Department of Zoology

University of Toronto

June 4, 1976

EXAMINATION

The Ph.D. examination for Mrs. Martha L. U. Gillette will take place on Monday, June 14, 1976 at 10:00 a.m., in Room 435E.

The committee in charge will be:

Dr. I. Tallan (Chairman)
Dr. R. P. Elinson
Dr. D. H. O'Day
Dr. M. F. Filosa (Supervisor)

The thesis is entitled "The role of the cell surface in aggregation of the cellular slime molds Dictyostelium discoideum and D. mucoroides: morphological and biochemical studies using Concanavalin A."

If any member of the examining committee is unable to attend, he should find a substitute and notify the Graduate Secretary (3477).

All members of the professorial staff are invited to attend.

/svf

UNIVERSITY OF TORONTO

SCHOOL OF GRADUATE STUDIES

PROGRAM OF THE FINAL ORAL EXAMINATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

OF

MARTHA LEE ULBRICK GILLETTE

2:00 p.m., Thursday, Oct. 14, 1976

Room 201, 65 St. George St.

THE ROLE OF THE CELL SURFACE IN AGGREGATION OF THE CELLULAR SLIME MOLDS DICTYOSTELIUM DISCOIDEUM AND D. MUCOROIDES: MORPHOLOGICAL AND BIOCHEMICAL STUDIES USING CONCANAVALIN A

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THE ROLE OF THE CELL SURFACE IN AGGREGATION OF THE CELLULAR SLIME MOLDS *DICTYOSTELIUM DISCOIDEUM* AND *D. MUCOROIDES*: MORPHOLOGICAL AND BIOCHEMICAL STUDIES USING CONCANAVALIN A

An abstract of a thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in the University of Toronto

Martha Lee Ulbrick Gillette, 1976

The present study was undertaken to examine the role of the cell surface in aggregation of the cellular slime molds, *Dictyostelium discoideum* and *D. mucoroides*. Aggregation was known to be mediated by chemotaxis to extracellular cyclic AMP secreted by the amoebae. As little cyclic AMP enters the amoebae, it was thought that it must act at the cell surface to direct chemotaxis. I examined the interaction of Concanavalin A (Con A), a plant lectin that binds to α-D-glucose- and α-D-mannose-like residues, as a means of probing the cell surface during aggregation.

Binding of Con A to the cell surface during development was demonstrated at both the light and electron microscopic levels. Con A receptors are redistributed in response to interaction with multivalent Con A and shed from the surface.

In order to investigate the functional nature of Con A receptors, vegetative amoebae were allowed to undergo morphogenesis on Con A agar. While fruiting body morphology remained unaffected, aggregation was delayed in both species.

The density of fruiting bodies formed in the presence of increasing concentrations of Con A was dramatically increased for D. discoideum but decreased for D. mucoroides. When the ability of amoebae to respond to exogenously supplied cyclic AMP was tested in the presence of Con A, it was found that both species showed a reduced chemotactic response.

Because membrane-bound cyclic AMP phosphodiesterase (mPDE) has been implicated in the chemotactic response of aggregating amoebae, the possibility that Con A affects aggregation by changing the activity of this enzyme through its interaction at the cell surface was examined. Within one hour of exposure of vegetative D. discoideum to Con A, mPDE is found prematurely at the surface of whole cells and in particulate membrane preparations at specific activity higher that that found in untreated cells during aggregation. mPDE activity remains high during the pre-aggregation period on Con A. The enhancement of mPDE activity appears to be a specific effect on that enzyme mediated by events at the cell surface. D. mucoroides shows neither long term nor high level mPDE stimulation.

Cyclic AMP, at high concentration, reverses the effect of Con A on inhibition of chemotaxis and mPDE activation in D. discoideum.

The importance of this work arises from the finding that the activity of a developmentally regulated enzyme can be influenced by molecular interactions at the cell surface. In the general sense, this contributes to our

knowledge of the role the cell surface can play in controlling development. In the specific case of cellular slime mold aggregation, it supports the hypothesis that events at the cell surface are important for aggregation as well as the notion that membrane phosphodiesterase (mPDE) is important in regulating aggregation. Furthermore, the results strongly suggest that the mPDE enzyme can be controlled by events at the level of the cell surface. The work points up important differences between two species of Dictyostelium which aggregate in response to cyclic AMP.

BIOGRAPHY

1945	Born, Lincoln, Nebraska, U.S.A.
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First Minor Subject:

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PUBLICATIONS

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THE ROLE OF THE CELL SURFACE IN AGGREGATION OF THE CELLULAR SLIME MOLDS *DICTYOSTELIUM DISCOIDEUM* AND *D. MUCOROIDES*: MORPHOLOGICAL AND BIOCHEMICAL STUDIES USING CONCANAVALIN A

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While writing this thesis in absentia I was particularly inspired by my husband, Dr. Rhanor Gillette, my parents, Dr. and Mrs. George Ulbrick, my children, Eben and Megan, and the developmental biology group at the University of California, Santa Cruz. Drs. Jeffrey Ram and Tony Fink advised me regarding statistical treatments and analysis of enzyme data. Thanks to Pam O'Neal for her skillful typing.

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LIST OF ABBREVIATIONS

AC = adenyl cyclase

5'-AMP = adenosine 5'-monophosphate

Con A = Concanavalin A

cyclic AMP = cyclic adenosine 3',5'-monophosphate

cyclic GMP = cyclic guanosine 3',5'-monophosphate

DAB-HP = diaminobenzidine-horseradish peroxidase treatment

ePDE = extracellular cyclic AMP phosphodiesterase

Fl-Con A = fluorescein isothiocyanate-conjugated Concanavalin A

α-MG = α-methyl-D-glucopyranoside

α-MM = α-methyl-D-mannopyranoside

mPDE = membrane-bound cyclic AMP phosphodiesterase

pmoles/min/mg = picomoles of substrate converted to product per minute of assay per milligram of protein, or one specific activity unit

INTRODUCTION

Recent studies ascribe to the cell surface a key role in controlling embryonic growth (Humphreys, 1972; Rutter et al., 1973; Rossi et al., 1975), differentiation (Weston, 1972; Rutter et al., 1973) and morphogenesis (Bennett et al., 1972; Robertson et al., 1972; Weston, 1972). Experimental evidence indicates that the cell surface can receive information from the environment in the form of diffusible molecules which interact with specific membrane-bound receptors (Rutter et al., 1973). It has been hypothesized that intercellular contacts also may be important in transmitting positional information to the cell during morphogenesis (Roth, 1973). These surface activities may generate signals which affect existing molecules or alter synthetic activities within the cell. Response of the cell to these internal changes may include cell division, changes in morphology or motility, or the cell surface itself may be modified. Surface modifications may include the appearance of new recognition or receptor molecules that may effect morphogenetic attachments or the aggregation of

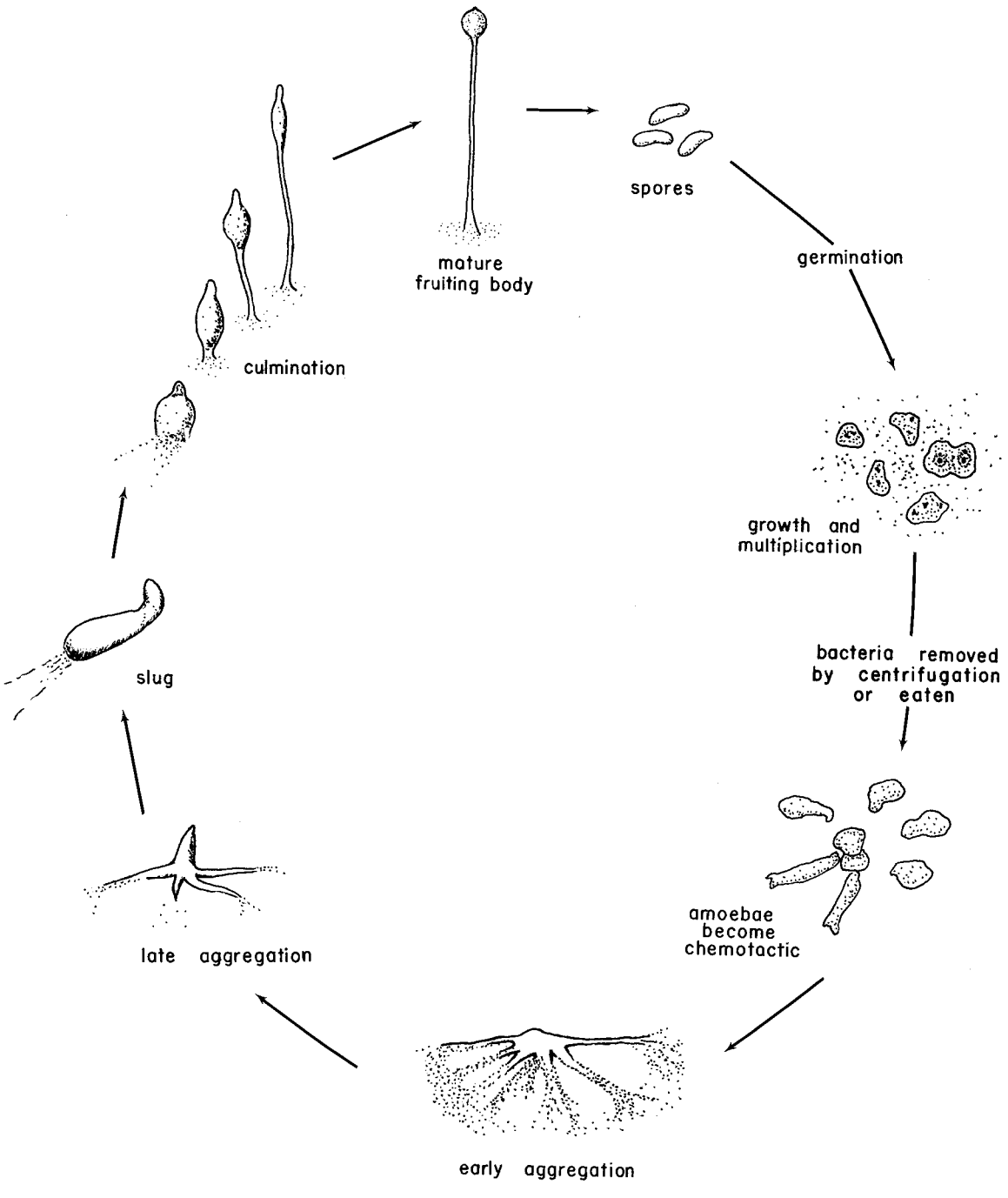
cells into tissues. Currently it is thought that such sequences of reciprocal interactions between the inside and outside of the cell guide differentiation along specific pathways (Moscona, 1974a). The consequence of molecular interactions at the cell surface is currently a subject of intensive research, and it was from this literature that the present study grew (Silvestri, 1972; Rutter et al., 1973; Moscona, 1974b).

The cellular slime molds present a developmental system useful for examining the nature of interactions at the cell surface which result in morphogenesis. This is because Dictyostelium forms a multicellular organism by aggregation of dispersed single cells. The life cycle begins with the germination of spores which have been liberated from mature fruiting bodies. A single amoeboid cell is released from each spore. Solitary amoebae feed on bacteria by phagocytosis, grow, and divide mitotically. This phase of vegetative growth comes to an end when the bacterial food supply has been exhausted or removed by centrifugation. The onset of starvation triggers the developmental phase of the life cycle. Post-vegetative amoebae enter an interphase period during which the single cells begin to

differentiate cytologically and become chemotactic. Subsequently, the solitary amoebae move together into multicellular aggregates called slugs (pseudoplasmodia, grexes). Each slug then differentiates so that anterior cells give rise to stalk cells and posterior cells to spores during the construction of a simple fruiting body (Fig. 1).

During the aggregation stage of morphogenesis solitary cells respond to an external signal with directed movements that result in the formation of a multicellular mass. Cyclic adenosine 3',5'-monophosphate (cyclic AMP) has been implicated as the intercellular signal directing chemotaxis of individual amoebae as well as in establishing the spatial relationships among groups of cells during aggregation. There is evidence that cyclic AMP acts without entering the cell (Malchow et al., 1972; Moens and Konijn, 1974) but interactions of this attractant with the cell surface and subsequent surface events are poorly understood. It is yet to be determined how this nucleotide can be modulated so as to give not only specific directional information for single cell movements, but also the spatial information required for the formation of a stellate aggregation pattern,

Figure 1. The life cycle of Dictyostelium discoideum. Not drawn to scale. The life cycle of D. mucoroides is similar except that the slug has no migratory phase and the stalk is being formed while aggregation is still underway.


mature
fruiting body
spores
germination
growth and
multiplication
bacteria removed
by centrifugation
or eaten
amoebae
become
chemotactic
early aggregation
late aggregation
slug
culmination

as well as for the regular spacing of these patterns. The present study was designed to examine the role of the cell surface in aggregation using a protein, Concanavalin A, which binds directly to the cell surface, and with the aid of this agent, to compare aggregation in Dictyostelium discoideum and D. mucoroides.

I. Chemotaxis and Aggregation

Arndt (1937) and many subsequent investigators observed that aggregating amoebae of D. discoideum move toward the aggregation center in a series of pulses which start centrally and move out. It is thought that aggregation is induced and controlled by propagation of a periodic signal through the population of post-vegetative, or interphase, amoebae (Gerisch, 1965; Cohen and Robertson, 1971). Some randomly distributed amoebae, perhaps with elevated metabolism, probably begin to emit a signal. Neighboring amoebae respond to these periodically repeated pulses by emitting a signal themselves about 15 sec after being signalled and then moving for 100 sec toward the original signal. During this time they are apparently refractory to further stimulation. The period between pulses is approximately 300 seconds. Such conditions of signalling and responsiveness generate the wave-like pattern of inward movement of amoebae observed at aggregation (Cohen and Robertson, 1971).

The earliest experimental work on aggregation showed that cellular slime mold amoebae move toward aggregation

centers by positive chemotaxis. Using a semipermeable membrane to separate aggregating cells from non-aggregating cells, Runyon (1942) observed that the latter took on the pattern of the former and aggregated directly beneath the established center. Bonner (1947) demonstrated that water flowing over an aggregation center carried downstream a factor which made non-aggregating cells aggregate; he named the factor "acrasin". The results suggested that amoebae orient to a gradient of acrasin, moving toward high concentrations. It was found that aqueous solutions of acrasin released from aggregating cells attracted sensitive amoebae only if applied at 10 sec intervals, but not 1 min intervals (Shaffer, 1953). Acrasin was shown to be degraded over the longer time period by a substance with enzymatic activity, acrasinase (Shaffer, 1956). The enzyme-substrate interaction seemed to be involved in the chemotactic response.

