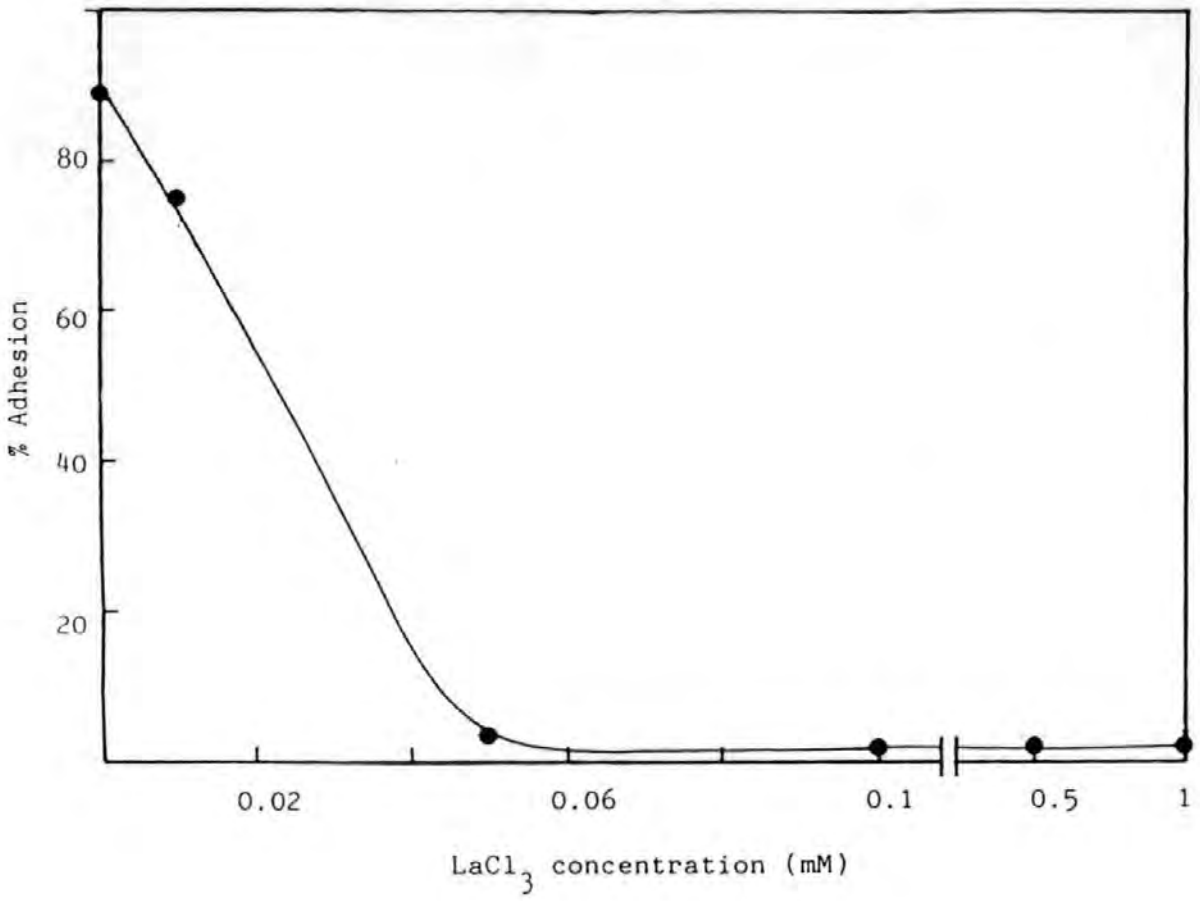


Fig 5.3. The effect of lanthanum ion concentration on the adhesion of D.discoideum amoebae to glass. Cells were pretreated for 30 min and then washed with BSS. Cells were allowed to adhere for 60 min. Values are the mean of triplicate samples.

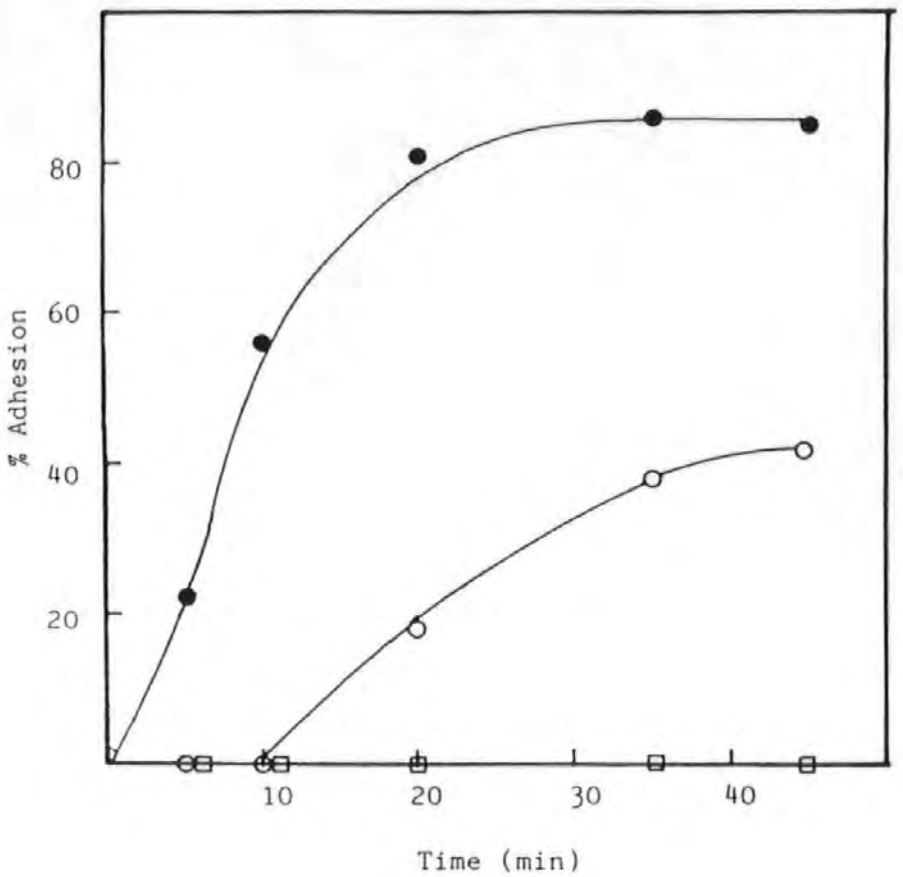
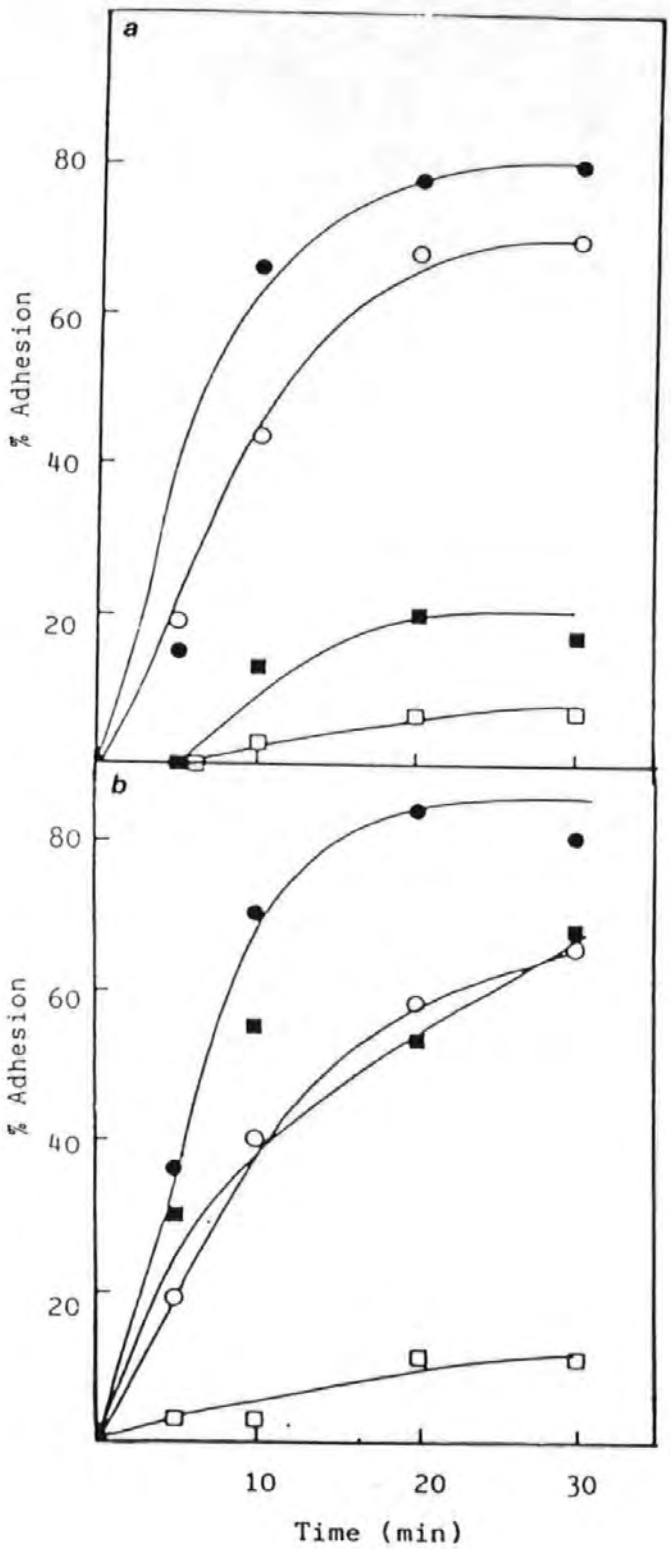


Fig.5.4. Time course of adhesion of D.discoideum amoebae to glass after 30 min pretreatment with 0 (●), 0.02 (○) and 0.1 (□) mM $LaCl_3$. Values are the mean of triplicate samples.

Fig.5.5. The effect of pretreatment with $LaCl_3$ on adhesion of *D.discoideum* to glass with subsequent washing in EDTA.

a) Cells preincubated for 15 min in 0.05 mM $LaCl_3$ (■ □) or BSS (● ○) and allowed to adhere in the presence of 5 mM EDTA (○ ■) or BSS (● □).

b) Cells preincubated fo 15 min in 0.05 mM $LaCl_3$ (□), 0.05 mM $LaCl_3$ and 5 mM EDTA (■) or BSS (● ○) and allowed to adhere in the presence of BSS (● □) or 5 mM EDTA (○ ■).


a
b
80
60
40
20
% Adhesion
10
20
30
Time (min)

for 15 min in 0.05 mM lanthanum chloride and then washed in BSS containing 5 mM EDTA. Only a small recovery in adhesion to glass was seen in the subsequent time course. This suggests that the lanthanum ions are very tightly bound to the cell surface or that they have been actually taken up by the amoebae. The effect is not due to the inability of EDTA to chelate lanthanum ions. This is demonstrated in fig. 5.5b, where amoebae were suspended in a mixture of 0.5 mM lanthanum chloride and 5 mM EDTA for 15 min before being washed and resuspended in BSS and assayed for their ability to adhere to glass. The lanthanum pretreatment in the presence of EDTA follows the same time course as the EDTA control.

The effects of calmodulin antagonists

Calmodulin has been isolated from D.discoideum and found to display many of the characteristics of calmodulins isolated from other sources (Bazari & Clarke, 1982). These workers also determined by indirect immunofluorescence, that calmodulin was located in the cortical cytoplasm of amoebae whether in suspension, attached to a substratum or phagocytosing yeast. As shown in fig. 5.6, trifluoperazine at a concentration of 10 ug/ml completely abolished the phagocytosis of bacteria. The adhesion of amoebae to glass was also strongly inhibited over the same

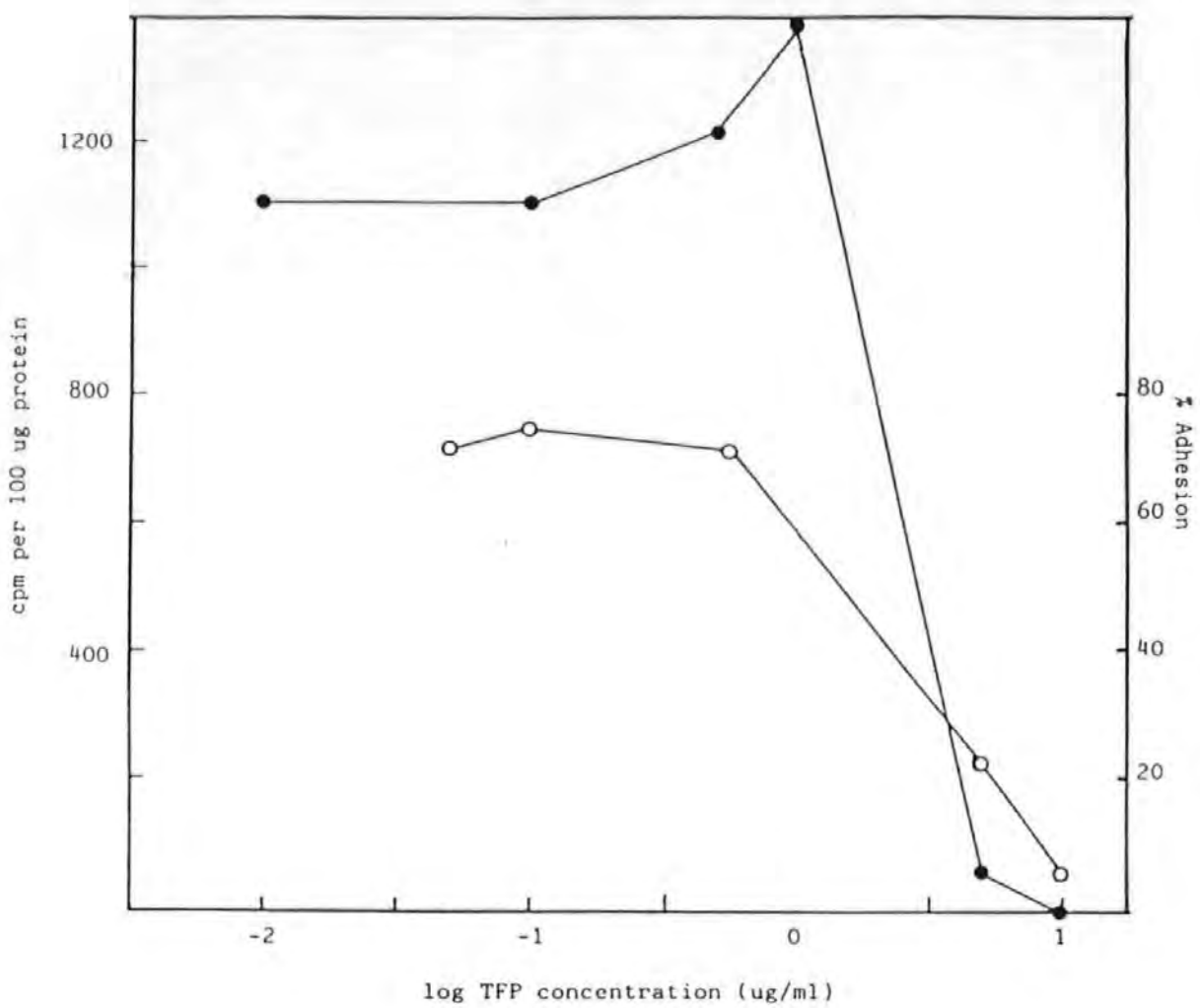


Fig. 5.6. Phagocytosis (●) and adhesion to glass (○) of D.discoideum amoebae in the presence of trifluoperazine (TFP). Phagocytosis was for 60 min and adhesion for 40 min. Values are means of replicate samples. Control value was 1.14 x 10^3 cpm per 100 ug of amoebal protein and 85 % for adhesion.

concentration range. Curiously, a reproducible rise in uptake was observed at a concentration of 1 ug/ml, being some 23% above control levels. No such rise in adhesion was seen at this concentration.

However, subsequent to these studies it was found that drugs of the phenothiazine group, including trifluoperazine, significantly inhibited the respiration of some cells (Corps et al.,1982; Vale et al., 1983). In the light of these reports the results using trifluoperazine have to be interpreted with caution.

Subsequently a drug reported to bind to and inhibit calmodulin function highly specifically was used. The specificity of compound 48/80 inhibition is based on its ability to completely inhibit calmodulin dependent stimulation of cellular enzymes at low concentrations, without reducing their calmodulin independent basal activities. Compound 48/80 is thought to act by complexing with calmodulin via ionic and hydrophobic interactions as a result of complementary structural features (Gietzen et al., 1983). As shown in fig. 5.7, compound 48/80 inhibited both phagocytosis and adhesion to glass in D.discoideum amoebae. Inhibition of phagocytosis increased upto the highest concentration used at 50 ug/ml of 48/80 in the incubation mixture. Over the range of concentrations used, inhibition of adhesion of amoebae to glass

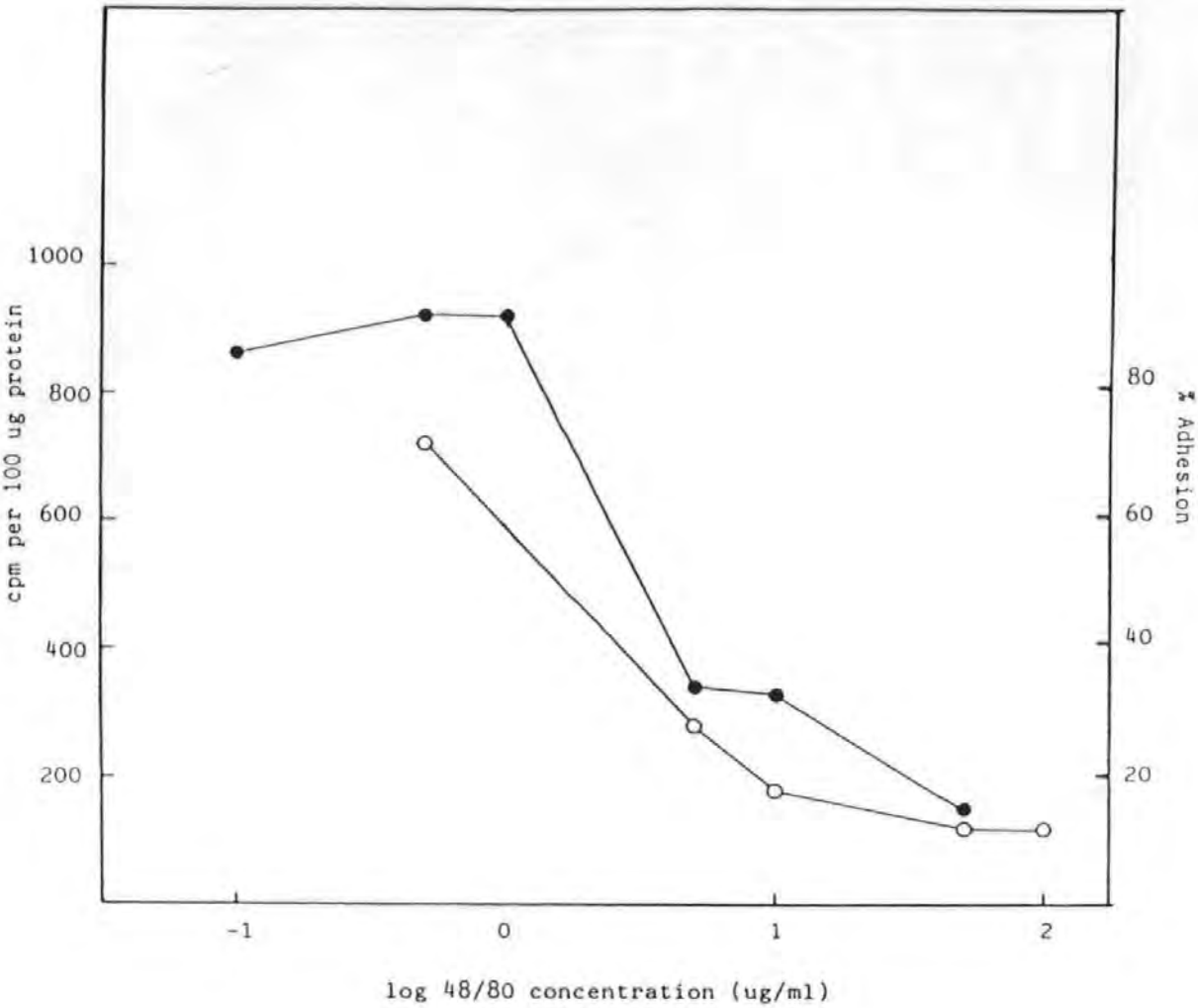


Fig. 5.7. Phagocytosis (●) and adhesion to glass (O) of D.discoideum amoebae in the presence of increasing concentrations of compound 48/80. Phagocytosis was for 60 min and adhesion for 40 min. Points are means of replicate samples. Control value for phagocytosis was 1.10×10^3 cpm per 100 ug of amoebal protein and 83 % for adhesion.