Work on the identity of acrasin was delayed until the development of a reliable bioassay (Konijn, 1965; Bonner et al., 1966) which allowed quantitation of the chemotactic response of slime mold amoebae to test solutions. Extracts of the food bacteria, Escherichia coli, proved strongly positive (Bonner et al., 1966; Konijn, 1969). When

chemotactic activity of various known substances was compared with that of the bacterial attractants, cyclic AMP was found to be highly active (Konijn et al., 1967). Subsequent analyses showed that E. coli secretes cyclic AMP and this is the component which attracts amoebae (Konijn et al., 1969).

In order to determine whether D. discoideum itself synthesizes cyclic AMP it was necessary to isolate acrasin which was free of acrasinase. Barkley (1969) succeeded in collecting enzyme-free acrasin, and in demonstrating that the chromatographic characteristics of D. discoideum acrasin were identical to cyclic AMP. Bonner and his colleagues (1969) showed that during the pre-aggregation period cells of D. discoideum release cyclic AMP extracellularly and that during that period the sensitivity of the amoebae to cyclic AMP increases 100 fold. This evidence that aggregating cells are sensitive to and produce cyclic AMP suggests that it is the chemotactic factor. Robertson et al. (1972) found that exogenous cyclic AMP applied in pulses initiates propagated waves of movement toward the source. Therefore cyclic AMP appears to act both as a chemotactic attractant and to relay or transmit the signal.

The acquisition of responsiveness to cyclic AMP and signalling capability is in keeping with the theoretical scheme for generating wave-like aggregation patterns. Two hours after plating washed vegetative cells they respond weakly to cyclic AMP. By 4 hr they demonstrate full chemotactic responsiveness but they are not able to relay the signal until 6 hr (Robertson et al., 1972). The ability to establish intercellular contacts and form streams appears at 8 hr, and depends on exposure of the amoebae to a gradient of cyclic AMP (Shaffer, 1957; Bonner et al., 1969).

The result of this sequence of signal propagation and acquisition of signalling capacity is that each cell will act as a local generator of the signal and will tend to move toward its nearest neighbor. As aggregation progresses,the cells move toward larger collections of cells where there are higher concentrations of cyclic AMP: first toward aggregation streams and then along the streams toward the original source of the signal, viz., the aggregation center.

II. Molecular Basis of Cyclic AMP Action during Aggregation

In order to understand how cyclic AMP can act as an attractant and transmitter which produces the pattern of cell movement observed during aggregation, it is necessary to examine the modulation of cyclic AMP levels as well as its molecular interactions with the cell. The amount of cyclic AMP present is regulated by the activities of the enzymes which control its synthesis and breakdown, adenyl cyclase and phosphodiesterase, respectively. As little extracellular cyclic AMP enters the cell during aggregation (Malchow et al., 1972; Moens and Konijn, 1974), the main site of action of extracellular cyclic AMP must be at the cell surface. Two components of the surface of the aggregating cell, membrane-bound phosphodiesterase and a distinct cyclic AMP-binding site, have been implicated as mediating the action of cyclic AMP in chemotaxis (Malchow et al., 1972; Malchow and Gerisch, 1974; Malchow et al., 1975).

A. Adenyl Cyclase Regulation

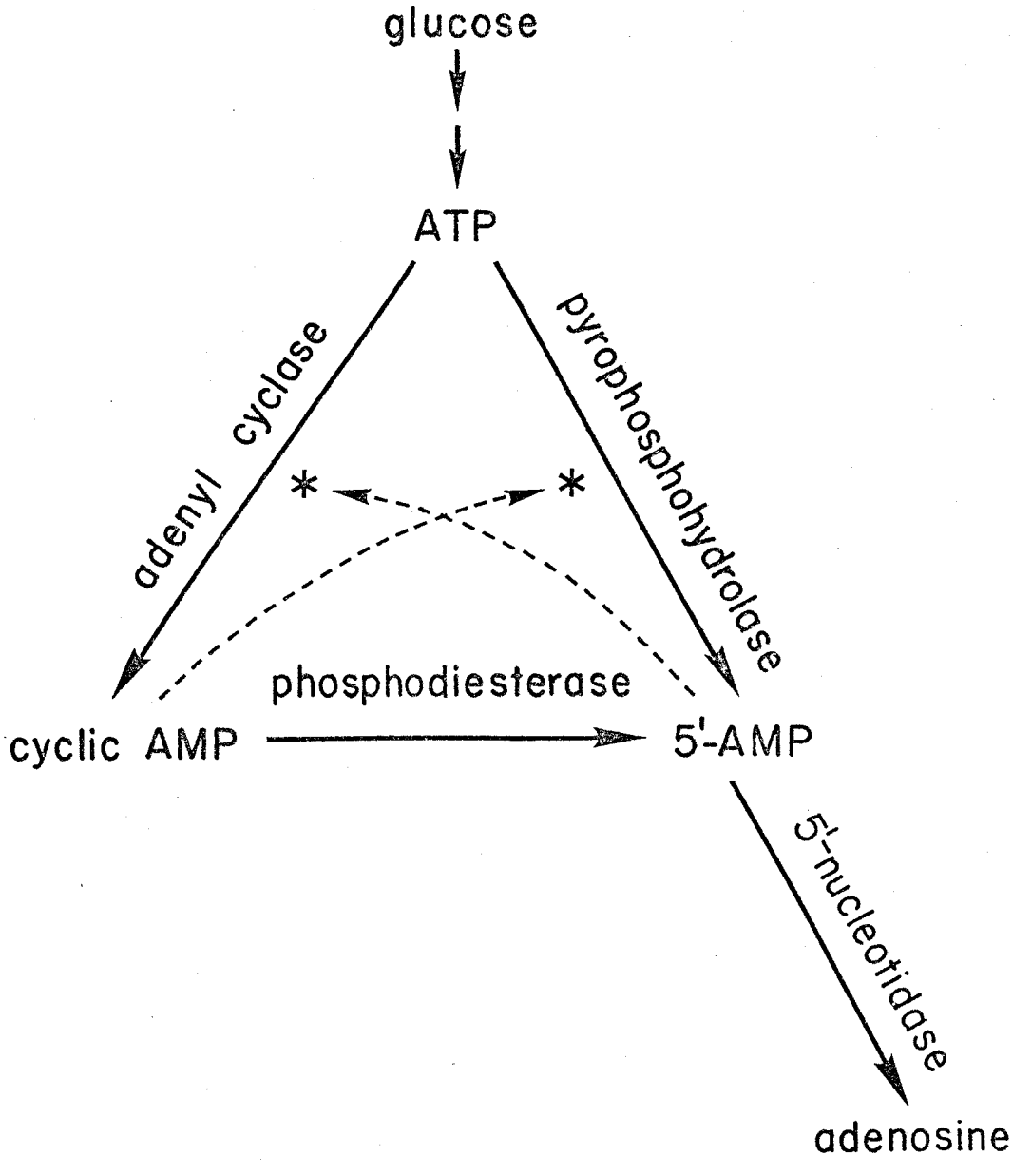
Adenyl cyclase (AC), the enzyme which synthesizes cyclic AMP, does not vary in specific activity from growth through the various stages of development, nor is there significant turnover of AC molecules (Rossomondo and Sussman, 1972). Thus, if AC plays a significant role in regulating cyclic AMP levels it must do so through modulation of its own activity. Early in development its activity may be regulated by compartmentalization of the enzyme so that its substrate is not readily available. AC requires 5'-adenosine monophosphate (5'-AMP) for its activation (Rossomondo and Sussman, 1973). This may explain its action during chemotaxis because breakdown of cyclic AMP produces 5'-AMP. Further, this enzyme shows unusual kinetics: the rate of activity drops off after 20 min incubation under assay conditions. This is not due to substrate or activator depletion nor to thermal inactivation of the enzyme (Rossomondo and Sussman, 1973). If this inhibition is transient, it may also be effective in controlling cyclic AMP production during aggregation.

The activity of AC may be linked to that of another enzyme, ATP pyrophosphohydrolase (Rossomondo and Sussman,

1973). This latter enzyme shows a requirement for cyclic AMP for its activation. One of the reaction products of ATP pyrophosphohydrolase is 5'-AMP, which stimulates AC activity. Therefore these two enzymes may constitute a self-reinforcing regulatory loop during aggregation. Such a loop would have a two fold effect in insuring that cells exposed to cyclic AMP produce cyclic AMP and that cells in a given area are entrained to a cyclic pattern of cyclic AMP production (Fig. 2). This type of enzymatic interaction may be the basis for signal transmission during chemotaxis.

Recently AC has been localized histochemically to the inner surface of the plasma membrane of aggregating D. discoideum amoebae (Farnham, 1975). This means cyclic AMP must be transported out of the cell to be effective during aggregation and agrees with the observation that an increase in intracellular cyclic AMP precedes the extracellular accumulation which peaks during aggregation (Malkinson and Ashworth, 1973). The mechanism of transport and release of cyclic AMP and their effect on signal generation are unknown.

Figure 2. Enzyme interactions involved in cyclic AMP metabolism. This demonstrates how adenyl cyclase and ATP pyrophosphohydrolase interact so that the product of each stimulates the other in a self-reinforcing pattern. (Derived from Rossomondo and Sussman, 1973.)

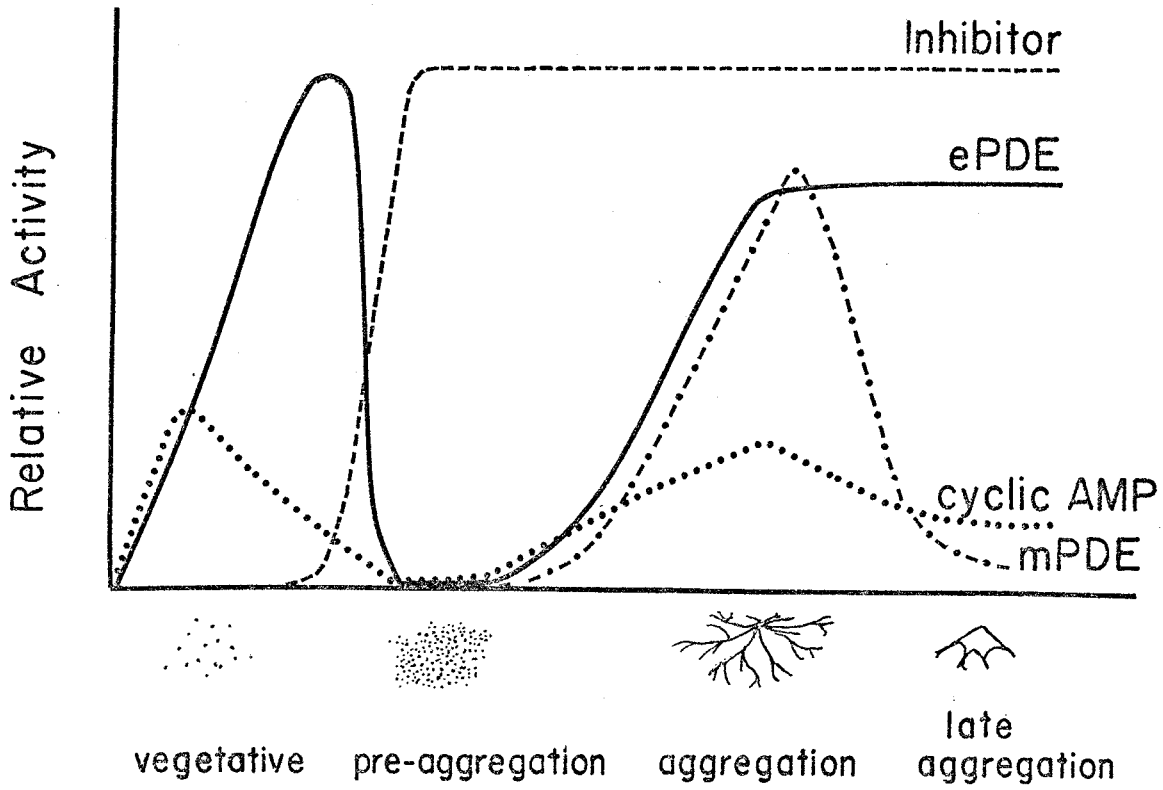

glucose
ATP
adenyl cyclase
pyrophosphohydrolase
*
*
phosphodiesterase
cyclic AMP
5'-AMP
5'-nucleotidase
adenosine

B. Phosphodiesterase Regulation

Cyclic AMP phosphodiesterase (PDE), which catabolizes cyclic AMP, varies in specific activity as well as spatial distribution in the period preceding and during aggregation. The life time of extracellular cyclic AMP is controlled both by soluble extracellular phosphodiesterase (ePDE) (Chang, 1968; Chassy, 1972; Pannbacker and Bravard, 1972) and by membrane-bound phosphodiesterase (mPDE) exposed at the cell surface (Pannbacker and Bravard, 1972; Malchow et al., 1972).

Extracellular PDE is secreted by the amoebae during the growth phase and accumulates in the medium (Chang, 1968). Thus although vegetative amoebae produce some cyclic AMP (Malkinson and Ashworth, 1973), it does not accumulate extracellularly due to hydrolysis by ePDE. As the amoebae approach the end of the growth phase, an extracellular inhibitor specific for the secreted form of PDE is produced in liquid culture (Riedel et al., 1973); this ePDE inhibitor has not yet been demonstrated in cultures incubated on agar (Bonner et al., 1972). It appears therefore that ePDE activity reaches a maximum at the end of cell growth, then falls off dramatically (Fig. 3).

Figure 3. Relative amounts of cyclic AMP and activities of the elements which regulate cyclic AMP levels from the vegetative phase through aggregation. (Derived from Malchow et al., 1972; Malkinson and Ashworth, 1972; and Riedel et al., 1973.)


Relative Activity
Inhibitor
ePDE
cyclic AMP
mPDE
vegetative
pre-aggregation
aggregation
late aggregation

Formation of the extracellular inhibitor is restricted to those species of cellular slime mold which respond chemotactically to cyclic AMP (Gerisch et al., 1972). Gerisch hypothesizes that the effective inactivation of ePDE by inhibitor during the transition period between feeding and aggregation must coincide with processes requiring elevated extracellular levels of cyclic AMP. As the cells approach aggregation active ePDE appears to accumulate again (Malkinson and Ashworth, 1973) suggesting that ePDE has some function in modulating cyclic AMP levels at that time.

Malchow et al. (1972) found that the activity of a membrane-localized phosphodiesterase (mPDE) which breaks down cyclic AMP is closely linked to aggregation. Activity of this enzyme is low at all times in development, except during aggregation when it is at its highest (Fig. 3). A series of aggregationless mutants were found to have reduced mPDE activities (Malchow et al., 1972) but no apparent defect in regulation of ePDE (Riedel et al., 1973). This finding coupled with the fact that highest mPDE levels normally occur at aggregation point to an important role for this enzyme during aggregation. Its location at the

cell surface suggests that it may participate in chemotaxis. This raises a question as to whether mPDE serves as a receptor of cyclic AMP, directing movements of the cell by its hydrolytic activity alone, or whether the enzyme is linked to a non-catalytic receptor with which it cooperates to effect chemotaxis.