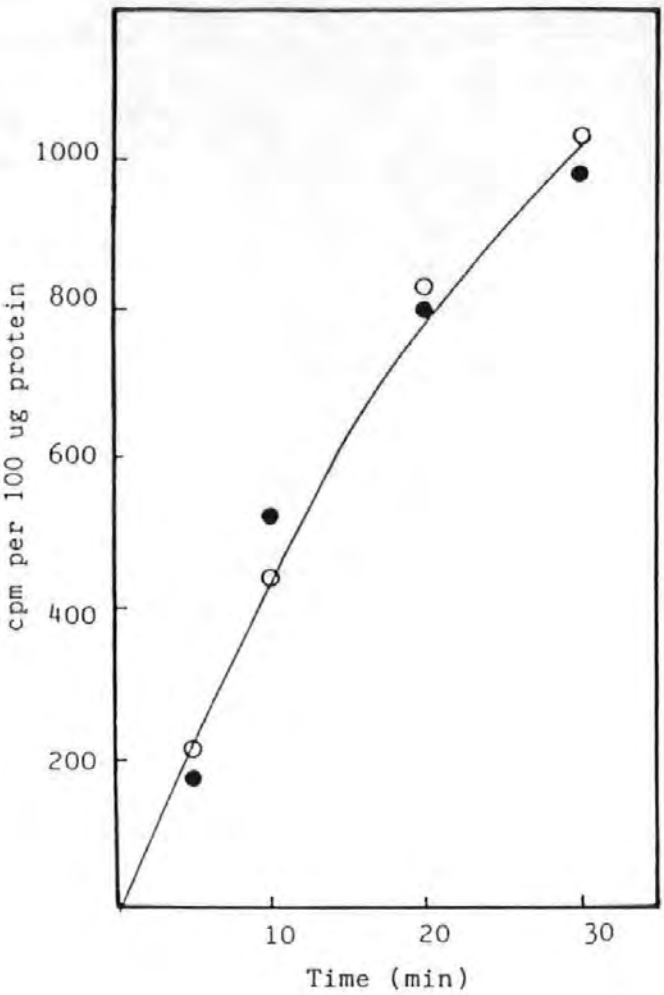


Fig. 5.8. The effect of cytochalasin B on the phagocytosis of E.coli by D.discoideum amoebae. (●) Control, (O) 10 ug/ml cytochalasin B.

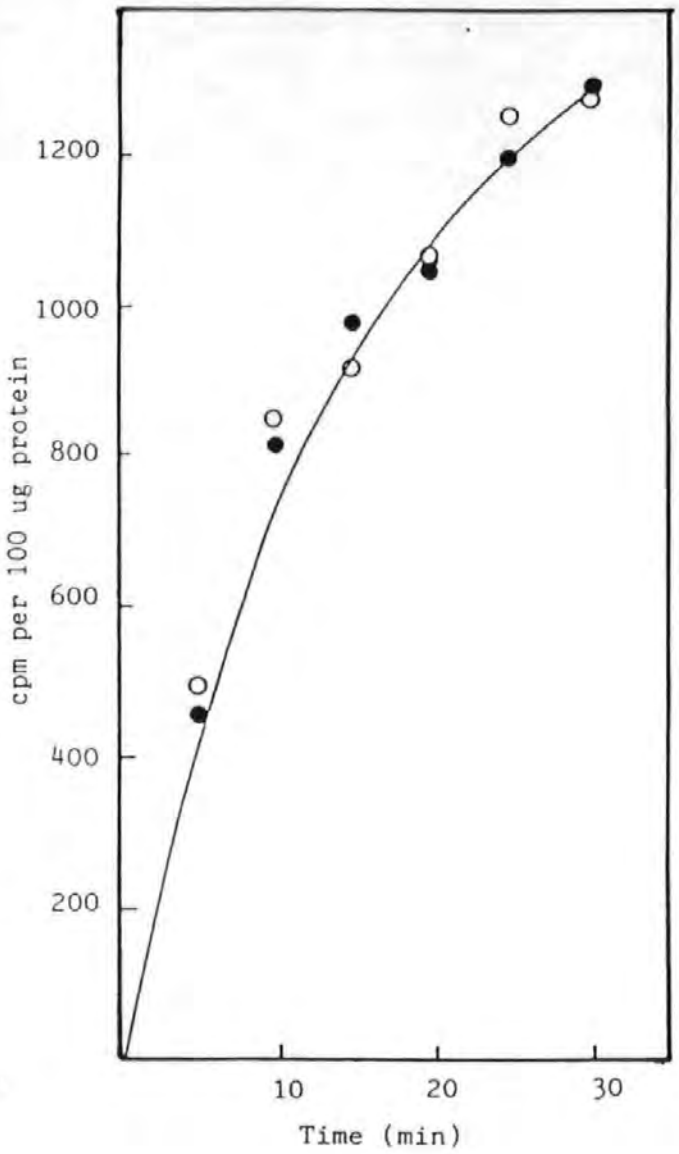


Fig. 5.9. The effect of colchicine on the phagocytosis of E.coli by D.discoideum amoebae. (●) Control, (○) 500 ug/ml colchicine.

dependence as that of phagocytosis. These results therefore suggest that calmodulin plays some role in the phagocytic and adhesive mechanisms as it seems unlikely that both trifluoperazine and compound 48/80 are acting non-specifically.

The effect of cytoskeletal inhibitors

Cytochalasin B at 10 ug/ml was tested for its effect on phagocytosis by D.discoideum. As can be seen from fig. 5.8 no inhibition of uptake was seen at this concentration over a 30 min time course. Microscopic observation of cells suspended in 10 ug/ml cytochalasin B revealed apparently normal cells that spread and moved.

The effect of colchicine on phagocytosis can be seen in fig. 5.9. It is clear that 500 ug/ml of colchicine had no effect on either the rate or final level of phagocytosis in D.discoideum. Concentrations of 1 mg/ml were also without effect.

Discussion

The ionophore A23187 is reported to disrupt intracellular calcium levels by the transport of these ions across the plasma membrane (Houslay & Stanley, 1982). This mechanism of action is supported by several

observations. Prince et al. (1973) reported that 10^{-6} M A23187 stimulated fluid secretion and calcium influx and efflux from insect salivary glands. Additionally, Prusch (1973) reported that A23187 stimulated the endocytic uptake of sucrose by Amoebae proteus, though only in the presence of external calcium ions, and suggested that this effect was due to the increased cytoplasmic calcium levels mediated by A23187. Such increased cytoplasmic calcium in D.discoideum would presumably lead to a wide ranging loss of control of functions modulated by this ion. The inhibition of phagocytosis by this ionophore clearly indicates that the integrity of the membrane with respect to calcium ions is essential for phagocytosis in these cells. However, the increase in cytoplasmic calcium mediated by this compound is presumably only seen in systems where extracellular free calcium concentration is very much greater than the levels of free intracellular calcium. This may not be the case for D.discoideum in BSS, (little being known about free calcium levels in these cells). It is possible therefore that there may even be some loss of intracellular calcium using this ionophore. There is also the possibility that A23187 might become incorporated into membranes other than the plasma membrane (Campbell, 1983). Mitochondria are known to take up calcium in some cells and play a part in regulating cytoplasmic calcium levels. Disruption of this function by possible incorporation of A23187 into

the mitochondrial membrane may affect other calcium mediated processes like phagocytosis. For these reasons, it is not possible to pinpoint the precise mechanism of inhibition by A23187. It is clear, however, that maintenance of normal calcium compartmentation is required for phagocytic activity in these cells.

Lanthanides have been shown to displace surface bound calcium from rabbit aorta cells and as a consequence of this, block the entry or exit of calcium from these cells (Van Breeman et al., 1973). This mechanism of action has been confirmed in several studies. Gorman and Thomas (1980) reported that lanthanum blocked the entry of calcium into molluscan neurons, while Gallitelli et al. (1985) reported the same effect in guinea pig ventricular cells. In addition Szasz et al. (1978) have shown that shape regeneration of calcium loaded erythrocytes is due to the extrusion of calcium and that cell shape can be "frozen" at any point during recovery, by the addition of lanthanum. The inhibition of calcium ion movement across the plasma membrane of D.discoideum amoebae by lanthanum ions has been reported by Europe-Finner and Newell (1985).

The present results were obtained by the pretreatment of amoebae with lanthanum ions. Therefore, inhibition of phagocytosis and adhesion by these ions is not reversed by washing in BSS. This may indicate

that either lanthanum ions are bound tightly to the cell surface or that they are taken up by amoebae. This latter possibility seems unlikely, however, based on the reported inability of lanthanum to cross membranes. Szasz et al. (1978) showed that lanthanum ions at concentrations below 0.25 mM do not penetrate fresh, intact erythrocytes. In addition, lanthanum inhibition of calcium entry into molluscan neurons is thought to be due to the binding of these ions to an external portion of the calcium channels. It is possible, therefore, that the observed inhibition of phagocytosis and adhesion by lanthanum is due to its ability to halt the movement of calcium across the membrane. It is apparent that attachment of amoebae to glass is also highly sensitive to pretreatment with lanthanum. It is possible that extrusion of calcium by these cells is also a requirement during attachment to glass. The failure of EDTA to reverse the inhibition of adhesion by lanthanum suggests that these ions bind tightly and irreversibly to the membrane. The striking similarity seen between inhibition of phagocytosis and inhibition of attachment suggests that lanthanum treatment is perturbing some mechanism involving calcium that is common to both processes.

The results using trifluoperazine and 48/80 indicate that the calcium binding protein calmodulin is involved in some way during phagocytosis of bacteria and attachment to glass. The inhibition of phagocytosis

agrees well with the data of Clarke (personal communication) who found that the phagocytosis of E.coli by D.discoideum (strain Ax3) was completely abolished at trifluoperazine concentrations of 12 ug/ml. She also detected an apparent rise in uptake at a trifluoperazine concentration of 4 ug/ml which is comparable to the rise in uptake at 1 ug/ml reported here. The basis of this phenomenon is unknown. The results using 48/80, showing significant inhibition of both phagocytosis and attachment of cells to glass, also implicate calmodulin in both of these processes.

Among the enzymes regulated by calmodulin is myosin light chain kinase which modulates actin-myosin interactions in non-muscle cells (Masuda et al., 1983). This actin/myosin complex is thought to be involved in many processes related to cell motility. The results presented here support observations by Bazari and Clarke (1982), who reported that D.discoideum calmodulin appeared to be involved in the attachment of cells to substrata and the phagocytosis of yeast, as implied by indirect immunofluorescent localisation of calmodulin. The involvement of calmodulin may extend to the formation and propagation of pseudopodia or possibly earlier stages of the phagocytic process such as attachment, in view of the inhibition of attachment to glass by calmodulin antagonists.

It is clear from the results presented here that cytochalasin B at a concentration that abolishes

phagocytosis in polymorphs (Todd-Davies, 1971), has no effect on the uptake of bacteria by D.discoideum. Although this might indicate a genuine insensitivity to cytochalasin B, it could also be due to the inability of this drug to pass across the plasma membranes. This is supported by the recent results of McRobbie and Newell (1985) who reported that ^{3}H-cytochalasin B fails to bind to intact amoebae and consequently has no effect on chemotactic movement. However, they reported that a considerable number of high affinity cytochalasin B sites were exposed when amoebae were lysed and incubated with ^{3}H-cytochalasin B and that these sites were probably actin microfilaments. This suggests that amoebae plasma membranes are impermeable to this drug.

Colchicine concentrations up to 1 mg/ml have no effect on phagocytosis by D.discoideum amoebae. This may indicate that microtubules are not involved in phagocytosis in this organism. However, the lack of inhibition of phagocytosis seen with high concentrations of colchicine, may reflect the impermeability of amoebae to this compound rather than the absence of microtubule involvement during phagocytosis. On the other hand, colchicine has been shown to inhibit the uptake of oil droplets by human polymorphs (Stossel et al., 1972), and Vasiliev (1982) reported that the spreading and locomotion of tissue culture cells appeared to be stabilised by a

microtubule dependent process. In support of the results presented here, Parish & Pelli (1974) found that preincubation of D.discoideum amoebae for 60 min in 1 mg/ml colchicine with an additional 30 min incubation during phagocytosis did not prevent the ingestion of latex beads but did prevent the normal exclusion of plasma membrane bound alkaline phosphatase from phagosomes. These results suggest that colchicine may indeed enter these cells but that phagocytosis itself is not dependent on microtubule assembly.

Chapter 6

The effects of substances interacting with cell surface components

Introduction

Phagocytosis is a process that is initiated at the cell surface with the attachment of the particle to the phagocyte. The nature of the surface components involved in phagocytosis in D.discoideum amoebae is poorly understood. The experiments described in this chapter were carried out in an attempt to determine the types of cell surface components involved.

The possible involvement of cell surface glycoproteins during phagocytosis by D. discoideum was investigated using the lectin concanavalin A (Con A). This lectin specifically binds D-glucose and D-mannose residues and has been shown to bind extensively to the plasma membrane of D.discoideum (West & McMahon, 1977; West et al., 1978).

The possibility of a role for glycoproteins in phagocytosis was further investigated using the streptomycete antibiotic tunicamycin , which has been shown to inhibit the N-glycosylation of proteins by blocking the synthesis of N-acetylglucosaminyl pyrophosphoryl dolichol phosphate (Lehle & Tanner, 1976). This in turn inhibits the transfer of core

oligosaccharides to asparaginyl side chains in secretory and integral membrane proteins (Struck & Lennarz, 1977). Thus, although tunicamycin acts intracellularly, an effect on cell surface glycoproteins is an important consequence of its action.

Finally, the susceptibility of phagocytosis by amoebae to treatment with glycosidic and proteolytic enzymes was investigated.

Results

The effects of Concanavalin A

It was not possible to study the effect of Con A on phagocytosis by including it in the assay mixture. This was because Con A caused extensive clumping of bacteria and these clumps resulted in contamination of the amoebal pellets during the washing procedure. This problem was overcome by pretreating the amoebae with Con A and removing excess lectin by washing the cells in BSS. It was found that during the pretreatment the amoebae themselves formed small clumps but that these were almost completely dispersed after washing and resuspension in BSS.

Preincubation of amoebae for 30 min with 100 ug/ml Con A caused almost complete inhibition of phagocytosis. A 50 % reduction in phagocytosis is

apparent with concentrations of Con A upto 20 ug/ml (fig.6.1). Cells treated with various concentrations of Con A exhibited swollen contractile vacuoles as reported by Hellio and Ryter (1980). These vacuoles were observed at concentrations of Con A as low as 5 ug/ml but did not affect all amoebae. At higher Con A concentrations larger numbers of cells displayed swollen vacuoles.

The effect of Con A on the adhesion of amoebae to glass was also investigated. Cells were preincubated in various concentrations of Con A for 30 min, washed twice in BSS and allowed to adhere to glass for 30 min. The results are shown in fig. 6.1, and indicate that cell adhesion to glass was less sensitive than phagocytosis at lower Con A concentrations (5-20 ug/ml). However, a rapid decrease in adhesion occurred between 20 and 50 ug/ml.

The specificity of Con A action on adhesion and phagocytosis in D.discoideum was confirmed by the reversal of inhibition when alpha-methyl mannoside was included in the preincubation mixture. Cells were preincubated in the presence of 100 ug/ml Con A and 150 ug/ml alpha-methyl mannoside for 30 min. Subsequently they were assayed for phagocytosis or adhesion over a 30 min time course. The results are shown in fig 6.2a and 6.2b. It is clear that the presence of alpha-methyl mannoside almost completely restored both phagocytosis and adhesion to control levels. It is

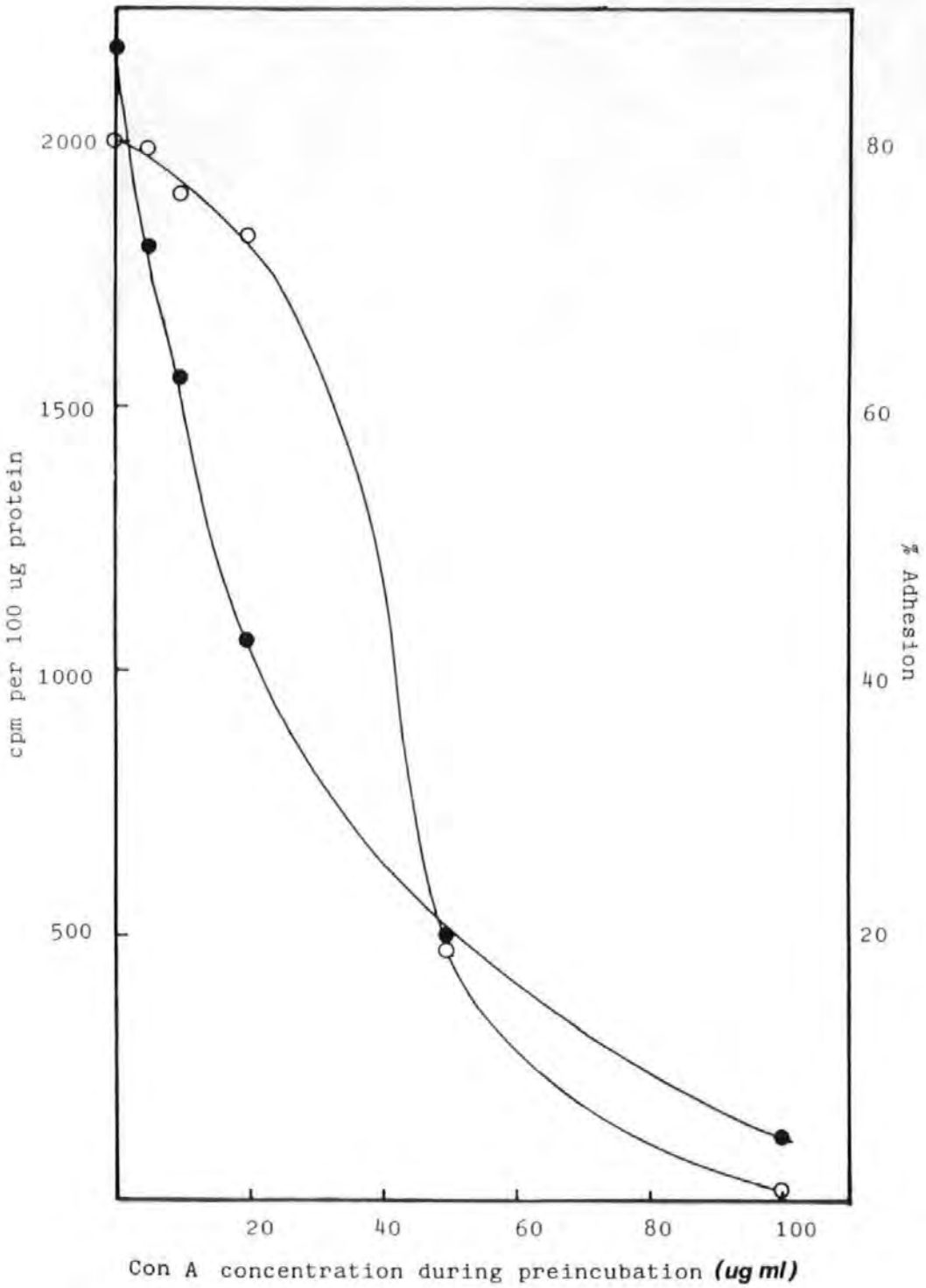


Fig. 6.1. The effect of 30 min preincubation in varying concentrations of concanavalin A on phagocytosis (●) and adhesion (O). After preincubation cells were washed twice in BSS. Phagocytosis was for 45 min and adhesion for 40 min.

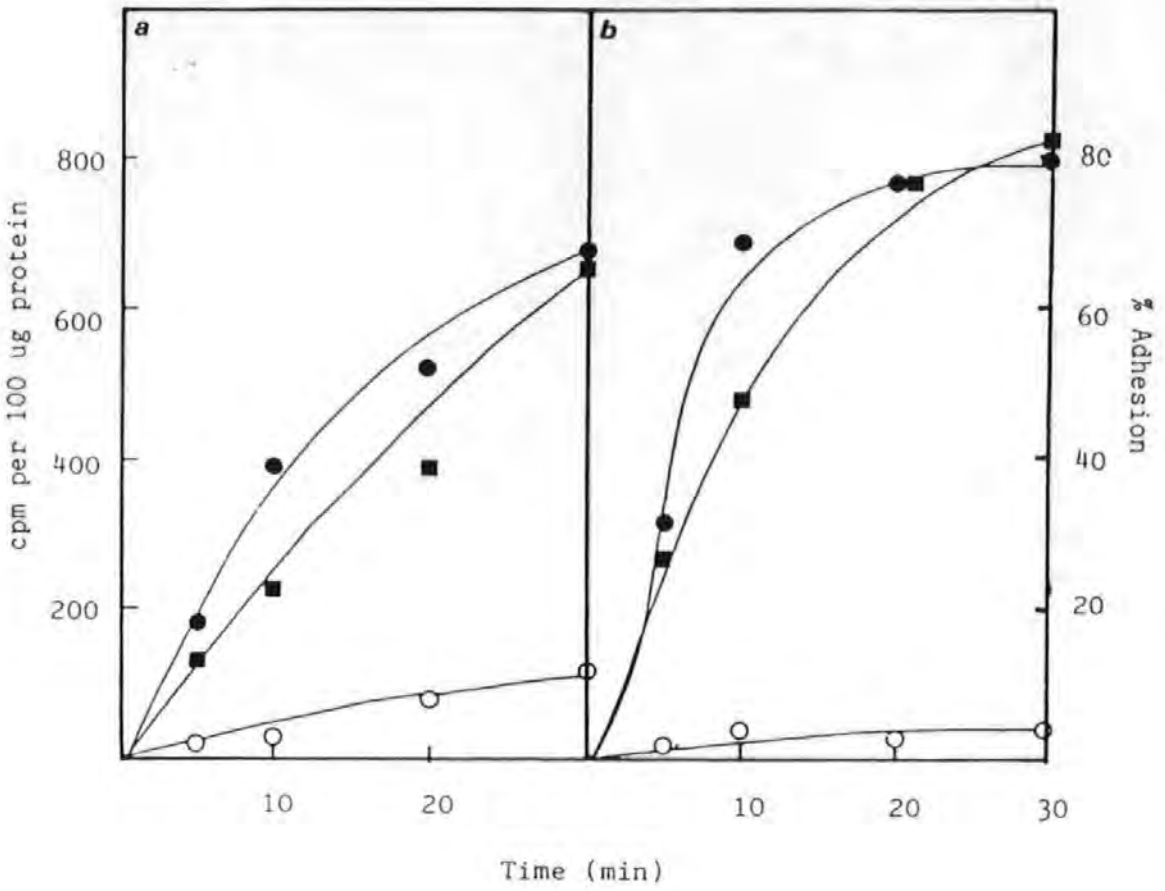


Fig. 6.2. Phagocytosis and adhesion of D.discoideum after 30 min preincubation in concanavalin A and/or alpha-methyl mannoside.

a) Phagocytosis. (■) Concanavalin A [100 ug/ml] + alpha-methyl mannoside [150 ug/ml], (●) Alpha-methyl mannoside [150 ug/ml], (○) Concanavalin A [100 ug/ml].

b) Adhesion. (■) Concanavalin A [100 ug/ml] + alpha-methyl mannoside [150 ug/ml], (●) Alpha-methyl mannoside [150 ug/ml], (○) Concanavalin A [100 ug/ml].

therefore likely that Con A inhibition of phagocytosis and adhesion is a genuine consequence of lectin binding to glucose or mannose residues at the cell surface of the amoebae.

The effects of Tunicamycin

Because of its mechanism of inhibition, any effects of tunicamycin on phagocytosis were likely to be observed only after long exposures of amoebae to the drug. For this reason, tunicamycin was added to cells growing in HL5-G medium and its effects on both cell numbers and phagocytosis were followed.

As can be seen from fig 6.3, a tunicamycin concentration of 1 ug/ml inhibits any increase in cell numbers after one doubling. However, the cells remained viable during the entire experiment, as judged by their ability to exclude trypan blue.

Tunicamycin present in the growth medium at 1 ug/ml significantly reduced phagocytosis, causing a sharp reduction during the first 10-15 h of treatment (fig. 6.3). However, phagocytosis could not be reduced to zero even after 34 hours exposure to tunicamycin, a residual phagocytic capacity (of approximately 30% of control values) remaining. The inhibition of phagocytosis was found to be non-reversible. Table 6.1 shows the phagocytic ability of cells exposed to 1 ug/ml tunicamycin for 10.5 hours, washed free of

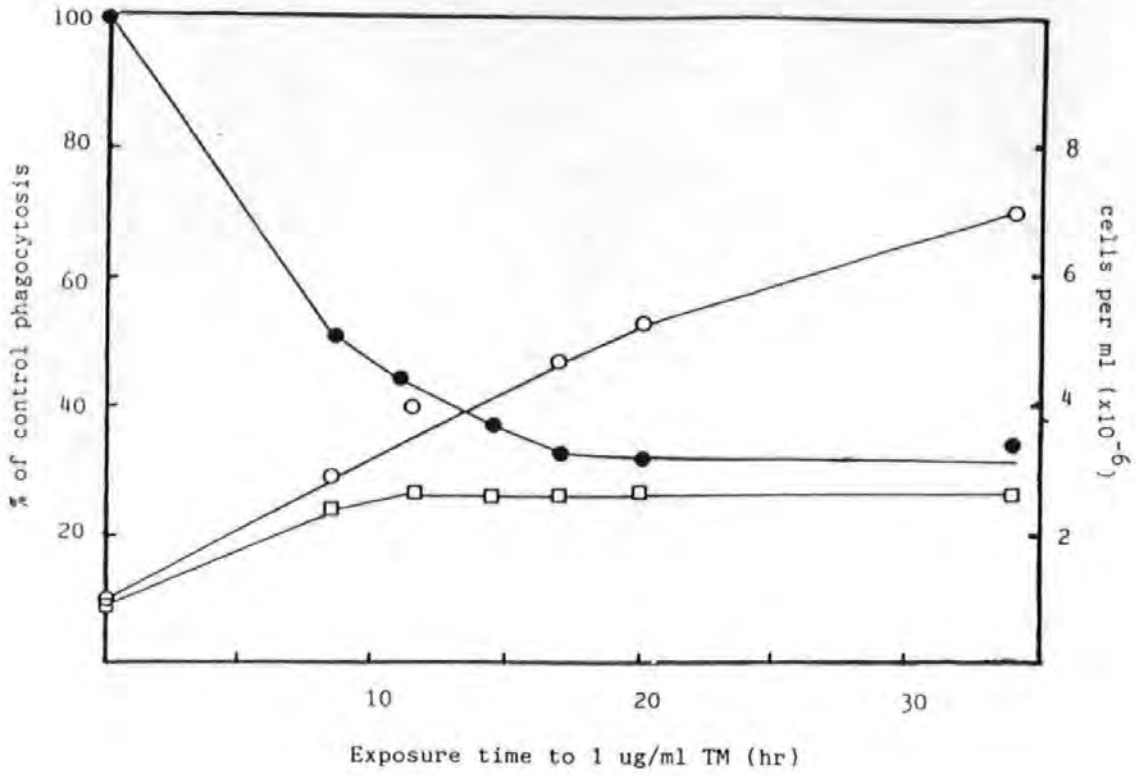


Fig. 6.3. Phagocytosis and growth of amoebae after increasing exposure time to 1 ug/ml tunicamycin. Cells were subcultured into fresh HL5/G containing 1 ug/ml tunicamycin. Phagocytosis and growth were assessed at time points up to 34 h after addition of tunicamycin. Points are the means of triplicate samples of amoebae at each time point. The control value was 1.6 x 10^3 cpm per 100 ug of amoebal protein. (●) Phagocytosis and growth (□) of cells exposed to 1 ug/ml tunicamycin, (○) Growth of control cells.

Table 6.1. The effect of removing tunicamycin after 10.5 h exposure to 1 ug/ml.

Time after removal of TM (h)	Cpm / 10^6 cells (Control)	(TM-treated)	% of Control
0	2980	834	28
6	2200	657	29
24	3120	687	22

Amoebae were treated with 1 ug/ml tunicamycin for 10.5 h. The antibiotic was washed free and cells resuspended in fresh growth medium. The cells were assesed at various time intervals for phagocytosis.

tunicamycin and resuspended in fresh growth medium. It is clear that there is no recovery of phagocytosis even 24 hours after the antibiotic has been removed. However there appeared to be some deterioration in the ability of cells to phagocytose at time periods greater than 24 hr (not shown). During this time the cells remained viable as observed by trypan blue exclusion.

The effect of 1 ug/ml tunicamycin on the adhesion of amoebae to glass was also investigated. As is apparent from fig. 6.4 there was no reduction in adhesion to glass in cells exposed to tunicamycin for 12.5 h. This compares sharply with a large decrease in phagocytosis seen in cells treated for 12 h (fig. 6.3). However, cells exposed to tunicamycin for 34 h exhibited a considerable reduction in adhesion although even after a long exposure to this drug adhesion is not reduced to zero. It therefore appears that phagocytosis by D.discoideum is more susceptible to inhibition by tunicamycin than is the ability of these cells to adhere to glass.

The effects of glycosidic and proteolytic enzymes

Glycosidases are able to liberate individual carbohydrate units of complex saccharide chains such as those found in glycoproteins. Beta-glucosidase from Phaseolus vulgaris is an exo enzyme able to hydrolyse beta-glycosidic linkages between D-glucose molecules

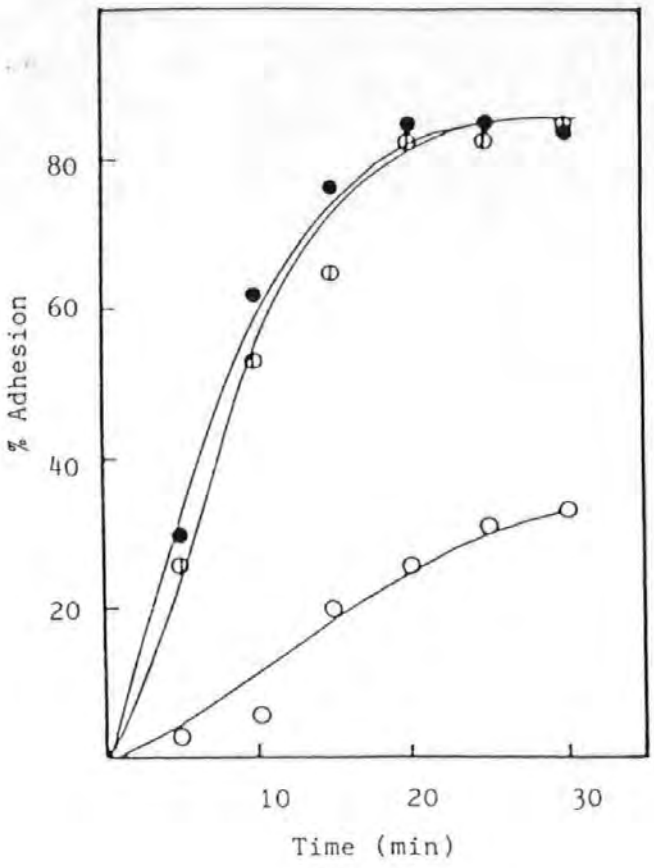


Fig. 6.4. The time course of adhesion of D.discoideum cells exposed for 0 h (●), 12.5 h (Φ), 34 h (O) to 1 ug/ml tunicamycin. Points are the means of duplicate samples.

(Agrawl & Bahl,1972). The effect of preincubation with this glucosidase on phagocytosis and adhesion was evaluated. Cells were preincubated for 30 min and then the enzyme washed free with ice cold BSS. Fig. 6.5 and 6.6 indicate that both phagocytosis and adhesion to glass were reduced after treatment with this enzyme. However, the reduction is small.

The sensitivity of phagocytosis to proteolytic activity was tested using three enzymes. Pronase E, a wide spectrum protease from Streptomyces griseus, caused no inhibition of phagocytosis when cells were preincubated at 1 mg/ml for 30 min (fig.6.7a). This was also the case for cells treated with 1 mg/ml trypsin under identical conditions, where no reduction in phagocytosis was detectable (fig.6.7b). However, preincubation of amoebae for 30 min with 1 mg/ml papain did result in a slight reduction in bacterial uptake, of approximately 30% as compared with control values (fig.6.7c).

It is possible that the resistance of phagocytosis to proteolytic treatment might be due to the presence of protein linked carbohydrate groups. Olden et al. (1972) have shown that sugar groups present on fibronectin prevent proteolytic degradation. Because of this possibility the effect of 30 min preincubation in 1 mg/ml pronase E on amoebae exposed to 1 ug/ml tunicamycin for 10 h was investigated. As shown in fig.6.8, phagocytosis by tunicamycin treated cells was

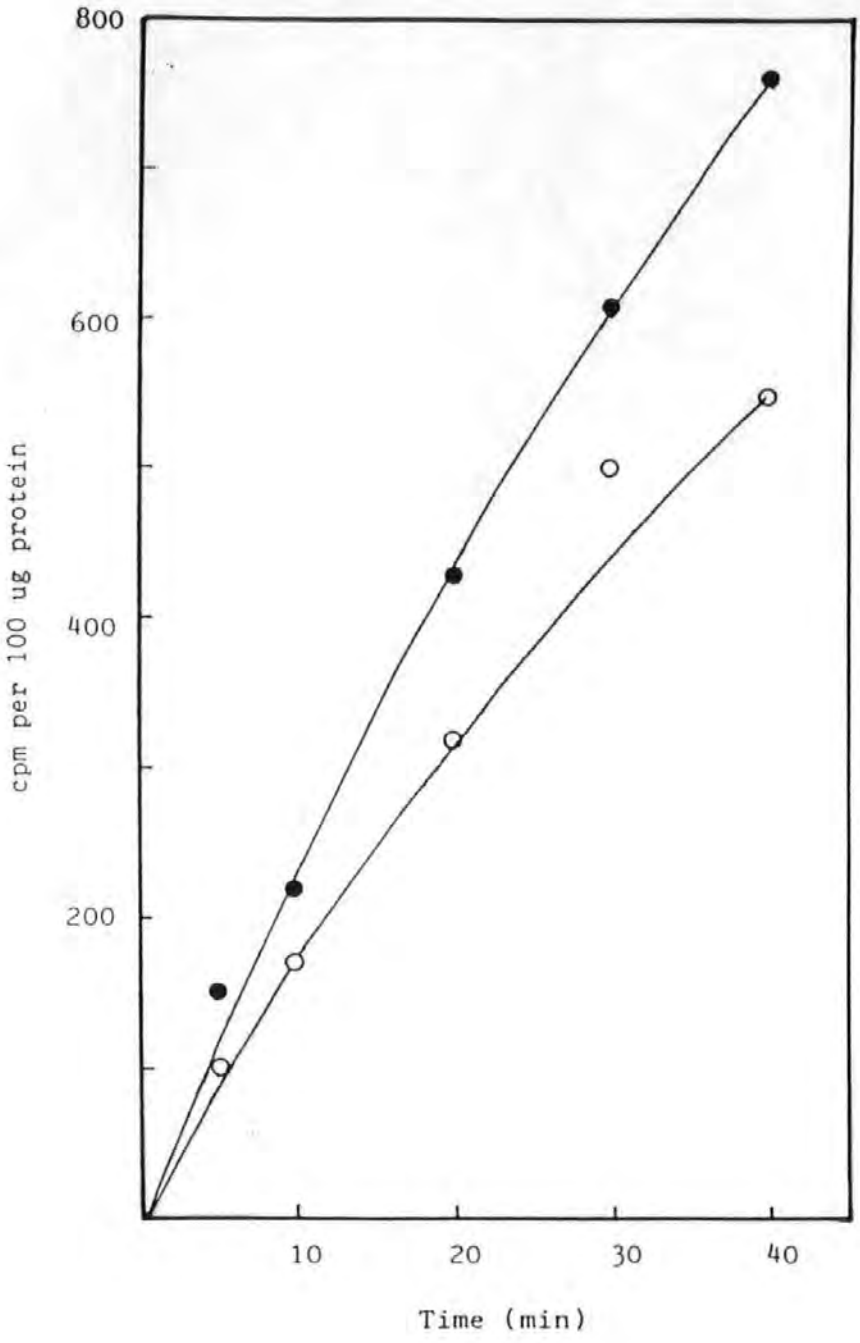


Fig. 6.5. The effect of beta-glucosidase on phagocytosis of E.coli by D.discoideumamoebae. Cells were preincubated for 30 min in 1 mg/ml beta-glucosidase (O) or BSS (●) and then washed twice in ice cold BSS. Phagocytosis was for 60 min.