In order to answer this question the specificity of cyclic AMP analogues for mPDE was compared with the chemotactic activity of the analogues (Malchow et al., 1973). The results show that cyclic AMP and cyclic 3',5'-guanosine monophosphate (cyclic GMP) are hydrolyzed by this enzyme at the same rate but cyclic GMP is 1000 times less active chemotactically than cyclic AMP (Konijn, 1972). Therefore effective hydrolysis by mPDE does not correlate completely with the level of chemotactic action. This suggests that receptor sites more specific than the mPDE catalytic site may be involved in chemotaxis. However, hydrolysis is necessary for chemotactic action (Malchow et al., 1972), which is in accord with the idea that mPDE is a necessary part of the chemotactic system.

C. Cyclic AMP-Binding Sites

Recently Malchow and Gerisch (1973; 1974) found that D. discoideum can bind [^{3}H]cyclic AMP for a brief period in the presence of excess cyclic GMP which shows a high affinity for mPDE. Binding is specific to cyclic AMP as [^{3}H]cyclic GMP and [^{3}H]5'-AMP are not bound. The bound cyclic AMP is released undegraded into the medium. The results demonstrate that aggregating cells have a surface binding site for cyclic AMP which is distinct from the mPDE catalytic site. The authors do not have good evidence that the ability of various analogues to inhibit cyclic AMP correlates exactly with their known chemotactic activity (Konijn, 1972; 1973), however, the interplay between a cyclic AMP-binding site and mPDE which is essential for chemotaxis may be too complex to be analyzed directly in this way.

The cyclic AMP-binding ability of cells is related to their developmental phase (Malchow and Gerisch, 1974). Growth phase cells bind no significant amount of cyclic AMP. Cyclic AMP binding increases as the cells approach aggregation. The time course of binding parallels closely,

and may precede slightly (by 1 - 2 hr) the increase in mPDE activity. In a non-aggregating mutant, cyclic AMP-binding sites and mPDE activity both remain low. Activity of the inhibitor of ePDE also remains low and contact sites normally responsible for end-to-end cell adhesions at the time of aggregation do not appear (Malchow and Gerisch, 1974). All four of these components of aggregation reappear in a spontaneous revertant suggesting that they are under common genetic control. Interestingly, all four are developmentally regulated factors that are either incorporated into the plasma membrane or secreted into the medium in association with the aggregation phase.

III. Spatial Patterns of Aggregation

While aggregation of the amoebae from a limited area may be understood in terms of movement toward one aggregation center, the aggregation of a population of cells takes on another dimension. Aggregation over the larger area involves the establishment of many territories, i.e., discrete areas each of which is influenced by one aggregation center. Aggregation territories have a non-random spacing which suggests that the presence of one center in a given area inhibits establishment of another center in that area. Under a given set of culture conditions territory size is constant over a range of cells densities (Bonner and Dodd, 1962), so it is spatial relationships rather than numbers of cells which determines the extent of the territory. There is evidence that elements of the chemotactic system have a role in establishing and maintaining each center within a given territory.

Bonner and his coworkers (1969) found that the density of centers may be artificially manipulated by altering cyclic AMP levels in the agar. D. discoideum amoebae consistently aggregate over reservoirs of cyclic AMP. The

observation that high cyclic AMP levels stimulate center formation was confirmed by plating cells on different concentrations of cyclic AMP agar. Up to a certain limit, increasing levels of cyclic AMP produce an increase in the number of centers, which results in many small fruiting bodies each with a minute surrounding territory. These results demonstrate that cyclic AMP has a role in establishing the aggregation center and its territory.

It has been hypothesized that the gradient of cyclic AMP around the center inhibits secondary center formation (Francis, 1965; Bonner et al., 1969). By extension, therefore, ePDE, which appears to have a role in maintaining the gradient (Bonner et al., 1969), may also be instrumental in maintaining the territory. In a survey of mutants defective in ePDE regulation, Riedel et al. (1973) found two classes of mutants with altered levels of growth phase ePDE production. One with excessive ePDE production failed to aggregate in streams and morphogenesis resulted in small fruiting bodies indicating a reduction of territory size. In another group of aggregation mutants ePDE activity was almost absent. These mutants produce extremely large aggregation territories with marked aggregation streams.

In both cases production of the extracellular PDE inhibitor was normal but other elements of cyclic AMP metabolism (AC and mPDE) were not examined. These results suggest that ePDE has a role in aggregation, and possibly in determining the size of aggregation territories.

IV. A Comparison of Aggregation in *Dictyostelium discoideum* and *D. mucoroides*

Both *D. discoideum* and *D. mucoroides* secrete and respond chemotactically to cyclic AMP during aggregation (Bonner *et al.*, 1972). *D. mucoroides*, however, produces about three times more cyclic AMP during the first six hours of development than *D. discoideum*. Normally *D. mucoroides* aggregates several hours before *D. discoideum*. As Bonner's study compared cyclic AMP production only during the first six hours of development, it does not reveal whether *D. discoideum* actually aggregates at a lower level of cyclic AMP than *D. mucoroides* or whether an equivalent amount of cyclic AMP accumulates by the time this species aggregates. It is possible that the rate or timing of cyclic AMP accumulation in *D. discoideum* differs from that in *D. mucoroides* and is responsible for this difference in the amounts of cyclic AMP assayed. A low rate of cyclic AMP accumulation may be related to the later time of aggregation in *D. discoideum*.

When pre-aggregation cells are plated on agar containing reservoirs of cyclic AMP, both species aggregate

over the sites where cyclic AMP is concentrated. However D. mucoroides collects over the reservoirs long before aggregation, indicating it has a high sensitivity to cyclic AMP earlier than D. discoideum (Bonner et al., 1969). This early sensitivity to cyclic AMP by D. mucoroides is due to an increase in cyclic AMP binding shortly after the cells are plated on plain agar; in D. discoideum it takes several hours before an increase in cyclic AMP binding is observed (Mato and Konijn, 1975).

Early production and sensitivity to cyclic AMP may explain Shaffer's observation (1962) that amoebae of D. mucoroides, unlike those of D. discoideum, gather in a locally dense collection of cells well before aggregation begins. At the time of aggregation, however, sensitivity of both species to cyclic AMP is high (Bonner et al., 1972). Chemotactic sensitivity of aggregating D. discoideum exceeds that of D. mucoroides, in apparent correlation with the higher density of cyclic AMP receptors in that species (Mato and Konijn, 1975).

Both D. discoideum and D. mucoroides secrete ePDE during growth which is inactivated by its specific inhibitor early in stationary phase. The inhibitors and ePDEs are

completely cross-reactive for both species (Gerisch et al., 1972). The levels of ePDE activity during development are more variable among strains than between species so there is no indication as to how relative levels might effect development. Membrane PDE has only been reported for D. discoideum, so its role in the development of each species has not been compared.

Patterns of aggregation are similar for D. discoideum and D. mucoroides. Initially amoebae move separately, directly to a center of a few cells in the case of D. discoideum or to the slightly larger initial collections of amoebae in the case of D. mucoroides. Very soon, however, the amoebae flow together in streams. Movement of amoebae in both species is characterized by pulses, with a wave-like pattern of inward movement over the territory of cells (Arndt, 1937; Bonner, 1944).

Although the territories set up by aggregating amoebae of both species are relatively constant over a great range of cell densities, the average territory size of each species is distinct. Bonner and Dodd (1962) found territories of D. discoideum to have a mean radius of 1.27 mm while those of D. mucoroides averaged a radius

of 0.53 mm. Assuming the territory is circular, this means the average territory of D. discoideum is more than three times larger than that of D. mucoroides. It is not known which element(s) of the aggregation machinery are responsible for this difference in the area over which cells are drawn to aggregation centers. It would seem that a careful comparison of the two species might elucidate the role that cyclic AMP and its regulation play in establishing spatial patterns during aggregation.

V. Concanavalin A as an Experimental Tool

Concanvalin A (Con A) is one of a class of proteins of plant origin called lectins. Lectins have as a general property the ability to bind specific simple sugars whether free or part of glycoproteins or glycolipids on the surfaces of cells. Given this property, lectins have gained wide use as molecular probes of sugar-containing macromolecules on the surface of mammalian adult and neoplastic cells (for review see Lis and Sharon, 1973).

A. Chemical and Physical Properties of Concanavalin A

Con A, a protein derived from the jack bean, is the most extensively studied of the lectins (Sharon and Lis, 1973). It binds specifically and reversibly with α-D-mannopyranoside, α-D-glucopyranoside, D-fructofuranoside, and sterically related structures, but not with other sugars (Agrawal and Goldstein, 1967; Poretz and Goldstein, 1970). Con A interacts with these sugars whether free or as α-linked non-reducing residues terminally exposed on branched polysaccharides (Goldstein et al., 1965). It will combine with configurationally appropriate sugar

residues of glycoproteins or glycolipids on the surface of cells and can be competed off by adding an excess of an haptenic sugar to the medium.

Con A has specific binding sites for transition metal ions and calcium ions in addition to each saccharide binding site (Yariv et al., 1968). Binding of each ion is necessary before saccharide binding can occur. Each set of sites is associated with a protein subunit (MW 25,000, Wang et al., 1971). At pH values between 5.6 and 7.0 the protein subunits associate into tetramers each of which has four saccharide binding sites (Kalb and Lustig, 1968). Under conditions where it is multivalent Con A can cross-link macromolecules containing the appropriate sugars.

B. Biological Properties of Concanavalin A

1. Binding to Cell Surfaces

Because of its specificity of binding, Con A is used to assay for the presence and arrangement of molecules containing its specific sugars on the cell surface. In this way Con A has been used to demonstrate surface differences between various cell types. Embryonic and

transformed, neoplastic cells have clustered arrangements of Con A receptors and are agglutinated by Con A (Moscona, 1971; Kapeller and Doljanski, 1972; Weiser, 1972; Shoham and Sachs, 1972; Rosenblith et al., 1973). Normal adult cells are not agglutinated by Con A and their Con A receptors are randomly distributed on the cell surface. These cells become agglutinable and their Con A receptors clustered upon proteolysis (Nicholson, 1972; Rosenblith et al., 1973). Recent studies have shown a correlation between Con A agglutinability, the presence of microvilli on the cell surface, and low cyclic AMP levels within the cell (Willingham and Pastan, 1975). These studies demonstrate that Con A can be used to study both the composition and topography of the cell surface.

Because changes in the distribution of Con A receptors can be induced by Con A, Con A has also been used to probe the mobility of carbohydrate-containing surface receptors on the membrane (Ambercrombie et al., 1972; Karnovsky et al., 1972; Unanue et al., 1972; Inbar et al., 1973a; de Petris and Raff, 1973; Loor, 1974). With appropriate receptor mobility, binding of multivalent Con A results first in clustering of the receptors which

it has cross-linked and then concentration of the Con A-receptor complexes at one pole of the cell in the form of a cap. This redistribution is not a common property of all membrane-bound receptors (de Petris and Raff, 1973; Loor, 1974); the potential of particular receptors for being aggregated and then capped by specific multivalent ligands depends on the mobility of that receptor in the membrane. Rearrangement of particular receptors by lectins has been associated with particular states of differentiation of the cell (Inbar et al., 1973a; Rosenblith et al., 1973) and appears to trigger metabolic changes within receptive cells (Novogrodsky and Katchalski, 1971; Toyoshima and Osawa, 1975).

2. Metabolic Effects Mediated by Binding

Con A is among the lectins which stimulate lymphocytes to undergo blast cell transformation and divide (Novogrodsky and Katchalski, 1971). Mitogenesis is accompanied by extensive morphological and biochemical changes. Among the earliest metabolic alterations are changes in lipid metabolism and turnover (Kay, 1968; Fisher and Muller, 1971), carbohydrate metabolism (Roos

and Loos, 1970), cyclic nucleotide levels (Hadden et al., 1972), and transport of ions and small molecules (Quastel and Kaplan, 1970; Peters and Hausen, 1971). Somewhat later there appear changes in RNA, protein, and eventually DNA synthesis (Cooper, 1971). Although the mechanism by which Con A exerts its mitogenic effect is obscure, it appears to act at the cell surface by rearranging specific receptors (Greaves and Bauminger, 1972; Greaves et al., 1972; Edelman et al., 1973).

Some lectins, including Con A, have marked insulin-like effects on isolated fat cell (Cuatracasas and Tell, 1973). Very low concentrations of Con A are as effective as insulin in enhancing the rate of glucose transport and in inhibiting epinephrin-stimulated lipolysis and adenyl cyclase activity in fat cells. Further, Con A can interact directly with the insulin receptor so as to competitively displace insulin. These effects are reversed by α-methyl mannopyranoside demonstrating that the effect is due to Con A interacting with its specific sugar. Because insulin is thought to exert its effect on the metabolism of these cells solely through surface interactions (Cuatracasas, 1969), it is likely that Con A

effects this system in a similar manner (Cuatracasas, 1974).

These studies demonstrate that lectins are particularly useful in examining the nature of the surface events that can mediate biochemical changes within the cell.

VI. Objectives of this Study

At the time the present study was undertaken I hypothesized that the cell surface might be important for aggregation in Dictyostelium. This seemed a reasonable assumption if during chemotaxis extracellular cyclic AMP is to guide the movement of the amoebae toward a signalling source. At the same time plant lectins, especially Concanavalin A, were becoming widely used to study changes in the surface of mammalian cells and the role of the cell surface in controlling metabolic events within the cell. It seemed logical to examine the interaction of Con A with the cellular slime mold as a means of probing the role of the cell surface during aggregation.

The present study was designed, therefore, first to examine the slime mold cell surface at all stages in development for the presence and distribution of Con A receptors as well as the potential for the redistribution of these receptors. Next, the effect of Con A on development was ascertained. When Con A was found to affect aggregation, its action on the chemotactic response of amoebae to cyclic AMP and on the phosphodiesterase located

at the cell surface were tested. Subsequent experiments were designed to elucidate the mechanism of the Con A effect. A comparison was made of the effect of Con A on D. mucoroides with that on D. discoideum, the most thoroughly studied species of slime mold. It was hoped that by correlating known differences in the aggregation machinery of the related species with differences in the Con A response more could be learned about the role of the basic chemotactic elements in aggregation.