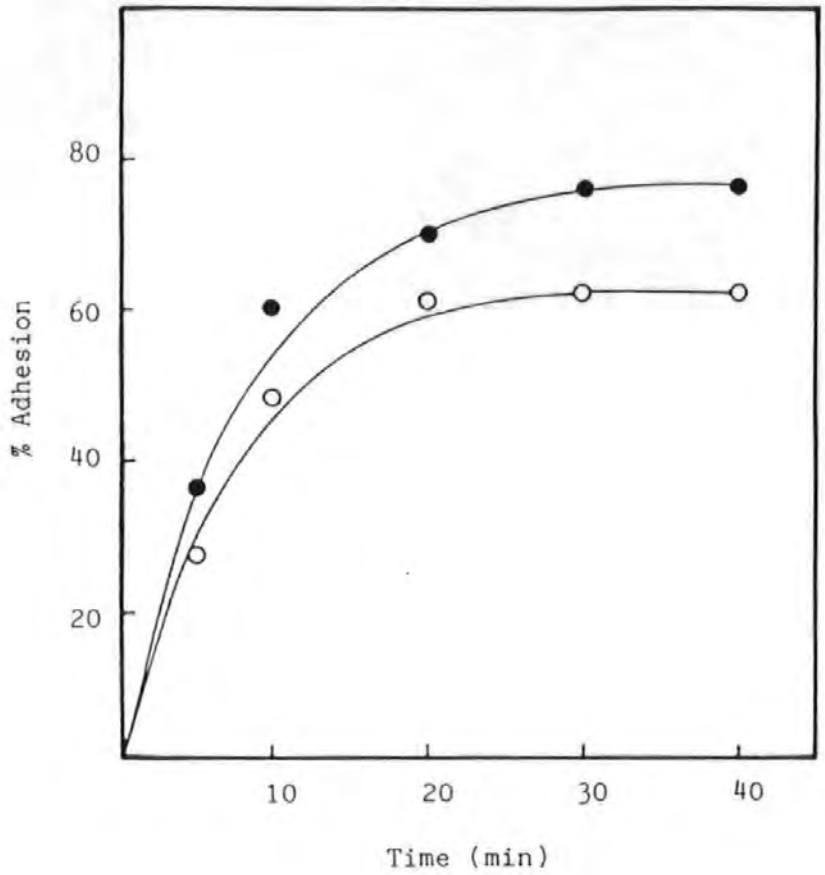


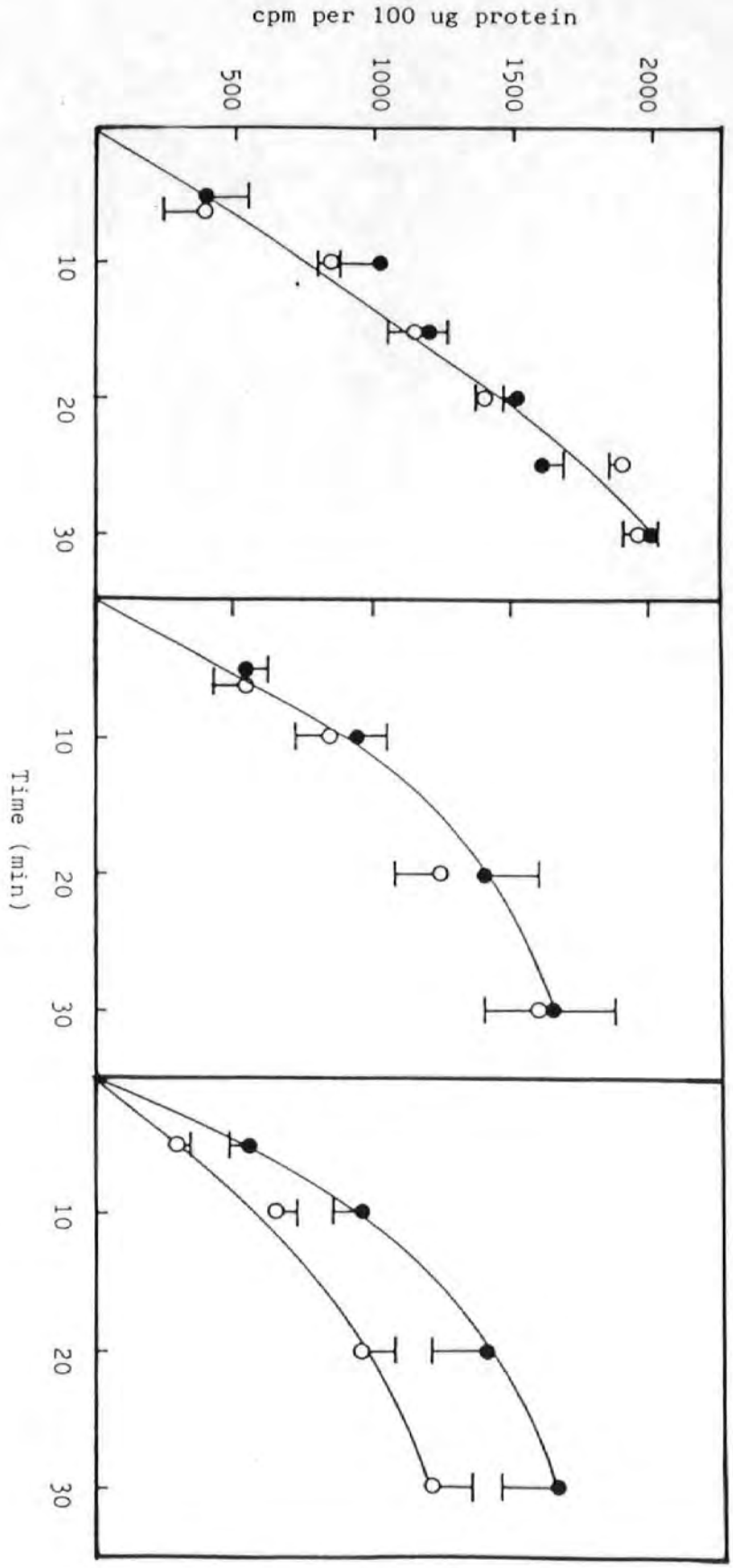
Fig 6.6. The effect of beta-glucosidase on the adhesion of D.discoideum to glass. Cells were preincubated for 30 min in 1 mg/ml beta-glucosidase (O) or BSS (●) and then washed twice in ice cold BSS.

Fig. 6.7. The effect of proteases on the phagocytosis of E.coli by D.discoideum. Cells were preincubated for 30 min in 1 mg/ml protease and then washed twice in ice cold BSS. Points are the means of duplicate samples from 4 different experiments. Bar = SEM.

a) Pronase E (o), Control (●)

b) Trypsin (o), Control (●)

c) Papain (o), Control (●)


cpm per 100 ug protein
500
1000
1500
2000
10
20
30
Time (min)
♀
♂

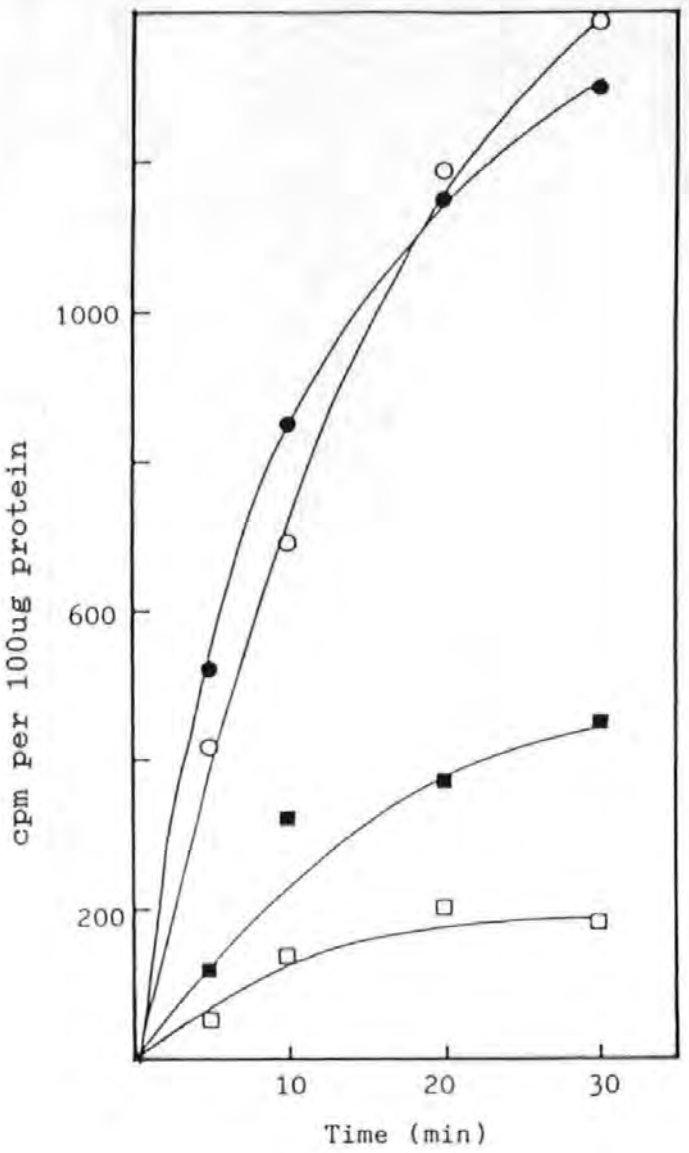


Fig. 6.8. The effect of pronase E on the phagocytosis of E.coli by D.discoideum cells exposed to tunicamycin. Cells were grown for 10 h in the absence (● ○) or presence (■ □) of 1 ug/ml tunicamycin and then preincubated for 30 min with 1 mg/ml pronase E (● □) or BSS (■ ○). Phagocytosis was assessed after washing all treatments twice in ice cold BSS.

further inhibited when exposed to pronase E as compared to cells treated with tunicamycin alone.

Discussion

Con A binds to glucose and mannose residues at the external face of the plasma membrane of D.discoideum, and has been shown to inhibit the uptake of yeast, latex beads and bacteria (Hellio & Ryter, 1980). However, these workers used only one concentration of Con A and monitored phagocytosis by an electron microscopic technique. In addition, adhesion of amoebae to glass was not investigated. The results presented here concerning phagocytosis, using a range of lectin concentrations and a quantitative assay substantially support previous findings with this lectin. Burridge and Jordan (1977), reported that Con A binds to many glycoproteins in the plasma membrane of vegetative cells of D.discoideum and other workers have estimated that 25-35 plasma membrane proteins bind Con A in these amoebae (West & McMahon, 1977; West et al., 1978).

The results presented here indicate that Con A inhibits both phagocytosis and adhesion to glass, suggesting that cell surface glycoproteins containing glucose or mannose residues are involved in these processes. In both cases it is clear that the Con A is acting specifically by virtue of the effect of including alpha-methyl mannoside with the lectin.

However, Hellio and Ryter (1980) found that Con A treated amoebae regained the ability to ingest bacteria 1.5 hours after the addition of the lectin, and that this occurred before any Con A binding proteins reappeared at the cell surface as measured by the ability of cells to bind this lectin. They interpreted this as evidence for the lack of any specific Con A binding being involved in phagocytosis. However, it has been reported that Con A treatment induces capping on treated D.discoideum amoebae (Condeelis, 1979). Therefore the inhibition may be due to the involvement of such large amounts of cytoskeletal components in the capping process that phagocytosis is rendered impossible.

The inhibition of adhesion to glass by Con A may also be a consequence of the utilisation of cytoskeleton in caps. The decreased sensitivity to Con A may indicate that either some proteins involved in adhesion do not bind Con A or that the depletion of cytoskeleton does not affect this process. However, these must remain as only tentative explanations.

The results obtained using tunicamycin treated cells suggest that there is some involvement of glycoproteins, with N-glycosidically linked carbohydrate moieties, during phagocytosis. Inhibition of cell growth may be due to the 30% drop in protein synthesis at greater than 3 h incubation in 3 ug/ml tunicamycin reported by Lam and Siu (1982). However

they report that no further inhibition of protein synthesis was apparent between 3 and 9 h. As this is the period where phagocytosis inhibition is increasing it seems likely that this is due to inhibition of protein N-glycosylation rather than impairment of protein synthesis. In the results presented here, at 10 hours incubation in 1 ug/ml tunicamycin, inhibition of phagocytosis is almost maximal. It is evident that complete inhibition of phagocytosis could not be achieved using tunicamycin. This residual resistance to the drug may be mediated by glycoproteins other than the N-glycosidically linked type or by lipids. It is interesting to note that both Lam and Siu (1982), and Yamada et al.(1982), reported that the EDTA sensitive binding site, (contact site B), that mediates cohesion between vegetative cells, is not affected during prolonged exposure to tunicamycin. The 10 h exposure to tunicamycin needed before maximal inhibition is seen may be a reflection of the rate of turnover of those cell surface components involved in phagocytosis. If these components were degraded and resynthesised rapidly in normal cells, then incubation in tunicamycin might be expected to reduce phagocytosis equally rapidly. However, the long exposure required before the final level of inhibition was achieved may indicate that cell surface components affected by this compound are degraded and replaced relatively slowly in axenically growing amoebae. Cells growing on bacteria,

on the other hand, might be expected to be inhibited far more quickly if cell surface components involved in the uptake of bacteria were internalised at a faster rate than in axenically growing cells. This might be a fruitful area for further study using tunicamycin.

The adhesion of cells to glass was also inhibited by tunicamycin but required longer exposure to elicit any inhibition. N-glycosidically linked glycoproteins may be involved in adhesion to glass. It is possible that the cell surface components involved in adhesion to glass may be turned over at a slower rate than those involved in phagocytosis. In summary, the results suggest that N-glycosydically linked glycoprotein(s) are involved in phagocytosis and adhesion of D.discoideum amoebae.

Treatment of intact cells by beta-glucosidase would be expected to hydrolyse exposed glucose residues from oligosaccharide chains at the cell surface. The slight inhibition of phagocytosis and adhesion to glass by glycosidase may indicate that cell surface components containing terminal glucose residues are involved in some way during these processes. However, the cells appear to be quite resistant to this treatment and it is difficult to draw firm conclusions without additional experimental information possibly using other Glycoside enzymes.

The treatment of intact D.discoideum amoebae with high concentrations of proteases has little effect on

phagocytosis by these organisms. Jerym et al. (1977) reported that chymotrypsin and pronase at concentrations of 3 mg/ml removed ^{14}C-leucine from pre-labelled intact amoebae and that development could be arrested with such treatments. They also found that at the same concentration, pretreatment with trypsin had no effect on development. Smart and Hynes (1974) reported that over 90% of peptides labelled by radioiodination can be removed by trypsin. However, this treatment only labels exposed tyrosine residues and therefore it is possible that only a small number of proteins were labelled and subsequently removed by trypsin.

The results presented here clearly show that neither pronase or trypsin at 1 mg/ml caused any significant drop in phagocytosis. Either the proteins digested by these enzymes are not involved in phagocytosis or the proteins involved are not susceptible to disruption by these enzymes. A third possibility, although unlikely, is that proteins are not involved at all. It has been suggested that the carbohydrate groups present in glycoproteins may serve a protective function against proteolytic degradation (Olden et al., 1972). In some support of this idea, cells that had been exposed to tunicamycin were found to be sensitive to pronase treatment so that phagocytosis was reduced below the level of tunicamycin treated cells alone. Although the degree of additional

inhibition was not large it may be that some glycoproteins involved in phagocytosis are resistant to pronase treatment because of the presence of protective carbohydrate groups. However, this must remain only a tentative suggestion.

The results using pronase and trypsin are consistent with those of Parish et al. (1977) who reported that plasma membrane proteins are only digested to a limited degree when intact cells are incubated with proteases. They also report that low papain concentrations used to treat intact vegetative amoebae produced a "new" protein band on polyacrylamide gels that is apparently derived from actin. They interpret this result as being due to the possible penetration of the plasma membrane by a portion of membrane associated actin, that is attacked by low papain concentrations. It is possible that the slight reduction of phagocytosis seen with papain treatment may be related to this effect. However, additional investigations are needed to lend support to this idea.

Chapter 7

The isolation of phagosomes and plasma membrane

Introduction

During phagocytosis psuedopodia are advanced around the particle until they meet at its apex and fuse, pinching off a section of membrane into the the interior of the cell. The resulting phagosome membrane is therefore derived from the plasmalemma. However, there is some evidence that the protein composition of D.discoideum phagosome membrane may differ from that of the bulk plasma membrane (Parish & Pelli, 1974). In addition, the attachment of different types of particles to the plasma membrane might involve recognition by specific receptors, possibly proteins. Therefore, the phagosome membrane surrounding different particles may differ in protein composition.