MATERIALS AND METHODS

I. Localization of Concanavalin A on the Cell Surface

A. Fluorescent Concanavalin A Method for Light Microscopy

1. Preparation of Fluorescent Concanavalin A

Concanavalin A (Con A; Sigma, Grade IV, salt-free, Lot 52C5510) was conjugated with fluorescein isothiocyanate (Sigma) according to the method of Tkacz et al. (1971). Conjugate was separated from unreacted material at 4°C on a column (18.6 X 1 cm) of Sephadex-G75 (Pharmacia). Active fluorescein isothiocyanate-conjugated Con A (Fl-Con A) and any remaining unconjugated Con A adhere to Sephadex. Unbound material was eluted with 1.0 M NaCl; bound materials were eluted with 0.1 M glucose in 1.0 M NaCl. The colored fractions were collected and dialyzed against 1.0 M NaCl to remove glucose. Optical absorbance of the center of the Fl-Con A peak showed a 280 nm: 493 nm ratio of 1.655. The amount of protein

present was estimated by the optical method of Warburg and Christian (1941). This Fl-Con A preparation was active when tested on murine erythrocytes; staining at 100 µg Fl-Con A/ml was inhibited by 5 X 10^{-2} M α-methyl-D-glucopyranoside (α-MG; Sigma). Aliquots of the Fl-Con A were stored at -20°C and diluted with slime mold saline (Bonner, 1967) or Sörensen's phosphate buffer (0.0167 M), pH 6.0, for use as a staining reagent.

2. Staining of Living Cells

Vegetative amoebae in the late logarithmic phase of growth were collected from liquid cultures (see III. A.) and washed free of bacteria with saline. These washed vegetative amoebae were either treated with Fl-Con A according to the method used for fluorescent antibody (Beug et al., 1973) or plated on 1% purified agar at 1 X 10^4 cells/mm^2. Pre-aggregation amoebae were scraped off the agar and dispersed in a drop of saline or Sörensen's phosphate within a vaseline ring on a microscope slide. One drop of Fl-Con A at 200 µg/ml was added making the final staining concentration about 100 µg/ml. A coverslip was placed on the wet mount.

In order to study multicellular stages of aggregation, microscope slides were covered with a thin layer of 1% Difco purified agar. Washed vegetative amoebae were spread on these and incubated in a humid chamber. Cells were stained in situ with Fl-Con A at 100 μg/ml and examined as wet mounts. Later stages of development were transferred from the culture dish to a slide using an eyelash wand, stain was applied, and the preparation was examined as a wet mount.

In some experiments Fl-Con A was preincubated 15 min with α-methyl-D-glucopyranoside (α-MG) or α-methyl-D-mannopyranoside (α-MM) (both from Sigma). The mixture was then applied to the cells making the final concentration 100 μg/ml Fl-Con A, 5×10^{-2} M α-MG or α-MM.

Fluorescence under ultra-violet illumination was examined using a Zeiss Universal microscope equipped with exciter filters BG 38/2.5 and BG 12/4 and barrier filter 50. Tri-X film (Kodak) was used for all photography.

B. Diaminobenzidine-Horseradish Peroxidase Method for Electron Microscopic Localization of Concanavalin A

Amoebae were grown and plated as those used for localizing Con A under the light microscope. After 1 hr on plain or Con A (300 µg/ml) agar, cells were collected and washed twice in Sörensen's buffer at 4°C. They were fixed for 30 min at room temperature in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. At this point some of the cells from plain agar were incubated in 300 µg Con A/ml buffer for 15 min. The cells were then treated with 3-3' diaminobenzidine-horseradish peroxidase reagent (DAB-HP; Barat and Avrameas, 1973) for 15 min to localize Con A. A control was processed in diaminobenzidine (DAB) alone to check for endogenous peroxidase activity. After a 1 hr post-fixation in 1% osmium tetraoxide in phosphate buffer, pH 7.4, the material was embedded in 1:1 Epon: Araldite. Thin sections were cut on a Reichert ultramicrotome by M. Idler and examined on a Zeiss EM-95 electron microscope with the help of M. Idler.

C. Agglutination Titre

The agglutination titre of the slime mold amoebae was performed according to the method used by Burger and Goldberg (1967) for vertebrate cells. To 0.1 ml cells (1×10^7 cells/ml) in 10 X 75 mm test tubes was added 0.1 ml Con A at various concentrations in saline. Agglutination was determined within 5 min of mixing cells with agglutinin by observing the cells microscopically and assessing the degree of clumping on a qualitative serological scale, 0 to +++++.

II. Morphogenetic Studies

A. Methods of Culture

The cells used in morphogenetic studies were grown on nutrient agar by incubating spores with Escherichia coli at 22°C in the dark. Before bacteria had been completely consumed, vegetative amoebae were collected in cold slime mold saline and washed with saline 3 - 5 times by differential centrifugation (500 X g, 4°C) to remove uneaten bacteria.

B. Preparation of Concanavalin A Agar Plates

Hydrophobic agar (1%) was prepared following the method of Konijn and Raper (1961) by dissolving Difco purified agar (Lot 510285) in slime mold saline; pH after boiling was 5.8. Then 3.6 ml of the molten sugar was pipetted into individual test tubes. α-MG had been added to the appropriate tubes. The unstoppered tubes were heated in a boiling water bath for 15 min, then transferred to a 50°C bath for 15 min. After this period tubes were removed singly, preweighed Con A was added, and the contents were mixed on a vortex generator. The Con A agar was then poured into sterile 35 mm Petri dishes (Falcon Plastics)

and allowed to solidify. The plates were stored inverted at room temperature, and used 13 - 15 hr after they were poured.

It is important to prepare the agar in this way so the liquid from the droplet of cells will be absorbed rapidly by the agar thereby producing a physical barrier between the deposited cells and the agar peripheral to them. The cells are thus restrained from leaving the drop unless strongly attracted by an external source (Konijn, 1970). This procedure allows the cells to move within a prescribed area so the cell density fixed at the time cells are deposited is maintained.

C. Preparation of Small Populations of Cells

Washed vegetative cells were counted with a Levy haemocytometer and the cell density was adjusted with saline to produce the appropriate test densities. Then 2 µl droplets of cell suspension were applied to the hydrophobic agar with a 10 µl Hamilton microsyringe from which the point had been filed. One drop of each of six cell densities was applied per dish. The drops were 5 mm apart and had a diameter of 2.6 - 2.8 mm. Each experimental class was

prepared in duplicate or triplicate. The inoculum was absorbed within 10 - 15 min, then the plates were incubated inverted at 22°C in the light.

Plates were removed periodically and examined under the microscope. Because the characteristic aggregation pattern was absent on Con A plates, the first sign of stalk formation was selected as a marker for positive aggregative response. Each experiment was repeated at least four times.

D. The Konijn Test for Response to Cyclic AMP

Responsiveness of D. discoideum to cyclic adenosine 3',5'-monophosphate (cyclic AMP) in the presence of Con A was tested according to Konijn (1970). Vegetative amoebae were prepared from nutrient agar cultures as described above (Section II. A.). Washed cells were deposited on hydrophobic agar in 0.1 µl droplets. Within the absorbed drop there were 3.7×10^3 cells/mm^2. About 25 droplets 4 - 5 mm apart were applied to each plate. Amoebae were incubated 2 hr at 22°C in the dark, stored at 4°C overnight and tested the next day. Incubation at 16°C about 45 min before exposure to room temperature prevented rounding of the cells (Konijn, 1970). Cyclic AMP (Sigma) in saline was applied with a

Hamilton microsyringe in a 0.1 µl drop 0.1 - 0.2 mm from the edge of each droplet of sensitized amoebae. Populations of cells, incubated at 22°C in the light, were examined microscopically at 30 min intervals. Movement of amoebae outside the perimeter of the drop was considered a response to cyclic AMP (Konijn, 1970). The rate of response was scored for cells incubated on various concentrations of Con A in the agar: 0, 50, 100, 200, 300, 400 µg Con A/ml; 200 µg Con A/ml + 2.5 X 10^{-2} M α-MG. Cyclic AMP at 10^{-3} M and 10^{-6} M was tested in three to five separate experiments.

The procedure for testing D. mucoroides was identical to that used for D. discoideum with the exception that cells were incubated only 10 min at 22°C before being placed at 4°C overnight, to compensate for the fact that D. mucoroides normally aggregates several hours before D. discoideum. Responsiveness to 10^{-3} M cyclic AMP was examined in three experiments.

E. Motility Studies

To examine the possibility that Con A might affect the ability of the amoebae to move, the rate of movement of individual amoebae incubated in the presence or absence of

Con A was determined. In order to achieve this it was necessary to plate the amoebae at densities low enough so that individual amoebae could be traced. This meant adjusting the Con A concentration to a level which delays aggregation but permits development to procede.

Washed vegetative amoebae were applied in 10^{-1} µl droplets to 1% purified agar in 36 mm Petri dishes or to agar to which 50 µg Con A/ml had been added. Amoebae concentration was adjusted so the final density was 1×10^3 cells/mm^2. In one experiment a density of 2.5×10^3 cells/mm^2 was studied. Cultures were incubated at room temperature in the light. Periodically, experimental and control plates were each placed on the stage of an Olympus microscope equipped with a camera lucida, and left undisturbed. The movement of individual amoebae was mapped at frequent intervals over a 30 min period.

III. Biochemical Studies

A. Methods of Culture

Amoebae used in biochemical studies were cultivated either on plates (II. A.) or in shaking liquid culture using Hohl and Raper's modification (1963) of Gerisch's original method (1959). The E. coli used in these cultures were grown at 20°C in 1% Difco nutrient broth for 17 - 20 hr in a gyrotary water bath shaker (New Brunswick Scientific) set at speed 7 (280 rev/min). The bacteria were washed twice by centrifugation (8000 X g, 10 min) with 0.0167 M Sörensen's buffer, pH 6.0, resuspended so that the optical density at 450 nm was 6.0, and shaken at 20°C at speed 5 (200 rev/min) for 24 hr. At the end of this period the suspension was adjusted so that the optical density at 450 nm equaled 5.2, the equivalent of 10^{10} bacteria/ml; the suspension was subdivided into the aliquots to be used for each experiment. These were centrifuged at 8000 X g for 10 min, the supernatant was decanted, and the bacteria were stored as a frozen pellet at -70°C.

To start cultures, the bacterial pellet was thawed and suspended in 40 ml Sörensen's buffer. Spores from

slime mold cultures not more than a week old were added to give a final concentration of 1 X 10^4 spores/ml. These cultures were incubated in the light at 22.5°C on a gyrotary water bath shaker set at 200 rev/min. When the optical density (at 450 nm) of bacteria washed free of amoebae had been reduced to about 40% of the original value, the amoebae had reached a point in the growth phase approximately two generations before the onset of stationary phase ($\sim$4.5 X 10^6 cells/ml). At this point the amoebae were harvested and washed in slime mold saline 4 - 6 times by centrifugation to rid them of remaining bacteria. Cell density was adjusted to 1 X 10^8 cells/ml and 0.1 ml was deposited on prepared plates. Amoebae were immediately spread with a glass rod to a density of 1 X 10^4 cells/mm^2. After the inoculum was absorbed by the agar, the cultures were transferred to the 22°C light incubator.

In one series of experiments plates spread with amoebae were transferred to a 4°C dark incubator for 10 - 12 hr. The plates were then moved to the 22°C light incubator. The time at which the cultures were placed at 22°C was taken as the zero time in development.

Cultures were allowed to develop in the light at 22°C until the appropriate stage, at which time they were collected and prepared for enzyme analysis. An exception to this procedure was the study using actinomycin D, where experimental and control plates were incubated in the dark at 22°C.

B. Preparation of Culture Plates

The Petri plates upon which amoebae were allowed to differentiate prior to enzyme analysis were prepared in the same manner as those used for development of fruiting bodies in the morphogenetic studies (II. B). For these experiments 5.4 cm diameter Petri plates (Falcon Plastics) were filled with 7 ml of agar in which any experimental compounds had been dissolved. Plates were stored 20 - 24 hr at room temperature before inoculation with amoebae.

C. Phosphodiesterase Assays

1. Assay *in vivo* of Whole Cells

In order to assay the total phosphodiesterase (PDE) activity of intact living cells, which represents PDE

accessible to extracellular cyclic AMP, cells were gently rubbed from culture plates flooded with Sörensen's phosphate. One plate was collected for each experimental class. The cells were washed three times in the cold by low speed centrifugations of 5 min each. After the final wash, cells were resuspended in Sörensen's phosphate and assayed according to Malchow et al. (1972) with modifications described here. Enzyme assays from control cultures were routinely run for 10 min with up to 5 X 10^6 cells/ml and were linear with respect to protein concentration and time. To maintain this linearity, samples from Con A-treated cultures were diluted. Two 0.1 ml aliquots from each sample were frozen for protein analysis (Lowry et al., 1951). For each assay 50 µl of cells were preincubated 5 min in a water bath at 19°± 0.5°C in order to temperature-equilibrate the system. The assay was begun by adding 150 pmoles [^{3}H]cyclic adenosine 3',5'-monophosphate (cyclic AMP, 20.7 Ci/mmol, Amersham Searle) in a 10 µl volume. Aliquots were removed periodically and the reaction stopped by making the sample 5% with respect to trichloroactic acid (TCA). Cyclic AMP, adenosine 5'-monophosphate and adenosine were separated on Whatman No. 1 paper in a 95% ethanol-1 M

ammonium acetate (70:30, v/v) solvent system. After 18 hr, the chromatograms were thoroughly air dried, and rectangles containing the reaction products cut out (Wang and Jones, 1959). They were counted in a Beckman 250 Liquid Scintillator in Spectrofluor liquid scintillant (4 gm PPO, 50 gm POPOP per liter toluene, Amersham Searle) for 10 min or until 1% standard error was reached.

2. Soluble Extracellular Phosphodiesterase

Soluble extracellular phosphodiesterase (ePDE) was collected under the same incubation conditions used to assay the total activity except that no substrate was added and the supernatant of the cell suspension was harvested by centrifugation, without addition of TCA. Enzyme activity in the supernatant was then assayed following the procedure used for whole cells.

3. Determination of Membrane Phosphodiesterase Activity

In order to determine what part of the total PDE activity assayed in intact living cells is due to membrane-bound phosphodiesterase (mPDE) exposed at the cell

surface, it is necessary to correct the results of the whole cell assay for ePDE activity which is secreted and accumulates during the assay. The method for calculating mPDE activity at the surface of living cells appears in the Appendix.

4. Particle-Bound Phosphodiesterase

For each experimental class cells were collected from three plates in 0.01 M TRIS·HCl buffer containing 0.02 M $MgSO_4$, pH 7.4. After washing three times by low speed centrifugation, the sample was resuspended in 0.3 ml of buffer and quickly frozen in acetone and dry ice to disrupt the cells. From this cellular debris the 27,000 X g sediment was obtained by 20 min centrifugation and washed three times (Malchow et al., 1972). The pellet was resuspended in 0.05 M TRIS·HCl containing 0.02 M $MgSO_4$, pH 7.4, two aliquots were removed for protein determination (Lowry et al., 1951), and the rest stored at -70°C.