To investigate this possibility, an attempt was made to isolate phagosomal membranes from two types of particles (polystyrene beads and fixed bacteria) so as to compare their polypeptide compositions by SDS polyacrylamide gel electrophoresis (PAGE). This required the development of a method for isolating ingested bacteria from phagocytes. The isolation of a bulk plasma membrane fraction was also attempted to

determine whether its polypeptide composition differed from that of the phagosome membranes.

In addition to SDS PAGE analysis, the respective activities of alkaline phosphatase, thought to be a plasma membrane protein (Green & Newell, 1974) and acid phosphatase, a lysosomal marker (Dean, 1977), were determined for each of the preparations.

Results

Phagosomes were isolated using two classes of particles, polystyrene beads and fixed bacteria.

Polystyrene beads

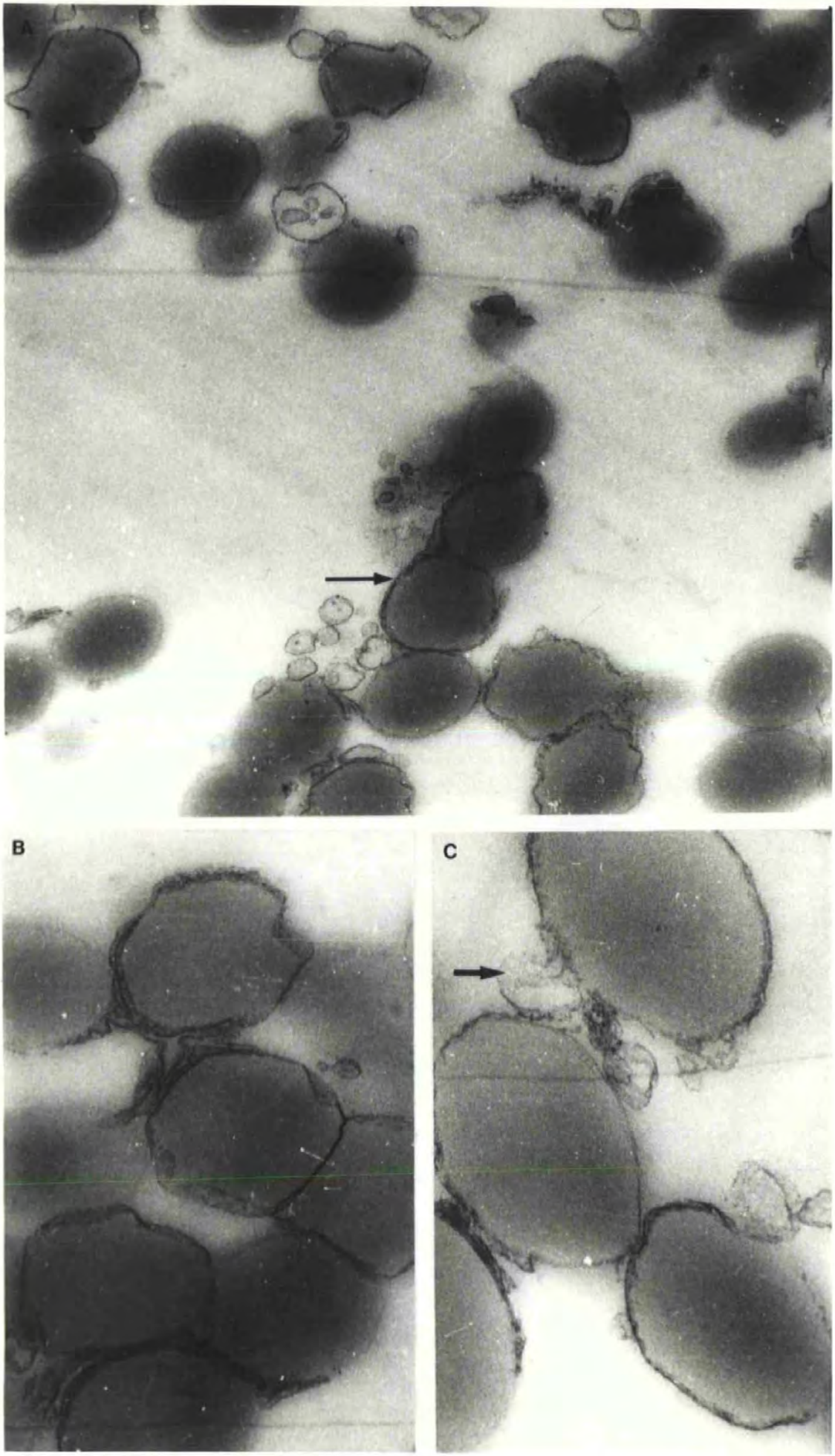
The method used to isolate internalised polystyrene beads from amoebae was essentially that of Korn (1974) who isolated phagosomal membranes from A.castellanii. In a modification of this method, D.discoideum amoebae were allowed to ingest 1 um diameter beads for 1 h while being agitated in growth medium. The amoebae were then separated from uningested beads by low speed centrifugation. Internalised beads were released by homogenisation of the amoebae and purified from the crude homogenate by ultracentrifugation on a discontinuous sucrose gradient. As can be seen from fig. 7.1a, a considerable purification of ingested beads has been achieved.

Fig. 7.1 Electron micrograph of polystyrene beads isolated from D.discoideum amoebae.

A) x 20,000 ⟶ Multiple membranes

B) x 35,000

C) x 40,000 ➟ Phagosome membrane extending from bead


A
B
C

Almost all the material obtained from the gradient consists of latex beads and these clearly have membranous material surrounding them. Higher magnifications are presented in figs. 7.1b and 7.1c. Solubilisation of these preparations in SDS loading buffer with subsequent electrophoresis on 13% polyacrylamide gels, resulted in reproducible protein profiles from different preparations. Fig. 7.2 illustrates those polypeptides that stained most intensely in preparations of phagosomes isolated using polystyrene beads. These polypeptides are the most densely staining bands and were present in all preparations. They are therefore referred to as major bands. A number of minor bands also appeared in these profiles but these varied between different preparations. Major polypeptides had apparent molecular weights of 23, 28, 42, 55, 72, 116, 130, 150 and 200 Kd (Fig. 7.2). Additionally, these membrane preparations were stained using the concanavalin A / peroxidase method, in order to visualise concanavalin A binding glycoproteins. In these preparations fifteen to twenty five bands were visible with two major bands of apparent molecular weights of 55 and 28 Kd, (fig. 7.3).

Table 7.1 presents the results of enzyme and protein analyses of three preparations of phagososmes from polystyrene beads. Alkaline phosphatase activity is absent in two preparations and where present is not enriched. Acid phosphatase activity was not detectable

Fig. 7.2 Polypeptides from phagosomes isolated using polystyrene beads.

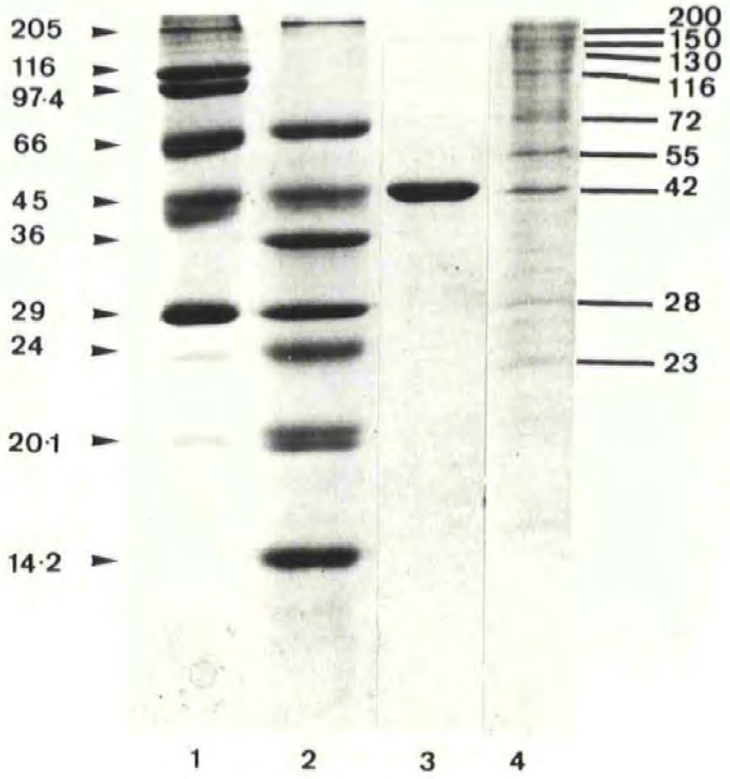
Lane:
1. High molecular weight standards.
2. Low molecular weight standards
3. Rabbit muscle actin (Sigma)
4. Phagosome preparation

Molecular weights are shown in Kd
Left hand values refer to standards (lane 1 & 2)
Right hand values refer to apparent molecular weights of phagosome polypeptides.

Fig. 7.3 Concanavalin A binding polypeptides surrounding polystyrene beads.
Major polypeptides are arrowed and apparent molecular weights given in Kd.

A) Result of Con A / peroxidase method used to stain phagosome preparation from polystyrene beads.

B) Diagrammatic representation of A.


205
116
97·4
66
45
36
29
24
20·1
14·2
200
150
130
116
72
55
42
28
23
1
2
3
4

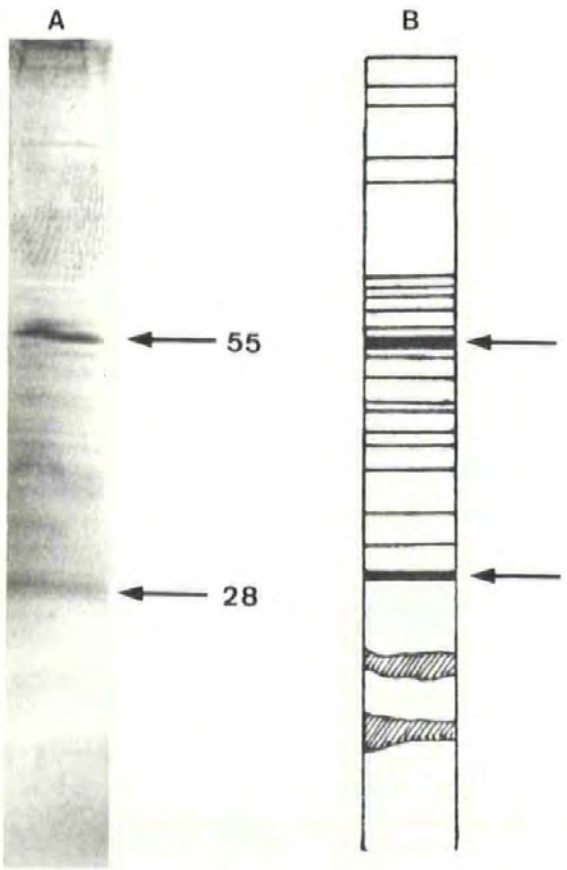

A
B
55
28

Table 7.1. Acid and alkaline phosphatase activities in phagosomes isolated using polystyrene beads.

Preparation	Protein		Alkaline phosphatase		Acid phosphatase	
	mg isolated	% yield	% yield	specific activity	% yield	specific activity
1. Homogenate	320	0.12	0.6	3.15 (0.85)*	0	0.605
Phagosome	0.4			2.69		0
2. Homogenate	270	0.09	0	1.97	0	3.59
Phagosome	0.26			0		0
3. Homogenate	294	0.10	0	0.41	0	4.68
Phagosome	0.31			0		0

* Relative specific activities

in any of these preparations. Protein yield as a percentage of homogenate protein is similar between preparations (0.096% - 0.125%) although a seven fold difference in alkaline phosphatase specific activities in the homogenate is apparent. This may be a result of varying degrees of degradation in these preparations. However, the range of these values is consistent with homogenate values reported by other workers during the isolation of plasma membrane (Gilkes & Weeks, 1977).

Fixed bacteria

Vegetative amoebae of D.discoideum feed by the ingestion of soil bacteria and it is possible that different membrane proteins are involved during the recognition and ingestion of different particles. Additionally, bacteria might be viewed as a more natural substrate particle for amoebal phagocytosis. However, solubilisation of phagosome membrane using SDS loading buffer, would have resulted in contamination of any isolated membrane polypeptides with polypeptides of bacterial origin. Furthermore a population of partially digested bacteria would be heterogeneous in its behaviour in the centrifuge. To resolve these difficulties, glutaraldehyde fixed E.coli were used as substrate particles. Fig. 7.4 shows the effect of fixing bacteria, for various time periods, on the subsequent solubilisation of bacterial proteins.

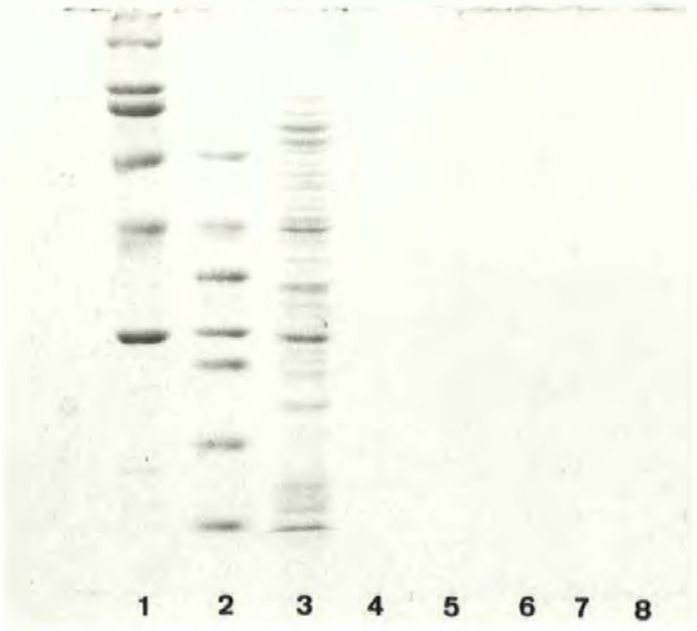


***Fig.* 7·4** The effect of various fixation times on the stability in SDS loading buffer of E.coli polypeptides.

Lane: 1. HMW standards

2. LMW standards

3. Unfixed E.coli

4. Fixed E.coli (1 h)

5. (2 h)

6. (3 h)

7. (5 h)

8. (24 h)

Treatment of unfixed bacteria with SDS loading buffer clearly causes extensive solubilisation of bacterial proteins (lane 3). However, fixation for 1 hour or longer in 2.5% glutaraldehyde results in complete resistance of bacterial proteins to solubilisation by boiling in SDS loading buffer (lanes 4 - 8). Bacteria fixed for 1 hour were therefore used as substrate particles for phagocytosis.

In order to demonstrate that fixed bacteria were ingested normally their rate of uptake was compared to that of unfixed cells. Fig. 7.5 shows that the rate of uptake of fixed bacteria was identical to that of normal cells. Confirmation that fixed bacteria were internalised can be seen in fig. 7.6a and at a higher magnification in fig 7.6b. It is also apparent from these electron micrographs that fixed bacteria are not digested over the time period of the incubation (1 h). Unfixed bacteria, however, are significantly degraded over the same time period (Glynn, 1981).

Amoebae were incubated with fixed bacteria for 1h. Internalised bacteria were separated from the cell homogenate by a bench centrifugation protocol to develop a rapid and simple isolation procedure. During the initial development of this procedure, low speed centrifugation was used and the preparation examined at each stage by light microscopy. Observations by light microscopy during the final stage of the isolation indicated that large numbers of bacteria were present