Assays for particle-bound mPDE were performed at 19°± 0.5°C in 0.05 M TRIS·HCl buffer containing 0.02 M $MgSO_4$, pH 7.4 (Malchow et al., 1972), using about 5 µg.

particle protein in 110 µl that included 200 pmoles [^{3}H]cyclic AMP (20.7 Ci/mmol, Amersham Searle). The procedure for the determination and for analysis of reaction products was as for whole cell mPDE (III. C. 1.). Under these conditions, the reaction rate was constant over at least 10 min (Fig. 4) and was a linear function of particle concentration (Fig. 5). To maintain this linearity, extracts from Con-A treated cultures were diluted and the assay time reduced. All assays on particle-bound mPDE were done within these limits.

5. Inhibitors of Membrane Phosphodiesterase

Particles from D. discoideum cells incubated alone or in the presence of Con A were prepared after 12 hr of culture, when control cells are in early aggregation and exhibit substantial mPDE. Particles from each class were assayed for PDE activity; additionally, one set of assay tubes from each class was adjusted to 5mM with respect to cyclic 3',5'-inosine monophosphate and another to 10 mM with respect to glutathione just before the preincubation period of the assay. These tubes were then analyzed as usual for the enzyme.

Figure 4. Hydrolysis of cyclic AMP by particle-bound phosphodiesterase as a function of time. The source of the particles was the 27,000 x g sediment of freeze-thawed D. discoideum cells harvested from plain agar at 0.5 hr of development (●) and 300 μg Con A/ml agar after 1.0 hr of development (⊗).

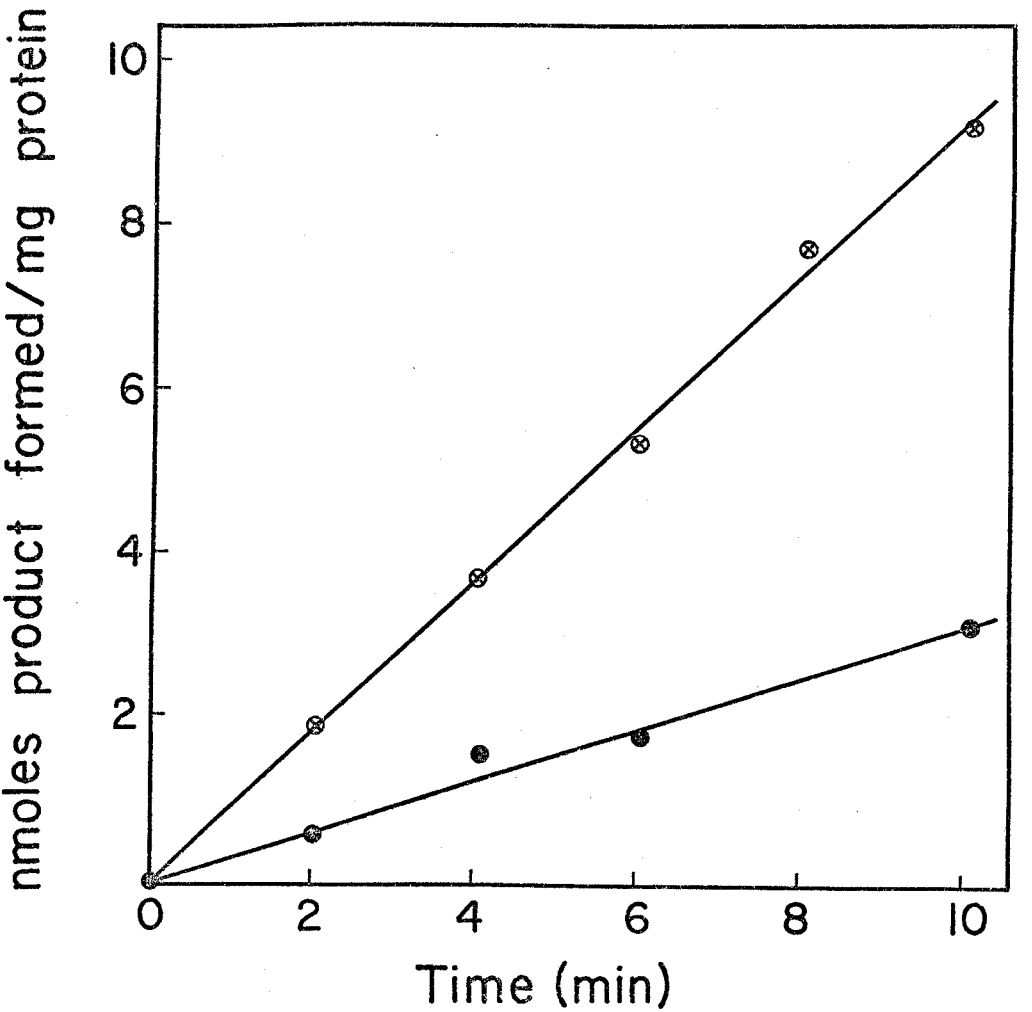
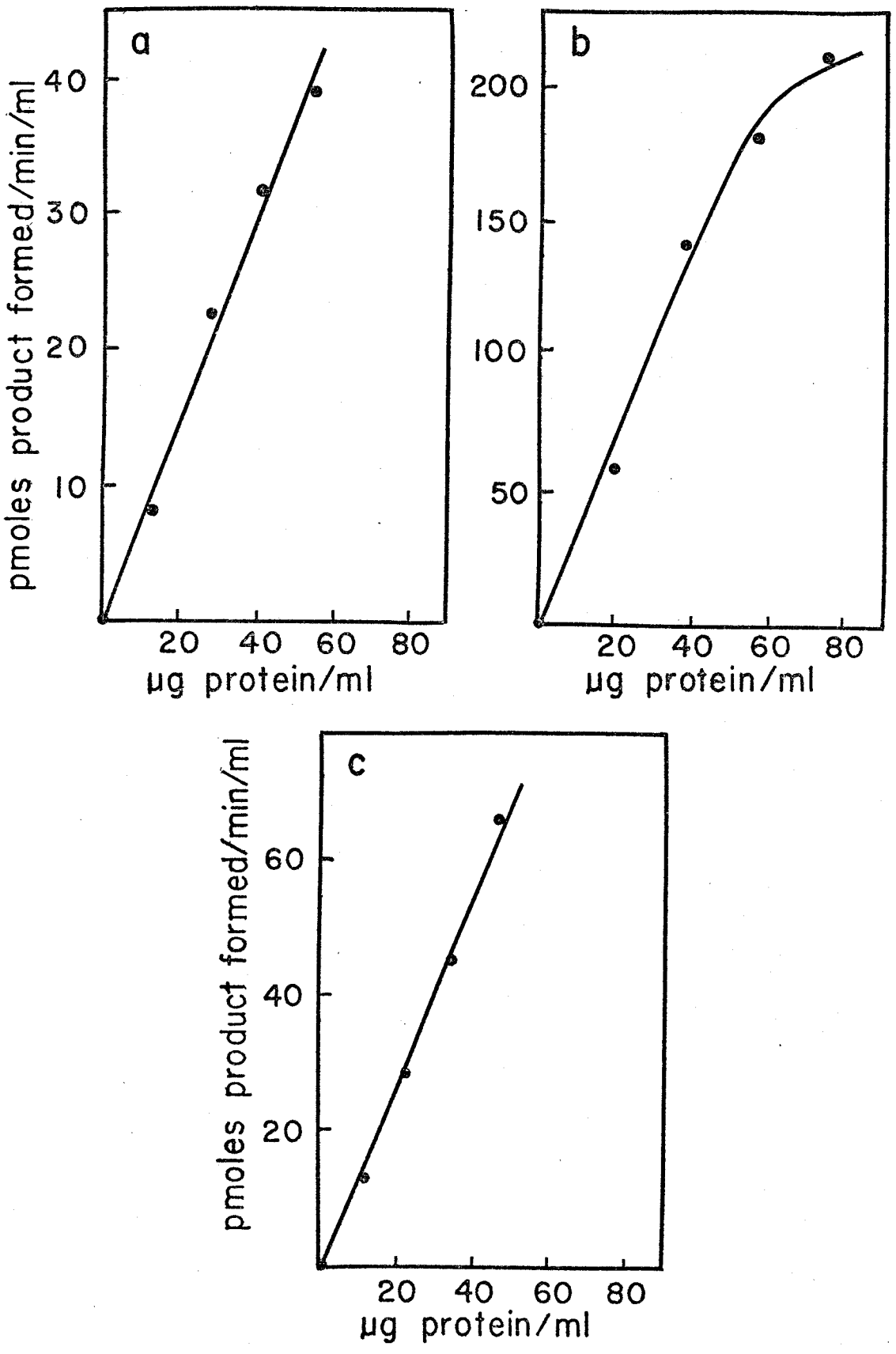

nmoles product formed/mg protein
10
8
6
4
2
0
2
4
6
8
10
Time (min)

Figure 5. Reaction rate of particle-bound phosphodiesterase as a function of particle protein concentration. The enzyme was assayed under the conditions described in the text. Particles were prepared from the following:
(a) D. discoideum collected after 12.0 hr on plain agar (early aggregation);
(b) D. discoideum collected after 12.0 hr on 300 μg Con A/ml agar (pre-aggregation);
(c) D. mucoroides collected after 6.75 hr on plain agar (early aggregation).


a
b
c
pmoles product formed/min/ml
μg protein/ml
10
20
30
40
50
100
150
200
20
40
60
80

D. Experiments concerning the Mode of Action of Concanavalin A

1. Inhibitor Studies

Two types of inhibitor studies were performed. The first examined the effect of the specific haptenic inhibitor of Con A binding, α- methyl-D-glucopyranoside (α-MG) on mPDE activity. The effect of this sugar was tested both alone and in the presence of Con A. This experiment was designed to reveal whether the action of Con A on the enzyme is due to the specific binding of Con A to the cells.

Secondly, metabolic inhibitors were utilized to determine what intracellular mechanisms might be involved. Both control and Con A-treated D. discoideum amoebae were incubated in the presence of cycloheximide (125 and 250 µg/ml agar; Sigma), a know inhibitor of protein synthesis in the cellular slime molds (Sussman, 1965), and actinomycin D (125 µg/ml; Sigma), which has been shown to inhibit RNA synthesis in D. discoideum (Sussman and Sussman, 1965). Particulate membrane preparations of cells incubated for 3 hr in the dark on actinomycin D in the presence and absence of Con A were analyzed for PDE activity.

2. Cells Incubated on Bovine Serum Albumin

To examine the possibility that Con A effects D. discoideum by a non-specific effect of an exogenous protein, amoebae were incubated on agar containing bovine serum albumin (BSA, Sigma) at 2.88×10^{-6} M, equimolar to Con A, assuming it exists in the tetravalent state with a molecular weight of 106,000 (Gunther et al., 1973). After 3 hr of culture, particles were prepared for PDE analysis.

3. Lactate Dehydrogenase Assay

Activity of the cytoplasmic enzyme lactate dehydrogenase was analyzed to study the possiblity that Con A effects a general metabolic stimulation of the cells. After three hours incubation of D. discoideum on plain or Con A agar, the contents of two plates were collected in 0.5 M phosphate buffer, pH 7.4. Samples were kept frozen overnight at -70°C, then thawed and centrifuged at 10,000 X g for 20 min. The supernatant was assayed according to Bergmeyer et al. (1963) using 2×10^{-3} M pyruvate (Sigma) as substrate in the presence of 8×10^{-3} M NADH (Sigma). The reaction was monitored on a Gilford spectrophotometer by following the change in optical density at 340 nm.

4. Phosphodiesterase Assays in the Presence of Concanavalin A

Con A was added to the assay mixture of whole cells and particulate preparations of cells not previously exposed to Con A to determine whether Con A changes the activity of mPDE by acting directly on the enzyme. As this should be a rapid effect due to direct association of Con A with the enzyme, the change in enzyme activity should be detectable within the 15 min incubation used in the assays. Con A was mixed with whole cells at 5 hr of development and with particles from 0.5, 4.5 and 7.0 hr of development so the final concentration was 500 µg Con A/ml. The assays were conducted and analyzed in the standard method used in this study.

5. Replating Experiments

In one series of experiments cells which had been allowed to develop for 5 hr were washed from the plates, collected by centrifugation and spread on plates of 1% purified agar or those containing 300 µg Con A/ml agar. After 1 hr, these cells were collected, as well as those cells which had remained undisturbed on plain agar or agar

with 300 μg Con A/ml for the whole 6 hr period. From each group a particulate preparation was made and each was assayed for phosphodiesterase activity.

6. Experiments on the Effect of Cyclic AMP in this System

Phosphodiesterase assays were performed on particulate preparations of cells which had been cultured 3 hr on the following substrates: 1% purified agar, agar containing 300 μg Con A/ml, agar containing cyclic AMP at 10^{-3} M, 10^{-5} M and 10^{-7} M, and agar containing both Con A (300 μg/ml) and cyclic AMP (10^{-3} M, 10^{-5} M and 10^{-7} M).

IV. Culture of Organisms

Throughout this study stock cultures of slime molds were maintained in association with Escherichia coli B on buffered nutrient agar (Bonner, 1967), 0.1% with respect to $MgSO_4 \cdot 7\ H_2O$. Dictyostelium discoideum was cultured at 22°C in the dark. The strain was originally obtained from J. Bonner's D. discoideum NC-4 and has been maintained in M. Filosa's laboratory for 15 yr with periodic cloning. Dictyostelium mucoroides, strain 7, clone 11, was maintained at 22°C as fruiting bodies in the light (G.E. 15W cool white).

RESULTS

I. Demonstration of Concanavalin A Receptors on the Slime Mold Surface

A. Light Microscopy of Living Cells Incubated in Fluorescent Concanavalin A

1. *Dictyostelium discoideum*

a. Staining during the Life Cycle

A preliminary survey was made to determine whether Con A receptors, molecules containing α-methyl mannose- or α-methyl glucose-like sugar residues, are present on the cell surface during any of the stages of the life cycle of *D. discoideum*. Nearly all stages of the life cycle display fluorescence when treated with 100 μg/ml Fl-Con A (Table 1). Fluorescence is reduced or abolished when cells are incubated in Fl-Con A which has been preincubated with α-methyl glucopyranoside (α-MG), therefore staining can be attributed to specific interaction of Fl-Con A with Con A receptors.

Table 1. Staining of various stages of the life cycle of *Dictyostelium discoideum* and *D. mucoroides* with fluorescent Concanavalin A*

Stage or structure	*D. discoideum*	*D. mucoroides*
Vegetative amoebae	+	(+) faint
Interphase amoebae	+	+ faint
Aggregating amoebae	+	+
Fruiting body		
Slug: Prestalk	+	+
Prespore	+	+
Sheath	+	+
Stalk cells	+	+
Spores	±	-
Empty spore case	+	+
Acellular debris	+	+

*The material was stained in a drop of saline containing 100 µg Fl-Con A/ml. Staining was studied in wet mounts using the fluorescence microscope (see Materials and Methods).

b. Localization and Fate of Fluorescent Concanavalin A Bound to the Cell Surface

The distribution of Con A receptors on the cell surface and redistribution of receptors resulting from interaction with Fl-Con A were examined during the period beginning when washed vegetative amoebae were plated on agar through development to aggregation (Table 2). Changes in the pattern of staining with Fl-Con A should reveal whether the cell surface is changing during development. Washed vegetative amoebae, whether incubated 15 min with Fl-Con A in an ice bath and washed before observation at room temperature or stained during observation at room temperature show localization of fluorescence in a cap within the first minute of staining. As the cell flattens out on the slide, it is clear that the fluorescence is localized to the uropod, that part of the cell which trails behind and is last released from the substrate as the cell moves forward (Fig. 6). It appears that the cell pinches off the fluorescent tail between 30-45 min of staining at room temperature.

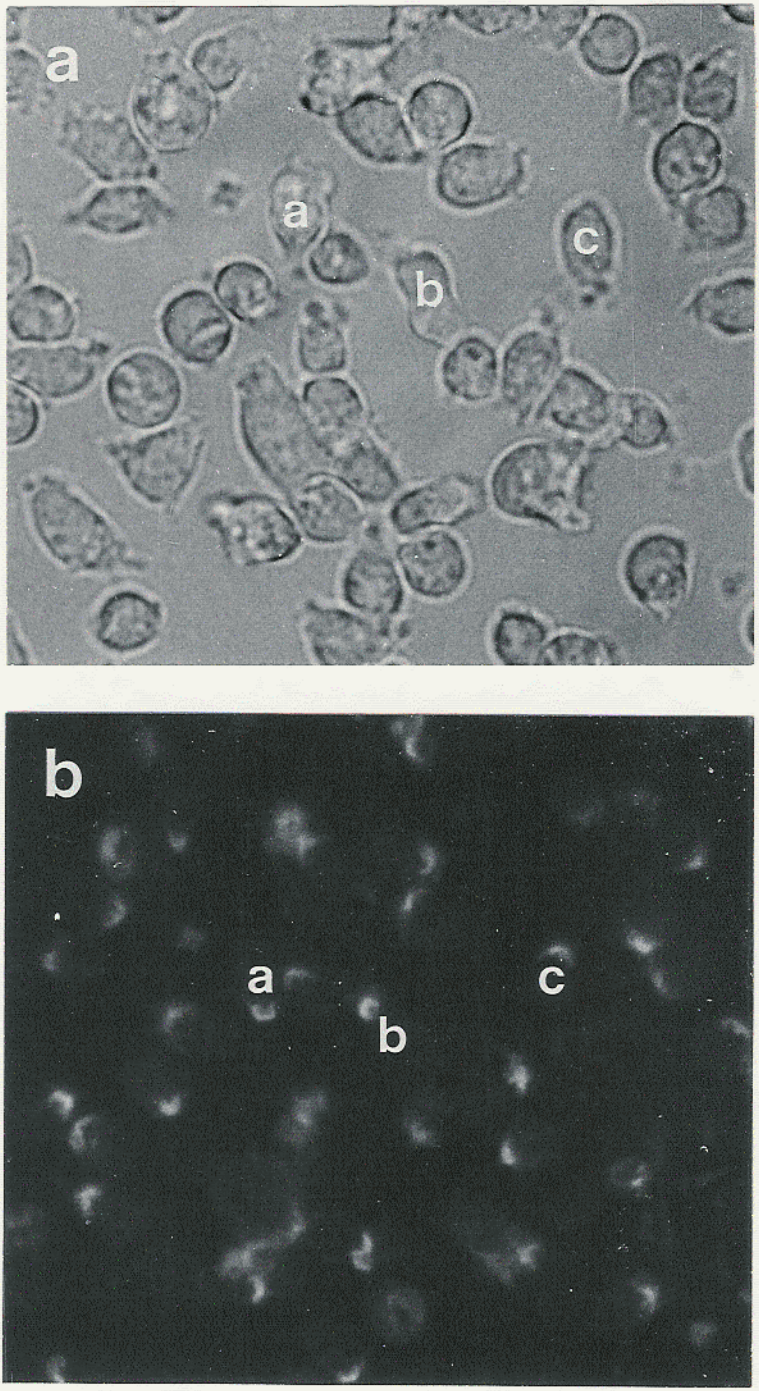
When the cell surface is examined with Fl-Con A at later developmental stages, the initial

Table 2. Localization and fate of fluorescent Concanavalin A on the cell surface of D. discoideum*

Hours after plating	Morphology	Diffuse	Cap	Intercellular contacts	Cap with spots	Diffuse with cap	Diffuse with cap and spots	Diffuse or ring with spots
0.0	Vegetative amoebae	No	1-45					
1.0	Elongate amoebae	No	3	10†	30, 90			
2.0	Amoebae with intercellular contacts	No 5	2 10		10, 30			
5.0	Reticular pattern of amoebae						15, 25, 60	
7.0	Clusters of amoebae					2	20	
8.5	Culsters of amoebae	2		2†		5		12, 24
9.5	Early aggregation	5†	6- 8 10-30†	10†		30		
10.5	Early aggregation	5†, 10†	15, 15†	10†				
19.5	Slug	10†, 30†						

*Cells were stained as described in Table 1 and Materials and Methods. The terms diffuse, cap, etc. refer to the pattern in which fluorescence is localized at the times indicated in the table. The times reported refer to the time in minutes after the cells are first treated with Fl-Con A that the staining pattern is observed. A† designates observations made on clusters touching cells; all other observations refer to single cells. The cell population sampled at 2 hr after plating exhibited two types of staining; both are reported. These observations are the result of four experiments.

Figure 6. Fluorescent caps at the uropods of living cells. These D. discoideum amoebae were removed from agar after 1.5 hr of development and incubated for 5 min in 100 µg Fl-Con A/ml saline. (a) Phase contrast microscopy; (b) Fluorescence microscopy, 2 min exposure. The letters indicate the same cells under the different forms of illumination. X3000


a
a
b
c
b
a
b
c

distribution of fluorescence is seen to have changed from what is observed initially in washed vegetative cells stained with Fl-Con A. The first indication that the cell surface is changing is observed after 2 hr incubation on plain agar (Table 2). Most cells removed from agar at 2 hr after plating and treated with Fl-Con A cap the stain initially, as did the washed vegetative cells. However, a few cells in the population are covered with diffuse fluorescence initially; subsequently these cells also cap the stain. When cells are removed from agar at 7 and 8.5 hr and treated with Fl-Con A, most initially show a diffuse fluorescence over the surface as well as stain concentrated in a cap at one edge. Therefore, as development proceeds the initial distribution of Con A receptors on the cell surface changes so that by late pre-aggregation stages a faint diffuse fluorescence appears simultaneously with a cap.

The cell surface also changes with respect to the way it redistributes bound Fl-Con A during development. Washed vegetative amoebae which initially cap Fl-Con A still have fluorescent caps after 45 min in the stain. However, cells which have been incubated for

1 hr on agar prior to exposure to Fl-Con A exhibit fluorescent spots as well as caps within 30 min of application of stain. Pre-aggregation cells removed from agar and stained redistribute Fl-Con A in a similar manner so that the following sequence of fluorescent patterns is seen: diffuse ----→ cap ----→ cap with fluorescent spots (Fig. 7). Where cells are in contact with each other, capping is followed by localization of fluorescence at intercellular contacts where the stain remains when fluorescent spots appear.

At 5 hr and for the rest of the pre-aggregation period this pattern of redistribution of stain takes place on a background of diffuse fluorescence over the cell surface. The cell forms a cap, then spots while continuing to exhibit diffuse fluorescence. By 8.5 hr the cap disappears and only fluorescent spots are present in addition to diffuse fluorescence (Fig. 8). This shows that the potential of Con A receptors for redistribution by Fl-Con A is different from earlier stages both because the cap disappears when spots appear and because the cap and spots form while the cells are diffusely fluorescent. It appears that as the cell approaches aggregation only

Figure 7. The sequence of fluorescent patterns observed on pre-aggregation amoebae of D. discoideum stained with Fl-Con A. Cells were placed in 100 µg Fl-Con A/ml saline and observed as wet mounts.

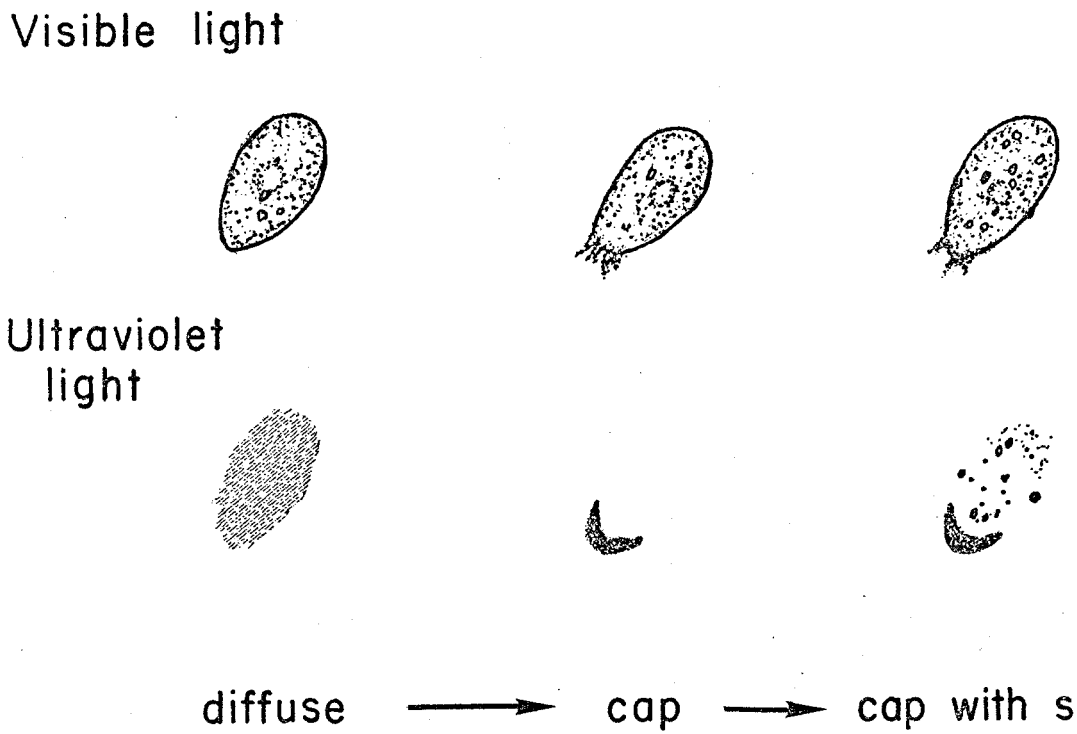
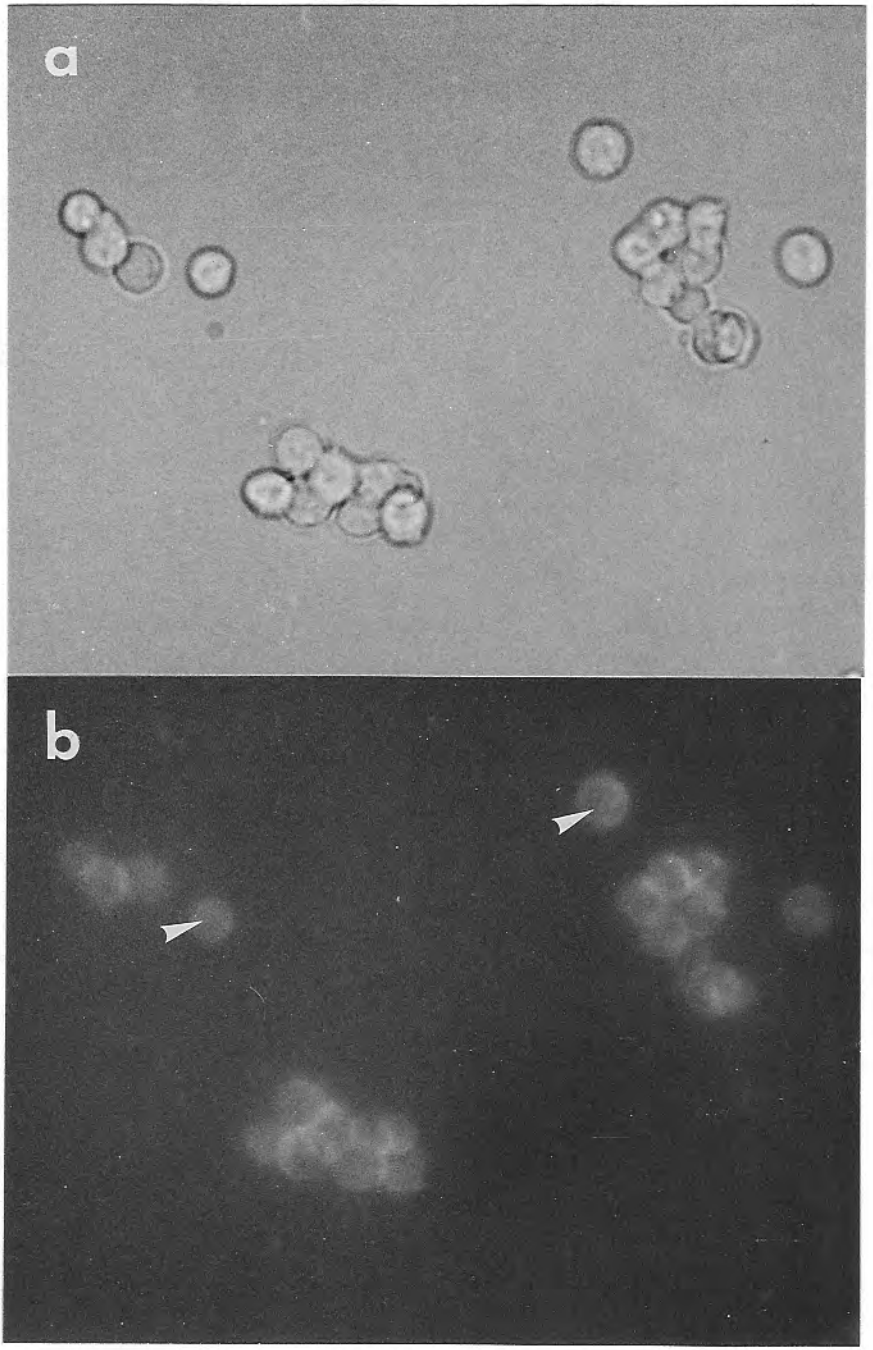

Visible light
Ultraviolet light
diffuse
cap
cap with spots

Figure 8. Diffuse and ring fluorescence with spots on pre-aggregation amoebae of D. discoideum. Cells were removed from plain agar after 8.5 hr of development and incubated for 20 min in 100 μg Fl-Con A/ml saline. (a) Phase contrast microscopy. (b) Fluorescence microscopy, 4 min exposure. Arrows point to fluorescent spots. X2000


a
b

some of the Con A receptors are free to move in response to Fl-Con A while others are immobile in the membrane and thus remain dispersed.