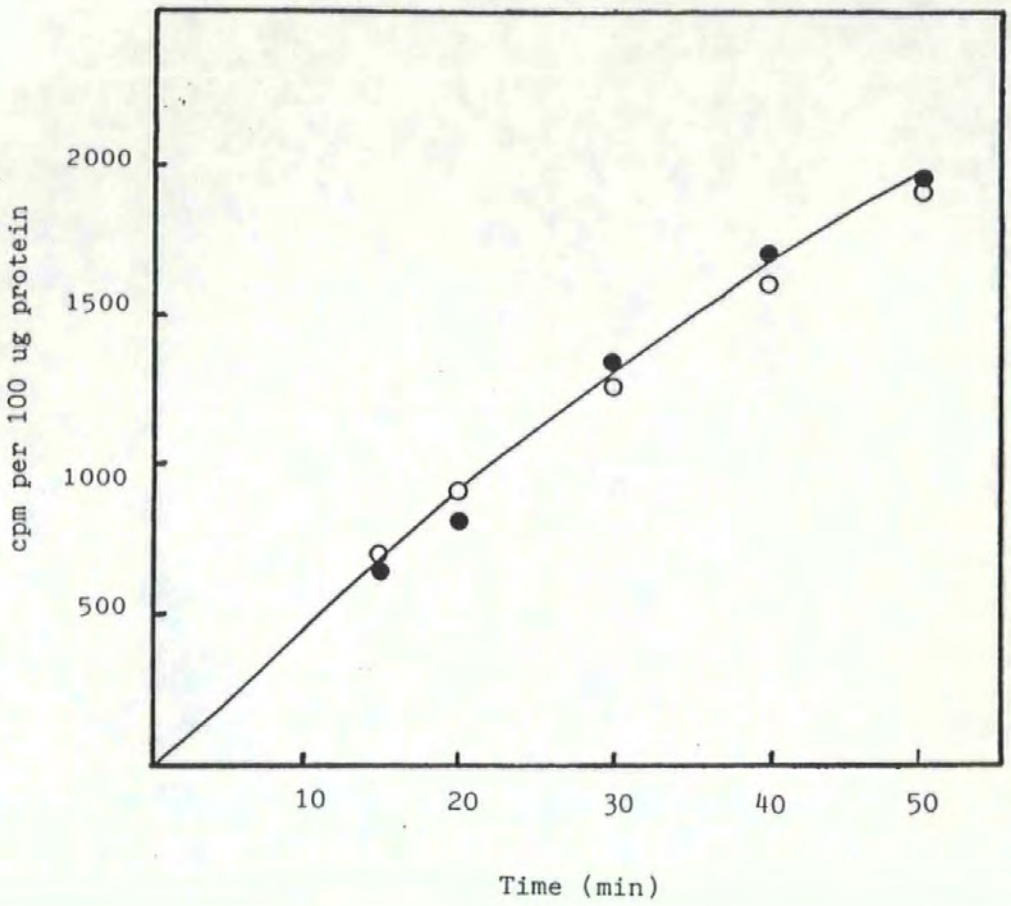


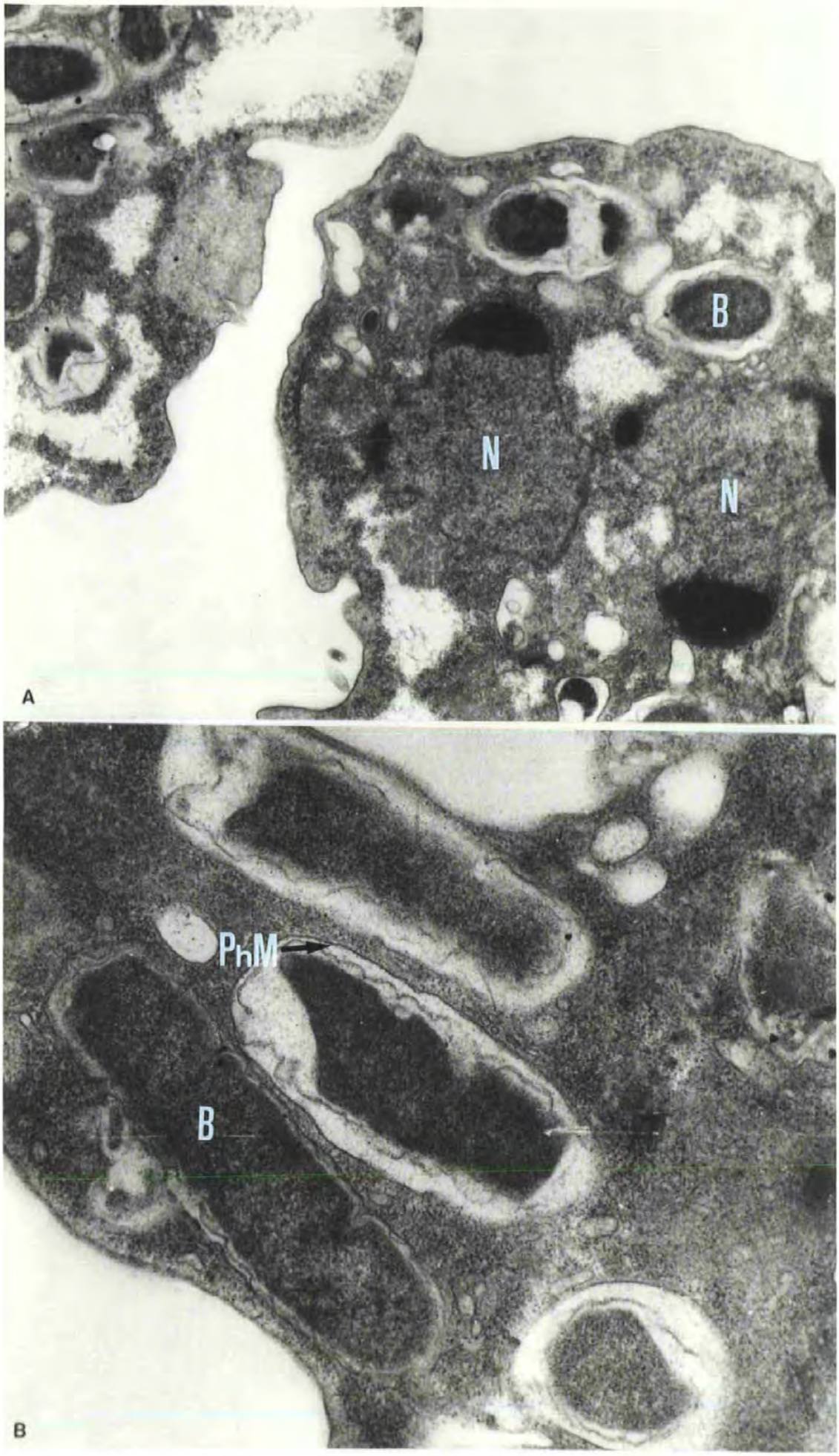
Fig. 7.5 The phagocytosis of 1 h glutaraldehyde fixed (●) and unfixed (○) E.coli by D.discoideum.

Fig. 7.6 Electron micrograph of Dictyostelium discoideum amoebae after phagocytosis of 1 h glutaraldehyde fixed E.coli

A) x 15,000

B) x 25,000

N= nucleus, B= bacteria, PhM= phagosome membrane


B
N
N
A
PhM
B
B

with no visible contamination. The bacteria are clearly seen in fig. 7.7a and phagosomal membranes are evident around them. However, these electron microscopic observations also indicate that the preparation, although consisting primarily of bacteria, also contains some contaminating material of cellular origin. It is therefore possible that some of the polypeptide bands present in SDS profiles are not derived from the phagosome membrane. The membrane surrounding bacteria does not appear to be as tightly apposed to the bacterial surface (fig. 7.7b), in contrast with membrane surrounding some polystyrene beads (fig. 7.1b). Some phagosomes containing two bacteria are clearly visible (arrowed in fig. 7.7a), a situation which frequently occurs *in vivo* (Glynn, 1981). In spite of the possible contribution to the polypeptide profiles by cellular contamination, the preparations gave consistent banding patterns from different preparations. Major polypeptides of apparent molecular weight 14, 16, 17, 28, 29, 32, 40, 42, 51, 55, 72, 116 and 200 Kd were present in all preparations (Fig. 7.8). Ten to fifteen Con A binding polypeptides in bacterial preparations were present after staining by the Con A/peroxidase procedure. Four of these were major bands and had apparent molecular weights of 60, 55, 42 and 39 Kd (fig. 7.9).

An enzyme analysis of two preparations is shown in Table 7.2. Alkaline phosphatase was present in both of

Fig. 7.7 Electron micrograph of isolated phagosomes containing 1 h glutaraldehyde fixed E.coli

A) x15,000 → Contaminating material

⇨ Phagosome containing two bacterial cells

B) x30,000 → Phagosome membrane

C) x30,000 ⇨ Bacterial membrane

D) x35,000 → Phagosome membrane

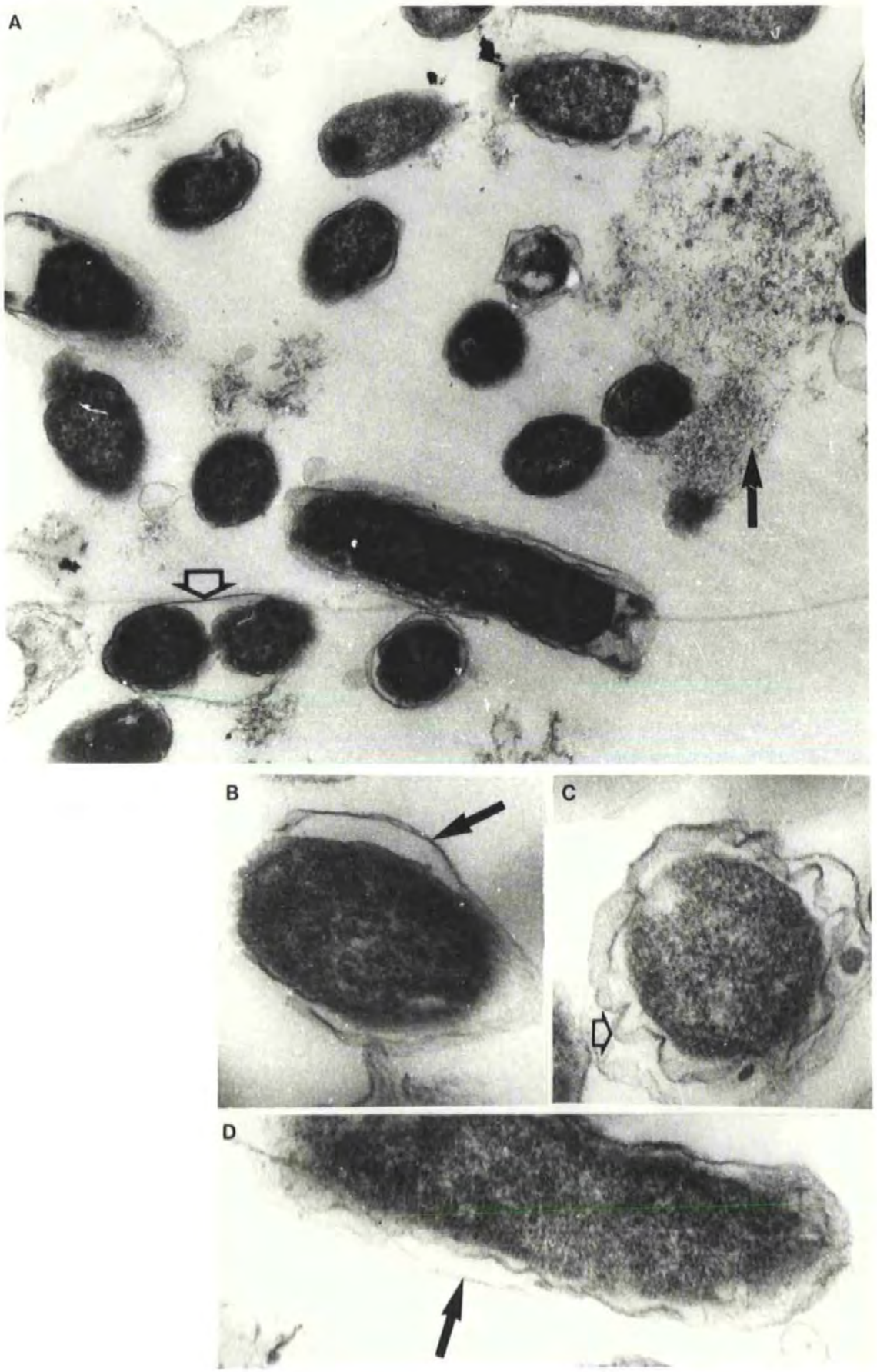

A
B
C
D

Fig. 7.8 Polypeptide profile from phagosomes isolated using glutaraldehyde fixed E.coli

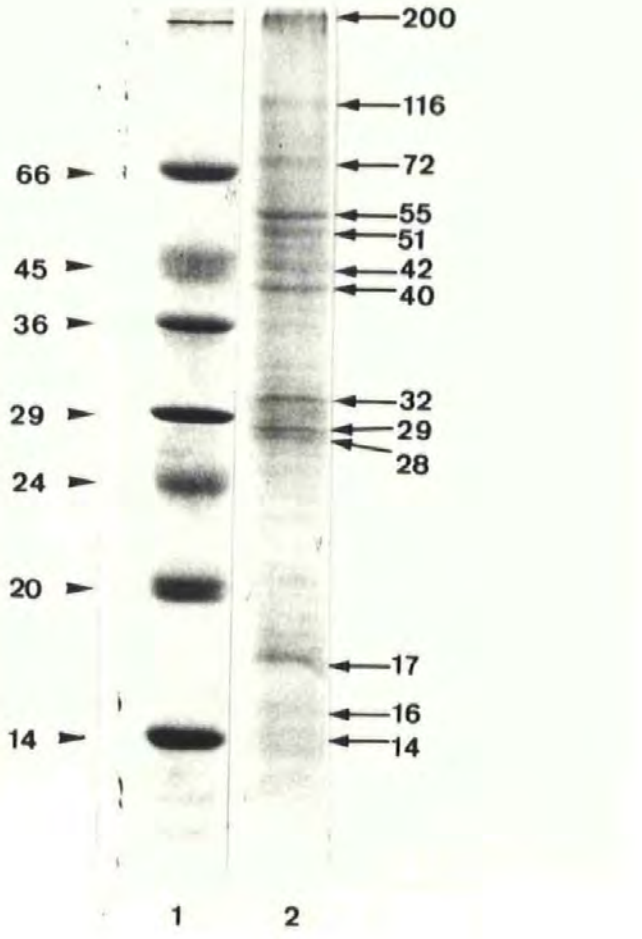
Lane: 1. Molecular weight standards.
2. Phagosome polypeptides.

Values are apparent molecular weights in Kd

Fig. 7.9 Concanavalin A binding polypepetides from surrounding glutaraldehyde fixed E.coli

A) Results of Con A / peroxidase method used to stain a phagosome preparation from around glutaraldehyde fixed E.coli

B) Diagrammatic representation of A.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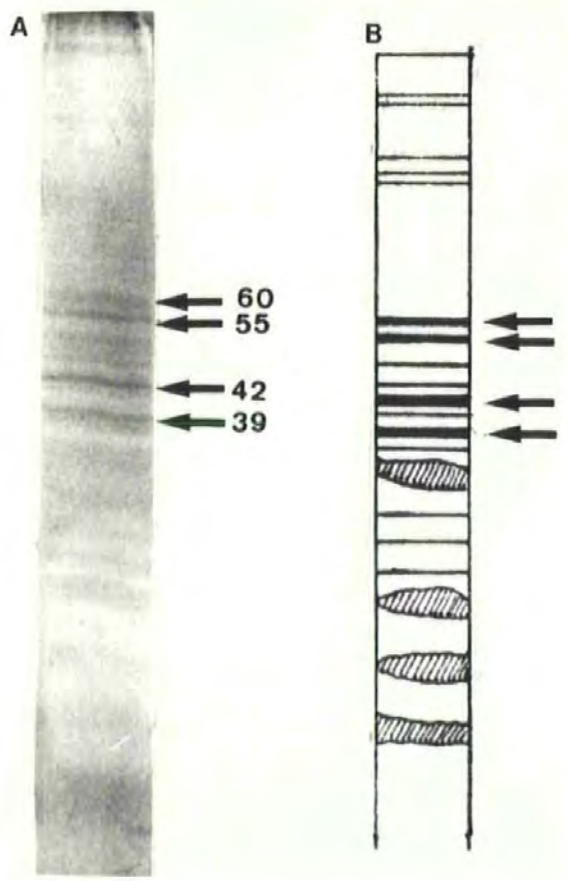

A
B
60
55
42
39

Table 7.2. Acid and alkaline phosphatase activities in phagosomes isolated using fixed E.coli

Preparation	Protein		Alkaline phosphatase		Acid phosphatase	
	mg isolated	% yield	% yield	specific activity	% yield	specific activity
1. Homogenate	312			2.15 (1.89)*		0.5 (6.12)*
		2.2	0.67		2.15	
Phagosome	7			4.07		2.15
2. Homogenate	74			1.0 (1.5)*		3.60 (0.6)*
		4.1	1.20		0.04	
Phagosome	3			1.47		2.02

* Relative specific activities

the isolates analysed and enrichment values of 1.5 and 1.9 were obtained (as compared to the homogenate). Acid phosphatase was also present in both preparations. However, homogenate specific activities differed considerably between the two preparations and may have been due to variable degradation of the homogenate activity. The protein yields were higher in these preparations than in those obtained with polystyrene beads (2-4 % of the homogenate protein as compared to 0.1 %).

Isolation of plasma membrane.

The method used for the isolation of plasma membrane from amoebae was essentially that of Rossamando and Cutler (1975). Cells were incubated with the lipophilic antibiotic, amphotericin B, which resulted in lysis of more than 85% of the cells. Plasma membrane was separated from other cell components on a sucrose gradient by ultracentrifugation. A plasma membrane preparation is shown in the electron micrograph in fig. 7.10. It consists of membranous material and is very similar to electron micrographs of preparations by Rossamando and Cutler (1975). Ten major bands with apparent molecular weights of 14, 15, 16, 17, 28, 29, 32, 55, 72 and 116 Kd appeared in plasma membrane polypeptide profiles seen in fig. 7.11. Glycoprotein staining by the Con-A peroxidase method

Fig. 7.10 Electron micrograph of a plasma membrane preparation isolated using amphotericin B

A and B) View of different fields from the same preparation. x50,000

⇩ membrane whorls

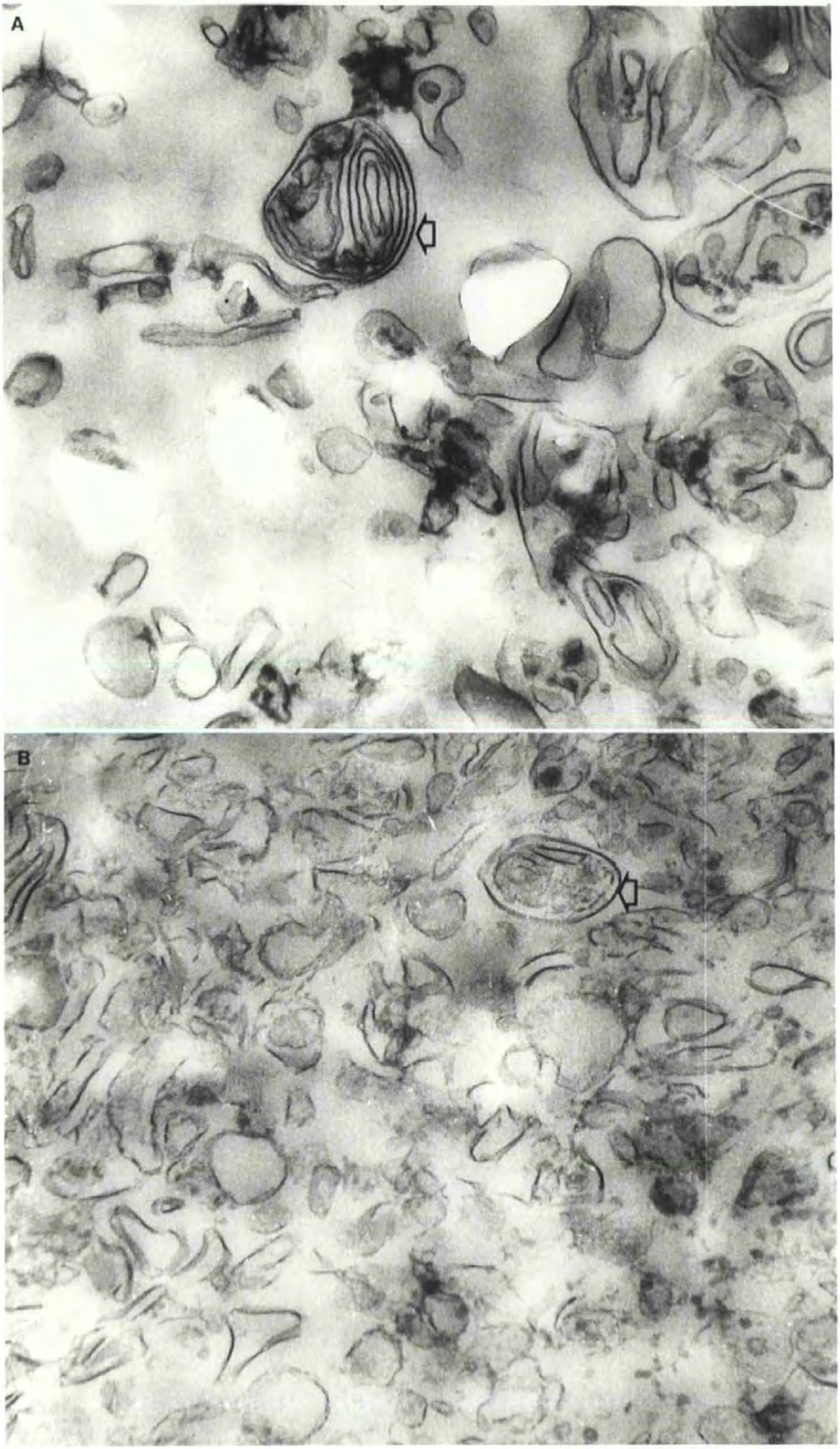
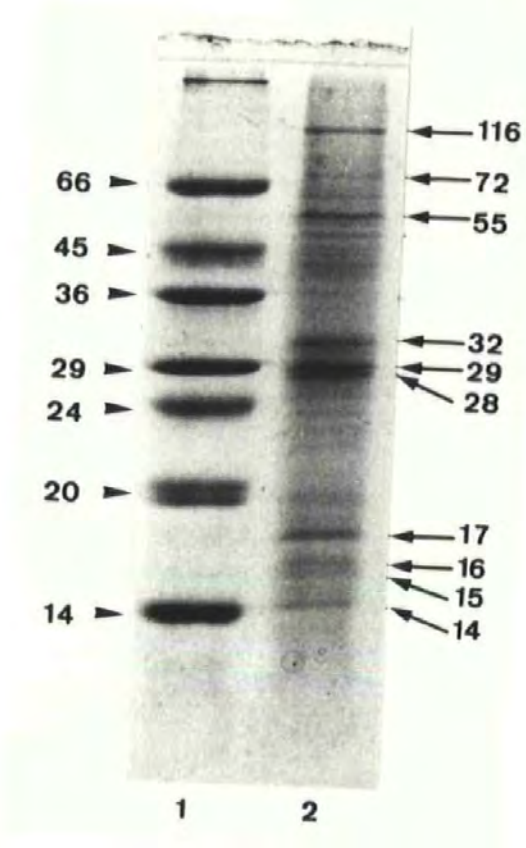

A
B

Fig. 7.11 Polypeptide profile from a plasma membrane preparation.