When aggregating cells are stained *in situ*, fluorescence is first detected on the outer edges of cells in the stream lines, probably because these edges are physically most accessible to the stain. Shortly after staining, these cells are faintly fluorescent over their surfaces, while highly elongate individual cells not yet in aggregation streams do not pick up the stain. Within 10 min the cells in the streams begin to come apart and round up. The fluorescence becomes confined to an outline of the cell, and by 15 min, to a bright cap. Isolated amoebae pick up stain as they round up in the presence of Fl-Con A. Fluorescence first appears as a cap on these cells 6 - 8 min after stain is applied. These cells cap more readily than the cells in aggregation streams which have already established intercellular contacts. By 30 min after staining the cells are elongate and motile, with a brightly fluorescent cap on the posterior uropod of each one. The rest of the cell surface may be faintly fluorescent.

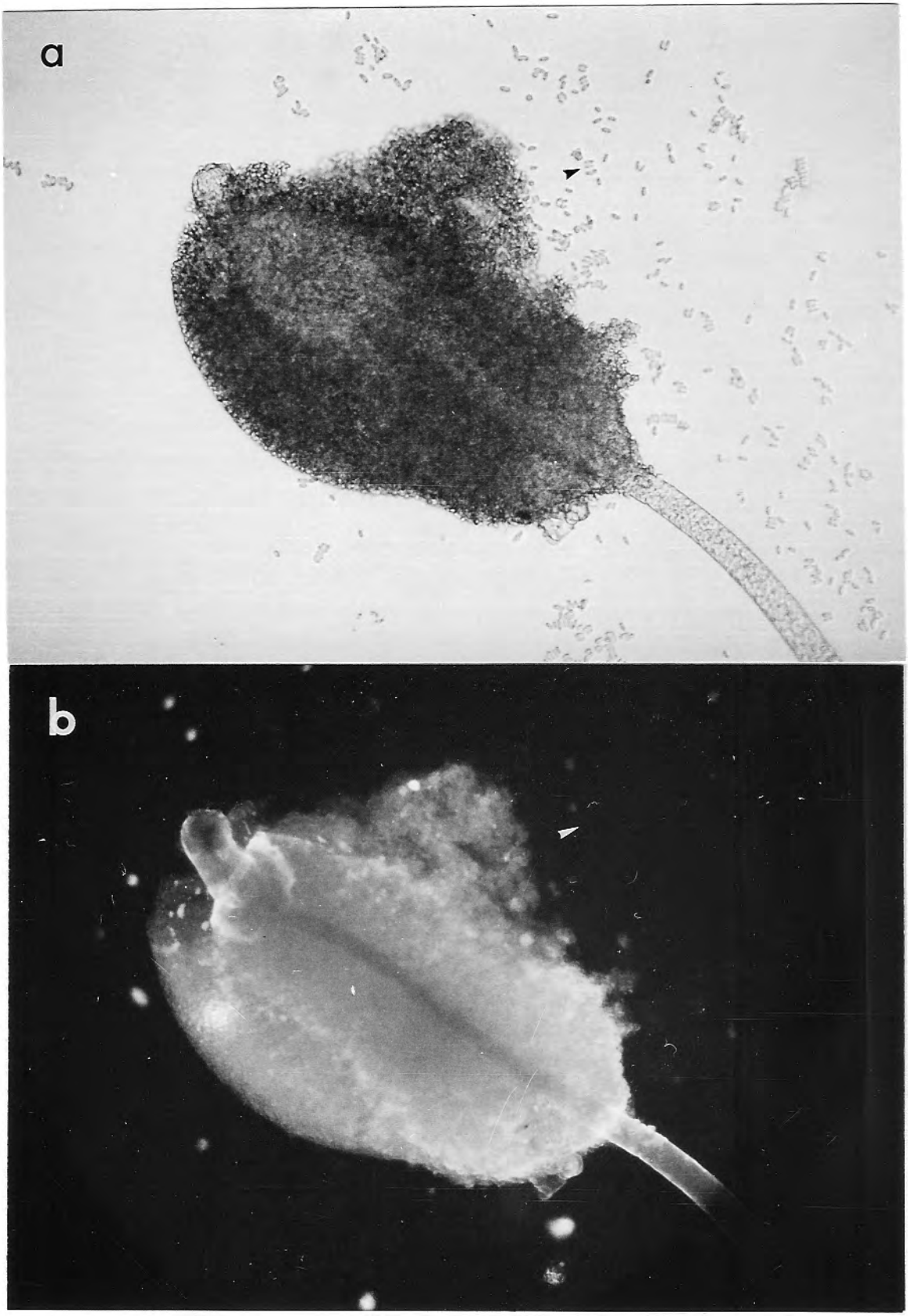
Cells from migrating slugs bind Fl-Con A but unlike earlier stages the fluorescence remains diffusely distributed over the cell surface. Fruiting bodies are composed of cells with non-fluid surfaces, namely the stalk cell wall and the spore case. The stalk cell wall as well as the stalk sheath bind Fl-Con A (Fig. 9). Spores from some cultures pick up the stain after a 10 min incubation. The stain appears evenly distributed over the surface of these cells; no change in the staining pattern appears with time.

c. Localization and Fate of Fluorescent Concanavalin A on Cells Incubated on Concanavalin A Agar

This series of experiments was performed in order to determine whether Con A-binding sites are saturated when cells are plated on Con A agar, and, if they are not saturated, to determine what effect prior binding of part of the available receptors by Con A from the agar has on the pattern of Fl-Con A binding and redistribution.

Amoebae were periodically scraped off 300 μg Con A/ml agar and observed for their ability to bind

Figure 9. Squash of a fruiting body of D. discoideum stained with 100 µg Fl-Con A/ml. (a) Light photomicrograph. (b) Fluorescence photomicrograph. The stalk, sheath and acellular material between spores bind Fl-Con A. Note the absence of fluorescence on spores in this preparation (arrow). X480.


a
b

Fl-Con A. The results show that all stages sampled bind Fl-Con A (Table 3) although fluorescence is less intense than on cells incubated for similar periods on plain agar. After 45 min on the Con A agar, cells are round, highly vacuolate and clustered in patches. These cells cap Fl-Con A within 15 min of stain application, and appear capped at the uropod with fluorescent spots at 30 min. They appear to redistribute Fl-Con A in a way similar to untreated cells of the same stage.

Cells kept on Con A agar for 2 hr exhibit diffuse staining 1 min after application of Fl-Con A. Some cells remain diffusely stained at 8 min while on others a faint cap is visible. By 15 min most cells have at least localized the stain to the posterior third of the cell in what might be termed a diffuse cap. The cap persists at 25 min and spots appear. Therefore, although the cap is less intense and well defined than in control cells of the same stage, cells kept on Con A for 2 hr appear to redistribute newly bound Fl-Con A in similar manner to controls.

By 5 hr the Con A treated cells have recovered normal morphology (see section II); however, at this point they demonstrate staining with Fl-Con A unlike controls of

Table 3. Localization and fate of fluorescent Concanavalin A on *D. discoideum* Amoebae incubated on Concanavalin A agar*

Hours after plating	Morphology	Diffuse	Cap	Cap with spots	Spots only	Diffuse with cap and spots	Diffuse or ring with spots	Ring with cap	Extracellular material
0.75	Cells round, highly vacuolate; clustered		<15	30					
2.0	Cells round, highly vacuolate; clustered	1 8	8 15		30				
5.0	Cells elongate, few vacuoles	3	3			13	60		
7.0	Clusters of amoebae				10, 20				10
9.5	Clusters of amoebae			1			7		1
21.5	Aggregation without stream formation	5, 20	30					40	5

*Cells were stained as described in Table 1 and Materials and Methods. The terms diffuse, cap, etc. refer to the patterns in which fluorescence is localized at the times indicated in the table. The times reported refer to the time in minutes after the cells are first treated with Fl-Con A that the staining pattern is observed. The cell population sampled at 2 hr after plating exhibited two types of staining; both are reported. These observations are the results of three experiments.

the same age. When cells that have been on Con A for 5 hr are treated with Fl-Con A, they still cap Fl-Con A rapidly (3 min). Spots appear with the caps by 13 min. At 60 min the caps have disappeared; the stain is localized to spots, in contrast to controls of the same stage. At 7 hr Con A-treated cells stain with Fl-Con A only in spots at 10 min post-staining. This is clearly a departure from controls: at 10 min of incubation all stages of controls still localize Fl-Con A as caps and later all controls possess caps when fluorescent spots have appeared. Much particulate debris present at this stage binds Fl-Con A. When 7 and 9.5 hr cells are removed from Con A agar, the cells remain clumped. The 9.5 hr cells localize Fl-Con A at the intercellular contacts as well as surface spots within 1 min of stain application. Single cells have spots or small caps over the uropod. By 7 min single cells have spots of fluorescence as well as fluorescent outline, a response comparable to isolated 8.5 hr controls cells stained for 24 min.

The latest observation of binding of Fl-Con A to cells incubated on Con A agar was made at 21.5 hr, when these cells are aggregating. Clumps of cells initially exhibit fluorescence over the cell surface. By 20 min individual cells are faintly fluorescent. Large amounts

of debris consisting of small particles, larger platlet-like debris and acellular sheath-like material is visible by its fluorescence when stained with Fl-Con A. After 30 min caps are evident, some of which are located at intercellular contacts. Eventually clumps of cells are outlined with fluorescence and a few spots appear.

Staining of D. discoideum incubated on Con A agar with Fl-Con A is compared with staining of untreated amoebae in the following summary:

a) Con A-treated cells are less brightly fluorescent when stained with Fl-Con A at all stages;

b) caps are less compact at 2 hr and 5 hr in Con A-treated cultures;

c) spots form more quickly and are more numerous in Con A-treated cultures, especially at late pre-aggregation stages, than for controls; also, only in Con A cultures do spots appear in the absence of any other pattern of staining;

d) Con A-treated cells produce debris by 7 hr which binds Fl-Con A; this is not found in controls;

e) at 45 min and again at early aggregation the pattern of redistribution of fluorescence is similar to controls.

2. Dictyostelium mucoroides

a. Staining during the Life Cycle

Various stages of fruiting body morphogenesis in D. mucoroides were stained with Fl-Con A to determine whether Con A receptors are present on the cell surface. The results using Fl-Con A (Table 1) indicate that most stages possess a sufficient number of Con A receptors to fluoresce in the presence of 100 µg Fl-Con A/ml. However, the vegetative and pre-aggregation amoebae stain very faintly with Fl-Con A, suggesting there are fewer available Con A receptors on these cells than on aggregating amoebae or than on D. discoideum amoebae of the same stages. Spores lack accessible Con A binding sites on the external surface as evidenced by their failure to bind Fl-Con A, but empty spore cases pick up the stain indicating the presence of Con A receptors on the internal surface.

b. Localization and Fate of Fluorescent Concanavalin A Bound to the Cell Surface

The distribution of Con A receptors on the surface of D. mucoroides cells and redistribution of these receptors due to interaction with Fl-Con A was examined during the developmental period from vegetative amoebae to aggregation. Washed vegetative amoebae show very faint fluorescence, first diffuse, then capped by 8 min after staining (Table 4). A similar pattern of staining occurs at the other pre-aggregation stages observed. Staining is faint when compared with D. discoideum and caps are less well defined.

As amoebae begin to aggregate, they at first stain over the entire surface, then within 5 min localize Fl-Con A in a bright fluorescent spot (tight cap) at their posterior ends. They continue to exhibit this pattern of staining, with a similar time course for cap formation until late aggregation. Aggregation streams are disaggregated in the presence of Fl-Con A in the same period it takes for capping to occur. The exaggerated elongate shape of amoebae in the stream lines is lost as the cell contacts are broken.

Table 4. Localization and fate of fluorescent Concanavalin A on the cell surface of D. mucoroides*

Hours after plating	Morphology	Diffuse	Ring and diffuse	Cap	Diffuse with cap
0.0	Vegetative amoebae	3		8	
0.75	Elongate amoebae		5-10	12-30	
2.0	Clusters of amoebae	3-10		20	
6.0	Early center formation			5≥30	
7.0, 7.5	Early aggregation	1-3		4≥40	4≥40
8.5	Aggregation	1		4≥35	
10.0	Late aggregation	2	5-40†	25-40†	

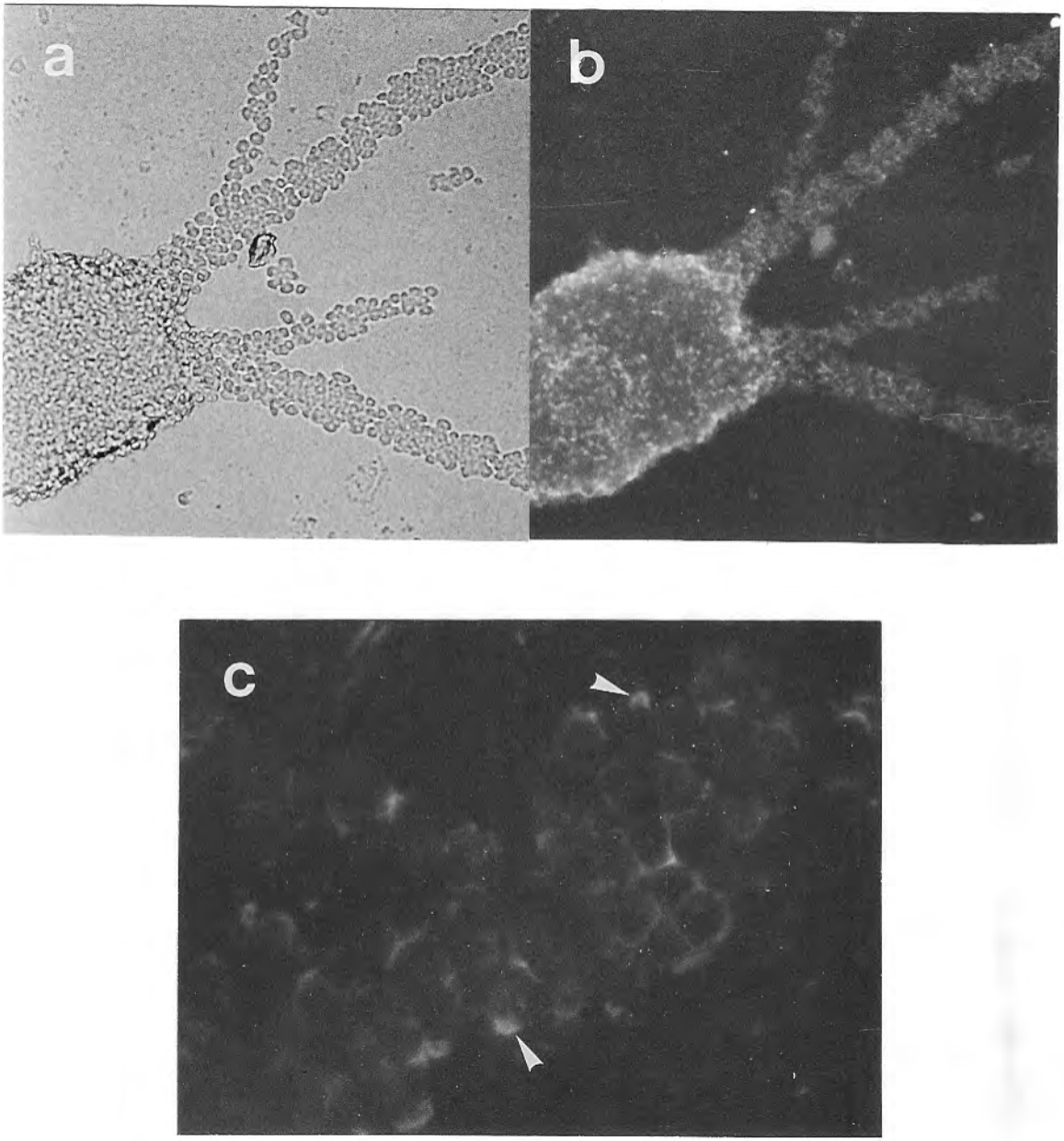
*Cells were stained as described in Table 1 and Materials and Methods. The terms diffuse, cap, etc. refer to the pattern in which fluorescence is localized at the times indicated in the table. The times reported refer to the time in minutes after the cells are first treated with Fl-Con A that the staining pattern is observed. A † designates observations made on clusters of touching cells; all other observations refer to single cells. These observations are the results of two experiments.