Lane: 1. Molecular weight standards

2. Plasma membrane polypeptides

Values are molecular weights in Kd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was also carried out on these preparations. However, heavy background staining prevented the clear visualisation of any glycoprotein bands.

The alkaline and acid phosphatase activity of two plasma membrane isolates are shown in Table 7.3. Enrichment of alkaline phosphatase was evident in both of these preparations. However, it is likely that some degradation of the homogenate activity took place during the time course of the isolation and this would account for the improbably high ratio of specific activities in preparation 1. In contrast, acid phosphatase was not enriched, relative specific activities ranging from 0.5-1.0.

A series of comparisons of the polypeptide profiles from different preparations is illustrated in figs. 7.11 to 7.14.

Discussion

The appearance of the polystyrene bead isolate in fig 7.1a is typical of the preparations shown by Korn (1974). However, some membranous material is apparently not associated with the beads. This might represent contaminating non phagosomal membrane but it could also represent material whose attachment to the phagosome fell outside the plane of the section. In fig. 7.1b at a higher magnification phagosome membrane can be seen surrounding the beads, in some cases tightly apposed to

Table 7.3. Acid and alkaline phosphatase activities in isolated plasma membrane preparations

Preparation	Protein		Alkaline phosphatase			Acid phosphatase		
	mg isolated	% yield	% yield	specific activity		% yield	specific activity	
1. Homogenate	240	1.4	---	0.1	(145)*	---	2.15	(1.2)*
Plasma mem.	3.3			14.4			2.49	
2. Homogenate	51.4	1.8	3.05	5.18	(15.5)*	---	3.77	(0.52)*
Plasma mem.	0.9			80.3			1.94	

* Relative specific activity

the bead surface, although a dense material possibly multiple membrane layers, does appear to surround some beads within the phagosome (fig. 7.1c). A similar material present around polystyrene beads ingested by Acanthamoeba castelanii is reported by Korn and Weisman (1967). Polypeptide profiles from different preparations displayed a small number of consistent bands illustrated in fig. 7.2, although protein yields were always low (Table 7.1). A polypeptide of molecular weight 42-43 Kd was consistently present as a major band in these preparations. This was presumably actin, which constitutes 95% of the polypeptide of this molecular weight in D.discoideum (Alton and Lodish, 1977).

In addition to PAGE analysis, enzyme characteristics were also investigated. Neither acid or alkaline phosphatase was measurable in these isolates. This could be due to the low yield of material in these preparations. However, Parish and Pelli (1974) have reported that alkaline phosphatase, normally associated with the plasma membrane, is excluded from phagosomes formed during the ingestion of polystyrene beads. This colchicine-inhibited exclusion could account for the undetectability of the enzyme in the present preparations. On the other hand, Parish and Pelli (1974) also found considerable acid phosphatase activity associated with their phagosomes, presumably due to fusion with lysosomes prior to isolation. The

fact that acid phosphatase is also undetectable in these preparations may therefore point to low yield as the source of the problem.

The isolation of phagosomes using fixed bacteria is a novel method that was initiated and partially developed during the course of this study. Separation of bacteria from the crude cell homogenate was attempted without density gradients using a bench centrifuge and as a preliminary step achieved a considerable enrichment of bacteria (fig. 7.7).

Major differences in polypeptide composition are apparent between phagosomes isolated using fixed bacteria and those isolated from polystyrene beads, as shown in fig. 7.12. Polypeptides of apparent molecular weight 29, 32, 14, 16 and 17 Kd present as major bands in phagosomes prepared from fixed bacteria are absent or very reduced in phagosomes from around polystyrene beads. Additionally, a greater number of minor bands appear in phagosomes from fixed bacteria. A polypeptide of apparent molecular weight 42-43 Kd was always present as a major band in preparations from polystyrene beads and less conspicuously in phagosomes derived from bacteria. Comparison of concanavalin A binding glycoproteins present in phagosome membrane from around polystyrene beads or fixed bacteria indicated additional differences between these two preparations. Two major bands at apparent molecular weight of 55 and 28 Kd, (fig 7.3), were present in

Fig. 7.12 Comparison of polypeptides from phagosomes isolated using fixed E.coli or polystyrene beads.

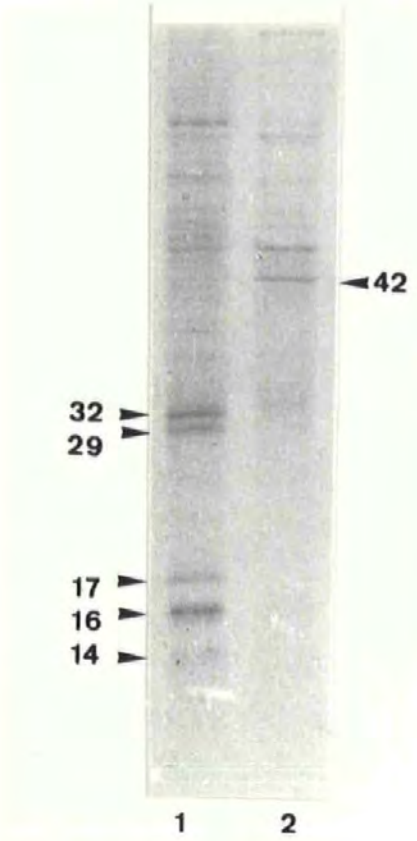
Lane: 1. Phagosomes from around fixed E.coli.
2. Phagosomes from around polystyrene beads.

Values refer to apparent molecular weights in Kd of polypeptides that differ between the two preparations.

Fig. 7.13 Comparison of polypeptides from phagosomes surrounding polystyrene beads and plasma membrane polypeptides.

Lane: 1. Phagosomes from around polystyrene beads.
2. Polypeptides from plasma membrane

Values refer to apparent molecular weights in Kd of polypeptides that differ between the two preparations.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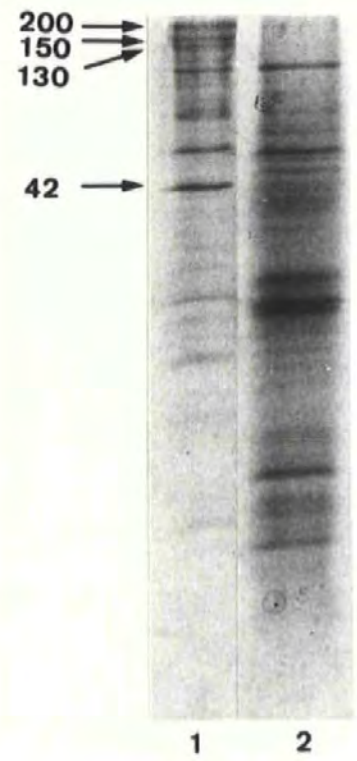
200
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polystyrene bead phagosomes while only the 55 Kd band was evident in phagosomes from fixed bacteria (fig. 7.9) Additionally, glycoproteins of an apparent molecular weight of 42, 39 and 60 Kd, (fig 7.9), appear to be major bands in bacterial phagosomes. Phagosome preparations from fixed bacteria also contain both acid and alkaline phosphatase activities, in contrast to those from polystyrene beads (Table 7.2). The presence of acid phosphatase is consistent with phagosome-lysosome fusion. It is unlikely to be due to contamination of the bacterial pellet by free lysosomes since the centrifugal forces employed were insufficient to sediment such small particles.

The data for alkaline phosphatase are more difficult to interpret. The specific activity in the final preparations is not much greater than in the crude homogenate. This could be due to contaminating cellular protein or could reflect the segregation of the enzyme from phagosomal membrane, as has been proposed for polystyrene beads. Phagosomal preparations from polystyrene beads are likely to be more pure than those from fixed bacteria, because of the centrifugal procedure used. The use of fixed bacteria for this purpose may have potential, however, if suitable density gradient methods can be developed to free them from contaminants. Although some investigation into the use of sucrose gradients was carried out in an attempt to increase the purity, these techniques could not be

fully developed within the time limitations of this work. Until this is achieved it will remain uncertain whether there are genuine differences in phagosomal proteins surrounding polystyrene beads and bacteria. The results obtained here are, however, consistent with this possibility.

The preparation of D.discoideum plasma membrane has been reported by a number of workers (Green & Newell, 1974; Jerym et al., 1977; Siu et al., 1977; Rossamando & Cutler, 1975; Jacobson, 1980; Gilkes & Weeks, 1977). An isolation of D.discoideum plasma membrane by Siu et al (1977) revealed major bands present at apparent molecular weights of 74, 55, 29 and 27.5 Kd. These may be equivalent to polypeptides of 72, 55, 29 and 28 Kd which appear as major bands in the plasma membrane profile presented in fig. 7.11. Additionally, two of the four low molecular weight bands of apparent molecular weight 17 and 14 Kd may correspond to polypeptides of apparent molecular weight 18 and 13 Kd in the preparations of Siu et al (1977). Using a Con A stabilisation technique, Luna et al. (1984) and Parish et al. (1977), isolated plasma membrane from D.discoideum. This method involves the isolation of plasma membrane associated with Con A after it has capped on the cell surface. Both actin and myosin were major constituents of the polypeptides present in these preparations in contrast to the apparent absence of actin and myosin in the plasma

membrane isolates presented here (arrow a and c in fig. 7.14). This discrepancy may be explained by the results of Condeelis (1979) who demonstrated that large amounts of these proteins are associated with Con A caps. It is therefore probable that the presence of these proteins in the plasma membrane preparations of these workers is due to the preparative technique used. However, the association of actin with isolated plasma membrane from A.castelanii has also been demonstrated, both ultrastructurally (Pollard & Korn, 1973) and by PAGE (Korn & Wright, 1973). It is possible that the absence of actin in the preparations here may be due to amphotericin B treatment and the method of isolation, although the lack of actin in plasma membrane preparations has also been reported by other workers (Jerymn et al., 1977). They reported that little or no actin is present in plasma membrane isolated from vegetative amoebae of D.discoideum but noted a sudden appearance of actin in the plasma membranes of cells allowed to develop for 2 h.

Alkaline phosphatase has been demonstrated to be a plasma membrane marker in D.discoideum (Green & Newell, 1974). These workers reported a 35 fold enrichment of this enzyme in comparison to the homogenate in plasma membrane preparations from this organism. Parish and Pelli (1974) also reported that 40-50% of the total activity of D.discoideum alkaline phosphatase is located on the external face of the plasma membrane. It

Fig. 7.14 Polypeptide profiles from plasma membrane and phagosomes from around fixed bacteria.

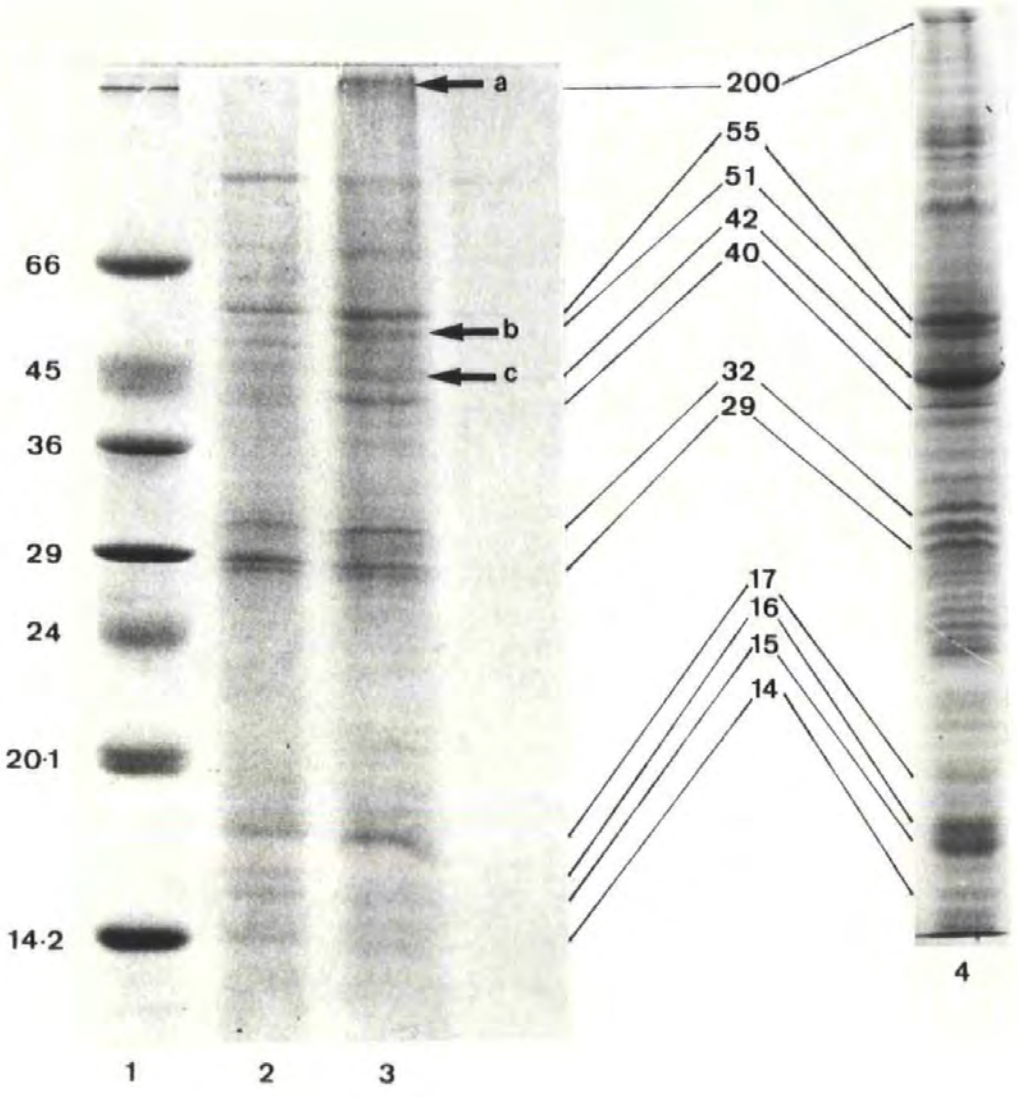
Lane: 1. Molecular weight standards

2. Plasma membrane

3. Phagosome polypeptides from around fixed E.coli

4. Whole amoebae preparation (from a different gel) Equivalent bands are marked

arrow a and c refer to the probable position of myosin and actin respectively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is clear from Table 7.3 that considerable enrichment of alkaline phosphatase has been achieved in these preparations. However, the purity of the preparation cannot be ascertained from this result alone. Acid phosphatase activity is clearly not enriched but is nevertheless detectable. This residual activity may indicate some contamination from a lysosomal origin.

A comparison of phagosome polypeptides from polystyrene beads and plasma membrane polypeptides indicate that actin and myosin are either absent or very much reduced in plasma membrane preparations (fig. 7.13). Additionally, two polypeptides of apparent molecular weight 130 and 150 Kd are absent in plasma membrane. Again, there are far more bands in plasma membrane preparations than polystyrene bead phagosomes. Furthermore, the same major bands of apparent molecular weight 14, 16, 18, 29 and 32 Kd that appear in phagosomes from fixed bacteria are also present in plasma membrane but are absent in phagosomes from polystyrene beads. In contrast differences between phagosomes from around polystyrene beads compared with plasma membrane or phagosomes from around bacteria were more marked. The surface characteristics of polystyrene beads are more hydrophobic than those of bacteria. The tight apposition of some phagosome membranes to polystyrene beads seen in fig. 7.1b, and noted by Korn and Weisman (1967) and Korn (1974) in phagosomes from A.castellanii may reflect this hydrophobic nature. If

so, then differences in surface properties may help to explain some of the differences seen in phagosome polypeptide compositions from around these particles as compared to plasma membrane or phagosomes prepared from fixed bacteria. Further study in this area may reveal if these differences really reflect changes in membrane composition during the ingestion of different particles.

A comparison of bulk plasma membrane with phagosomes from fixed bacteria reveal considerable similarity in the polypeptide profiles (fig 7.14). It would seem unlikely that contamination present in these two types of preparation would be similar, as the isolation procedures involved are very different. In addition, it is clear from fig. 7.14 that these two preparations are markedly different from whole amoebal protein preparations (fig. 7.14, lane 4). This suggests that specific enrichment of the same polypeptides has taken place during the preparation of these two membranes. This similarity is what might reasonably be expected, of course, and it may illustrate that contamination did not contribute greatly to the major polypeptide profiles in these two preparations. However, further investigations using a wider range of marker enzymes are required to determine the full extent to which these preparations are contaminated. Polypeptides of lysosomal origin are probably present in these phagosome preparations, and this is supported

by the apparent enrichment of acid phosphatase (Table 7.2). Additionally, a protein of apparent molecular weight 51 Kd appears to be present in phagosomes derived from around fixed bacteria but is much reduced in bulk plasma membrane (arrow b in fig.7.14), and is absent in phagosomes from around polystyrene beads. In conclusion, the marked similarity between plasma membrane and phagosome membrane derived from bacteria, does appear to suggest that extensive changes in membrane polypeptide composition (visualisable by SDS PAGE) does not take place with the internalisation of plasma membrane during the phagocytic uptake of bacteria.

Chapter 8

General Discussion

This chapter aims to summarise the main conclusions of the results presented in other chapters and discuss these in the broader context of other work concerning phagocytosis and adhesion.

It seems clear that the phagocytosis of E.coli by D.discoideum shows a requirement for divalent cations. The results are for the most part consistent with a cation binding site on the external face of the amoebal plasma membrane, and that this site is involved in phagocytosis. A divalent cation requirement for phagocytosis has been reported previously in both higher and lower phagocytes. Stossel (1973) found that divalent cations were required in the medium for full phagocytic activity during the ingestion of unopsonised particles by human leucocytes and macrophages. However, there was no such requirement with opsonised particles. In addition, Warr (1980) reported that the binding of non-opsonised yeast cells to lung macrophages was calcium dependent. Similarly, in lower cells such as Acanthamoebae, attachment and ingestion of glutaraldehyde fixed red blood cells is reduced in the presence of 0.5 mM EDTA (Rabinovitch & De Stefano,

1971). It is possible that lower phagocytes possess a non-specific site for the attachment of a wide variety of particles and that this site requires divalent cations for full activity. Higher phagocytes may retain such a site but in addition possess more specific sites (Fc and C3 receptors) that are not cation dependent.

In D.discoideum amoebae, a calcium binding site on the external surface of the plasma membrane has been demonstrated by Sussman and Boshwitz (1975) who claimed its involvement in cell:cell adhesion. The existence of a divalent-cation binding site raises the possibility that cation bridging is involved in the attachment phase of phagocytosis. A wide distribution of cation binding sites around the periphery of both the particle to be ingested and the phagocyte might allow a zipper like mechanism to mediate the stepwise apposition of calcium binding sites bridged by these ions. However, cation bridging would not be expected to require the expenditure of cellular energy, in contrast to the energy dependence reported for the attachment of these amoebae to almost all surfaces (Glynn & Carke, 1984). It is possible that attachment occurs in two stages, the first involving cation bridging which is then stabilised or "locked" by an energy dependent process, probably involving the cytoskeleton. The observation that attachment is both energy and cation dependent could be accomodated by such a model. This idea is consistent with the results of Garrod and Born (1971)

who reported that although cell-cell attachment in D.discoideum could occur at 1°C it could not be maintained on exposure to shear forces at this temperature. On the other hand, the attachment was stable to this treatment at a temperature of 24°C. Another important aspect of the phagocytic mechanism is that some form of trans-membrane communication must take place to signal to the cytoskeleton that attachment has occurred. The possibility that a cation binding site is involved in this is consistent with the findings of Maeda and Kawamoto (1986). These workers reported that a decrease in the occurrence of actin filament attachments to the cytoplasmic face of D.discoideum plasma membrane in the presence of 10 mM EDTA. It is possible that this may be as a consequence of removal of cations from the surface of the amoebae. At present, however, the nature of the cation requirement at the amoebal surface during phagocytosis is not clear and until additional investigations have been carried out these ideas must remain speculative.

A role for cations in other attachment processes such as the mutual adhesion of vegetative D.discoideum amoebae has also been reported (Beug et al., 1970). In addition, some undefined role for surface associated cations during the adhesion of these amoebae to glass has been reported by Gingel and Vince (1982). In the present work, EDTA caused only a slight (though consistent) inhibition of the adhesion of amoebae to

glass. The inability of EDTA to significantly reduce attachment of amoebae to other surfaces has been reported (Glynn & Clarke, 1984). It is possible, of course, that more than one attachment mechanism exists as proposed by Bozzaro and Roseman (1983) and Vogel et al. (1980). Alternatively chelating agents may only be effective when shear forces are applied during attachment of the cells. As no significant shearing forces are present during the adhesion of amoebae to glass (agitation being applied only after the cells have attached) then the effect of chelators may not be so marked. In contrast, in the phagocytosis assay, high shearing forces are present during the attachment of bacteria to amoebae. An examination of the effect of chelators on cell-glass adhesion in the presence of increasing shear forces may help to clarify whether the apparently slight effect of EDTA on this process indicates a real functional difference or is rather a reflection of the assay conditions.

The displacement of calcium from plasma membrane sites by lanthanum ions has been reported by Van Breeman et al., 1973, and the inhibition of phagocytosis by these ions is consistent with the involvement of a cation binding site in this process, as already suggested. However, lanthanum inhibition is far more severe than that seen using EDTA and in contrast to this chelator, pretreatment with low lanthanum concentrations abolishes adhesion of amoebae

to glass. The marked differences between the effect of cation chelators and lanthanum ions on adhesion could be explained by the fact that in addition to displacing surface bound calcium, lanthanum has been reported to irreversibly block the movement of calcium in and out of cultured aorta cells (Van Breeman et al., 1973) and Dictyostelium amoebae (Europe-Finner & Newell, 1985). Movement of calcium across the plasma membrane of D.discoideum amoebae has been demonstrated by Bumann et al. (1984) who reported that a transient increase in cytosolic calcium in chemoattracted amoebae was due to uptake of calcium external to the cell. Movement of calcium across the plasma membrane is also implicated by the results of De Chastellier and Ryter (1982) who reported that calcium rich electron dense deposits located predominantly in areas of microfilament formation, such as underneath phagocytic cups, may represent calcium channels that are involved in cytoskeletal activity. These same workers found that these deposits were drastically reduced if the cells were exposed to lanthanum ions. It is possible therefore, that inhibition of phagocytosis by lanthanum is due to the prevention of the normal movement of calcium ions across the plasma membrane. The possibility that this action would directly affect cytoskeletal activity might also explain the marked effect of these ions on adhesion of amoebae to glass as compared to the action of cation chelators. The

presence of a membrane located calcium pump has been suggested by Yin and Stossel (1982) who postulated a model of phagocytosis based on the local extrusion of calcium, as a prerequisite to cytoskeletal assembly during pseudopodium formation.

The modulation of calcium movement across the plasma membrane during phagocytosis may also be implicated by the inhibitory effect of A23187. The disruption of normal calcium compartmentation might be expected to disrupt the cytoskeleton. In support of this idea, Europe-Finner and Newell (1985) suggest that rapid polymerisation of actin in the cortex of D.discoideum following cAMP stimulation (McRobbie & Newell, 1984)) is triggered by liberation of calcium from large internal stores.

The disturbance of intracellular calcium-mediated functions also inhibits phagocytosis and cell adhesion. Many of the regulatory roles carried out by intracellular calcium ions are mediated by calmodulin. These roles include the regulation of cytoskeletal function, as indicated by the localisation of calmodulin in phagocytic cups (Bazari & Clarke, 1982). The inhibitory effects of trifluoperazine reported here were also observed in macrophages by Horwitz (1981).

Further investigations of the roles of both intracellular and extracellular divalent cations must undoubtedly be based on the use of radioactive isotopes such as ^{45}Ca. For example, pre-loading amoebae with

^{45}Ca would enable changes in the efflux of this ion during phagocytosis to be determined. The use of fluorescent calcium indicators such as BAPTA or Quin-2 (Tsien, 1980; Tsien et al., 1982) may also be useful for localising changes in intracellular concentrations during phagocytosis.

Phagocytosis involves local transmembrane communication between the cell surface, where particles attach, and the sub-plasmalemmal cytoskeleton. These functions are most likely performed by protein components of the plasma membrane. Many, perhaps most, of the plasma membrane proteins will be glycosylated, due to the post-translational processing to which they are subjected. This is consistent with the inhibitory effect of tunicamycin observed in the present work. A plasma membrane glycoprotein of molecular weight 126 Kd has been identified by Chadwick and Garrod (1983). These workers suggested that this molecule may account for the contact site B (CSB) activity which mediates divalent cation dependent cell-cell adhesion in these amoebae. They claimed that gp126 is directly involved the in phagocytosis of E.coli and the adhesion of amoebae to glass (Chadwick & Garrod, 1984). This claim was based on the inhibitory effects of treating amoebae with Fab fragments of anti-gp126 antibody. This hypothesis is consistent with the results presented here in several ways. The inhibition of phagocytosis by EDTA would be expected if CSB were involved in

phagocytosis and the effect of tunicamycin would also agree with this possibility . However, several lines of evidence are inconsistent with the suggestion that gp126 is the common basis for CSB activity, phagocytosis and adhesion. Firstly, both Yamada (1982) and Lam and Siu (1982) reported that treatment of D.discoideum amoebae with tunicamycin leaves CSB activity intact but that a glycoprotein of 127 Kd disappears from the plasma membrane of treated cells. Secondly, Takeuchi et al. (1983) reported that these amoebae lose the ability to phagocytose at the onset of aggregation, while McMahon and Hoffman (1979) stated that CSB activity changes little during development. Finally the inhibition of adhesion to glass reported by Chadwick and Garrod (1984) on exposure of amoebae to anti-gp126 antibody conflicts with results presented here concerning cell-glass adhesion after exposure to EDTA or tunicamycin. Both EDTA and tunicamycin are far less effective at reducing cell-glass adhesion than phagocytosis. It is possible that other molecules are involved in these two processes and that CSB activity itself may consist of components additional to gp-126. However, the involvement of glycoproteins is consistent with the results reported here and to a large extent with the results of other workers.

In this study amoebae were rendered sensitive to proteolytic treatment by prior exposure to tunicamycin. Inhibition of phagocytosis by tunicamycin may therefore

result from proteolytic degradation of surface molecules involved in this process that lack normal carbohydrate groups as a result of tunicamycin treatment. The inclusion of proteolytic inhibitors such as leupeptin during exposure of cells to tunicamycin may serve to clarify this idea. The lack of any significant inhibition of phagocytosis or adhesion by proteolytic enzymes indicates the involvement of cell surface components that are resistant to these enzymes. It has been reported (Olden et al., 1982) that the carbohydrate moieties of glycoproteins may serve a protective role in preventing the degradation of surface polypeptides. In support of this, Hirano et al. (1983) reported that the inhibition by tunicamycin of EDTA-stable adhesion in D.discoideum amoebae could be completely prevented by the inclusion of the protease inhibitor, leupeptin, during exposure of amoebae to 1 ug/ml tunicamycin. They concluded that the requirement for the carbohydrate moiety of the glycoprotein in cell adhesion is indirect in that it acts to protect the protein moiety from proteolytic degradation. Although it is possible that proteolytic degradation might be due to secreted enzymes, it seems more likely that carbohydrate groups may serve to protect membrane proteins from proteases present in phagolysosomes. This would be an essential requirement if proteins involved in phagocytosis were to be recycled to the plasma membrane. Alternatively, carbohydrate moieties may

simply serve to "tag" components that are destined to be recycled after internalisation during phagocytosis.

Results of electrophoretic analyses of phagosomes obtained from polystyrene beads and fixed E.coli suggest that there are differences in polypeptide composition between them. If confirmed, this is an important result since it indicates a flexible response to particles of different types. However, the interpretation requires caution for several reasons. Yields of protein from polystyrene beads were low, leading to faint bands on electrophoresis. This might be improved by using silver staining or, for even higher sensitivity, radioiodination of the amoeba surface and band detection by autoradiography. It would also be desirable to confirm the homogeneity of phagosomal preparations from fixed bacteria (and also plasma membrane preparations) by assaying for a wider range of marker substances. It may be that the method requires further refinements but it nevertheless shows considerable promise. The present results suggest little difference in polypeptide composition between phagososmes isolated from fixed E.coli and the "bulk" plasma membrane. It would be of interest for example, to compare the composition of phagosomes isolated using a gram positive bacterium with that from E.coli.

Appendix I

Composition of Minimal Medium

2 volumes	2% (w/v) Glucose
1 volume	x4 Minimal salts stock solution
1 volume	Distilled water

Composition of x4 Minimal Salts Stock Solution

Ammonium chloride	20 g
Ammonium nitrate	4 g
Sodium sulphate (anhydrous)	8 g
Dipotassium hydrogen orthophosphate	12 g
Potassium dihydrogen orthophosphate	4 g
Magnesium sulphate (hydrated)	0.4 g

Preparation and Autoclave conditions

The components were dissolved in water in the order listed and the solution made up to a final volume of one litre. Each salt was fully dissolved before the addition of the next. The pH of the solution was 7.2. Sterilisation was by autoclaving at 15 psi for 15 min.

Appendix II

Sucrose Density Gradients

A) The appearance of sucrose density gradients before and after centrifugation of the homogenate during the preparation of phagosomes using polystyrene beads.

Before %: Percentage sucrose concentrations
S: Sample homogenate

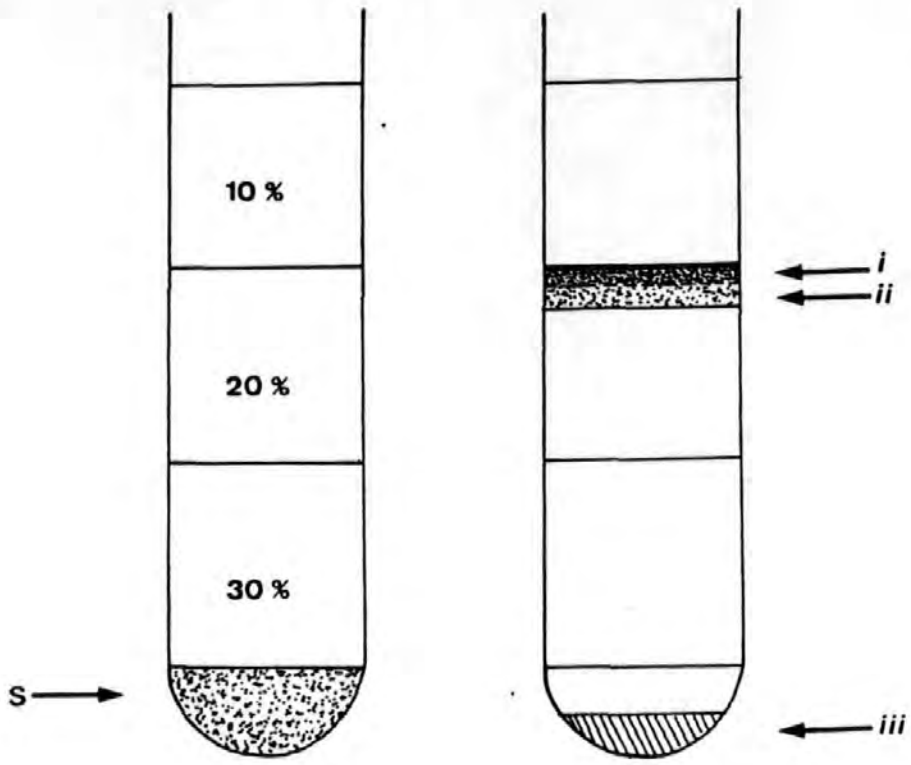
After i: Dense layer of polystyrene beads
ii: Diffuse layer of polystyrene beads
iii: Pellet

B) The appearance of sucrose density gradients before and after centrifugation of cell lysate during the preparation of plasma membrane.

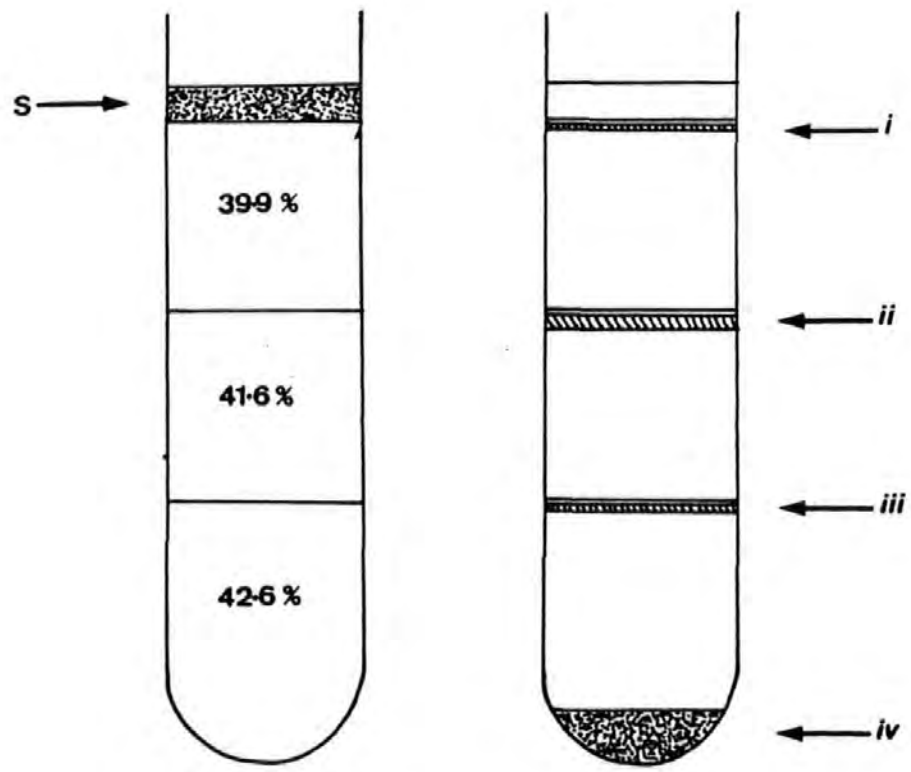
Before %: Percentage sucrose concentrations
S: Sample lysate

After i: Plasma membrane
ii: Unidentified
iii: Unidentified
iv: Pellet

A



B



Appendix III

Polyacrylamide Gel Stock solutions

Separation buffer	3.0 M Tris-HCl pH 8.9
Stacking buffer	0.5 M Tris-HCl pH 6.7
SDS	10% (w/v)
Ammonium persulphate	10% (w/v), freshly prepared
TEMED	As purchased
Acrylamide	30% (w/v) Acrylamide, 0.8% (w/v) methylene bisacrylamide

Volumes of stock solutions for the preparation of 13% and 4.5% gels

	Separation (13%)	Stacking (4.5%)
Separation buffer	5ml	---
Stacking buffer	---	5ml
Distilled water	6ml	13.2ml
SDS	200ul	200ul
Acrylamide/bis	8.7ml	1.5ml
Ammonium persulphate	100ul	100ul
TEMED	10ul	20ul

Electrode buffer

Tris	6 g
Glycine	28.8 g
SDS	2 g

In 2 l distilled water

Sample buffer

SDS	1 % (w/v)
Glycerol	10 %(v/v)
Mercaptoethanol	0.1 % (v/v)
Bromophenol blue	0.002 % (w/v)

In 0.05 M Tris-HCl pH 6.7.

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