Amoebae may round up briefly during the disaggregation process. Individual cells are elongate and move about during the next 40 min. It is noteworthy that formation of fluorescent spots is not observed in this species. The cells occasionally are observed to shed their fluorescent tails. The aggregation center does not disaggregate in Fl-Con A, but appears faintly fluorescent over its surface, probably due to the sheath material.

Late aggregation cells, which have been in streams for a longer period of time, initially show diffuse staining over their surface, then only in a ring at the rim of the cell. Capping is observed among the cells at the edge of streams by 25 min (Fig. 10). Cells more internal in the stream show ring staining. Disaggregation of thick streams of cells is not generally the case, probably because these cells are now covered by an acellular sheath or the Con A cannot penetrate to all the cells.

When early aggregation centers are treated with Fl-Con A in the presence of 0.05 M α-MG, the cells do not pick up the stain, although within 5 min the streams come apart and the cells begin to shorten. There is no disaggregation when the cells are simply flooded with saline.

Figure 10. Late aggregation center of D. mucoroides stained with 100 μg Fl-Con A/ml for 35 min. Notice that the cells in the stream lines have rounded and started to pull apart. (a) Light photomicrograph. (b) Fluorescence photomicrograph. X250. (c) Detail of a stream in (b). Fluorescence outlines cells in the stream; cells at the edge have capped (arrow). X3,000.


a
b
c

D. mucoroides cells incubated on Con A agar for 2 hr were treated with Fl-Con A. Individual cells are single and elongate initially, but start to round up as they become stained with Fl-Con A in a diffuse pattern at 6 min. By 15 min the cells are round although they no longer appear fluorescent. At this time the stain is confined to particulate debris. By 8.5 hr the cells on 100 µg Con A/ml agar show no fluorescence when treated with Fl-Con A although those on 300 µg Con A/ml agar show a very faint fluorescence which is difficult to localize.

B. Electron Microscopic Localization of Concanavalin A

D. discoideum amoebae incubated on plain agar for 1 hr and fixed before exposure to Con A show dense staining completely surrounding the cell at the surface when viewed in cross-section (Fig. 11). Cells incubated on Con A agar for 1 hr before fixation have localized the Con A to part of the surface (Fig. 12). In neither case is any Con A found internal to the cell. Controls exhibit no deposits of stain so the method can be judged specific for exogenously applied Con A bound to the cell.

Figure 11. Electron micrograph of a D. discoideum amoebae treated to localize Con A bound after fixation. Amoebae were removed from agar after 1 hr, pre-fixed with glutaraldehyde, incubated in 300 µg Con A/ml buffer, and then treated with DAB-HP reagent. Stain polymer completely outlines the cell; no stain is visible in the cytoplasm. X25,600.

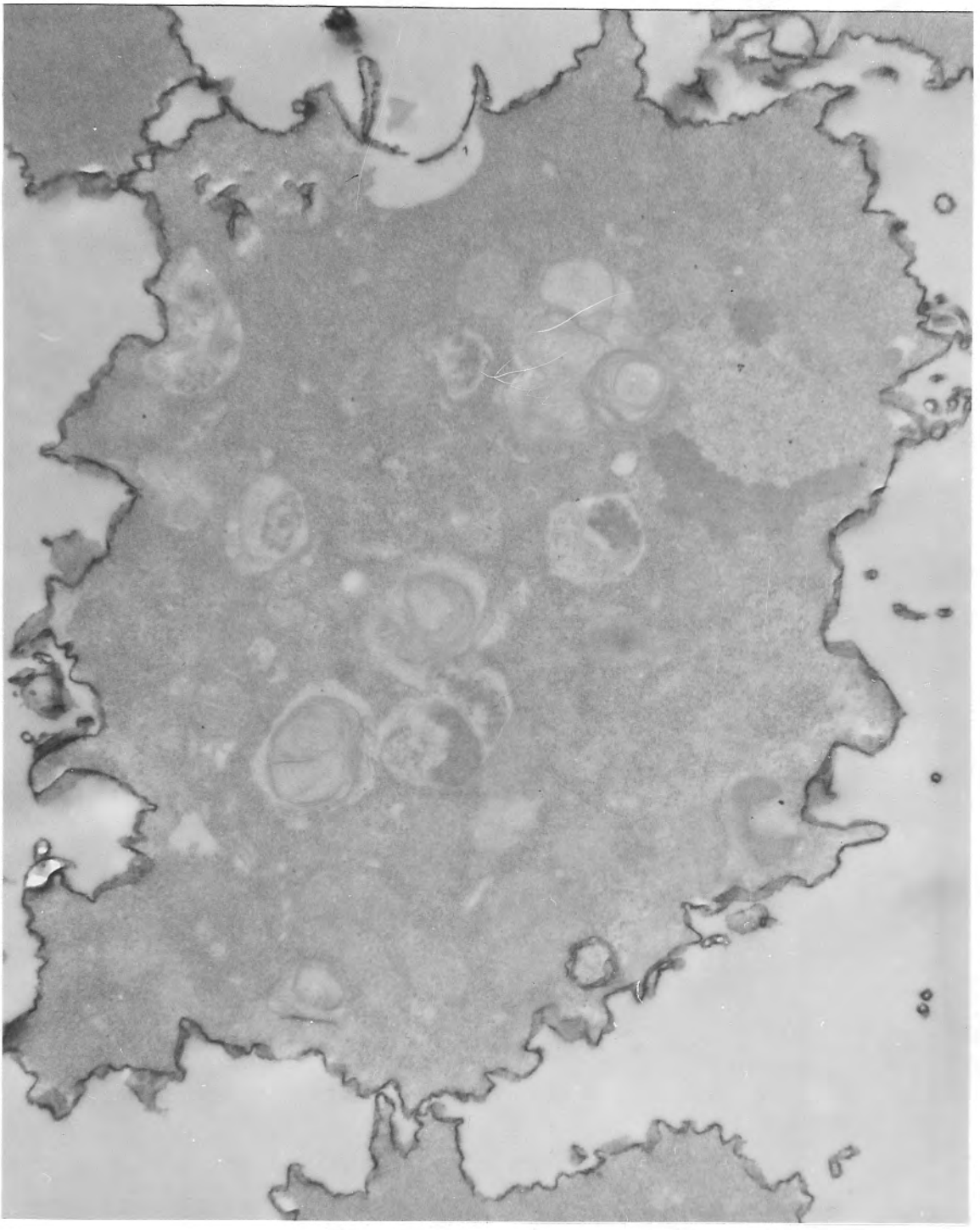
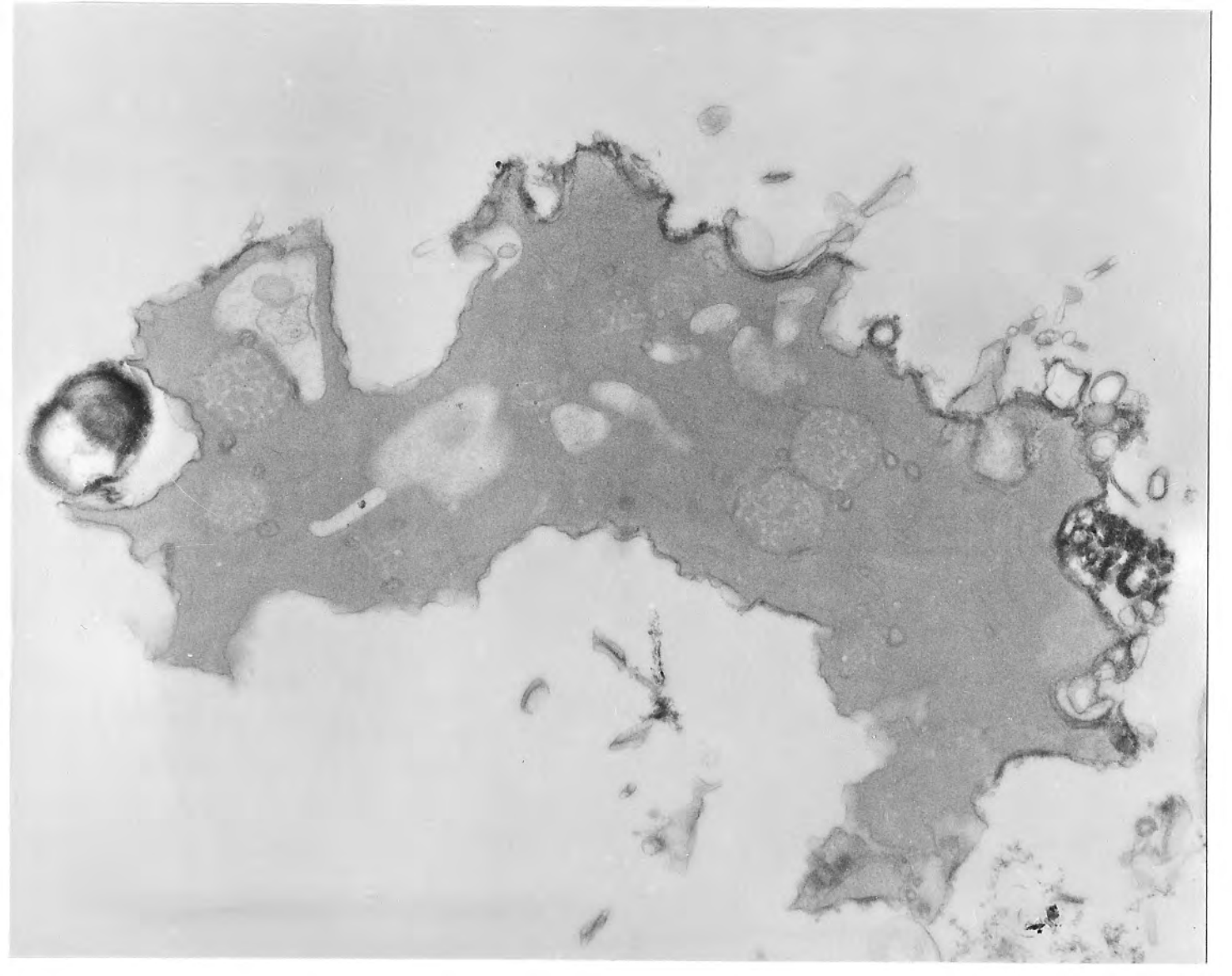


Figure 12. Electron micrograph of a D. discoideum amoeba preincubated on Con A then treated to localize bound Con A. Amoebae were incubated 1 hr on 300 μg Con A/ml agar, then fixed and treated with DAB-HP reagent. Stain polymer is localized to part of the cell surface; no stain is visible to the cytoplasm. X16,875.



C. Agglutination Titre of Washed Vegetative Cells

The results of agglutination assays performed on D. discoideum and D. mucoroides appear in Table 5. The titre is taken as the lowest concentration of reagent which clearly causes agglutination. For D. discoideum the titre is between 12.5 - 50 µg Con A/ml and for D. mucoroides between 25 - 50 µg Con A/ml. D. mucoroides is less completely agglutinated by higher concentrations of Con A than is D. discoideum. These results suggest that D. mucoroides has fewer sites by which Con A can cross-link cells to agglutinate them than does D. discoideum.

Table 5. Agglutination of washed vegetative amoebae by Concanavalin A*

Concanavalin A concentration	*D. discoideum*				*D. mucoroides*	
	Exp.1	Exp.2	Exp.3	Exp.4	Exp.1	Exp.2
400 µg/ml	+++++	+++++	+++++		+++	
200	+++++	++++	++++	++++	+++	+++
100	+++	+++	+++	+++	++	++
50	++	++	+	++	+	+
25	+	0	0	+	0	0
12.5	0	0	0	0	0	0
0	0	0	0	0	0	0
100 µg Con A/ml + 1.25×10^{-2} M α-MG	0	0	0	0	0	0

*Vegetative cells were removed from plates and washed with saline. 0.1 ml of cells (1×10^7 cells/ml) was mixed with 0.1 ml of Con A producing the test concentrations of Con A listed above. The degree of agglutination was assessed microscopically within 5 min according to the following qualitative scale:
+++++ = >90% of the cells agglutinated; ++++ = 75 - 90%;
+++ = 50 - 75% agglutinated; ++ = 25 - 50% agglutinated;
+ = 10 - 25% agglutinated; 0 = <10% agglutinated.

II. Morphogenetic Studies

A. Effect of Concanavalin A on Slime Mold Development

1. Dictyostelium discoideum

Having demonstrated that Con A receptors are present on slime mold amoebae during development, it was decided to examine the possibility that continuous exposure of cells to Con A might affect slime mold development. When D. discoideum is allowed to undergo morphogenesis on Con A, the fruiting bodies formed are completely normal. However, Con A produces several striking effects on development:

a) the morphology of the cells and of aggregation is characteristically altered;

b) aggregation is delayed;

c) the index of aggregate formation is increased at high cell densities;

d) there is a marked increase in the number of fruiting bodies formed per unit area.

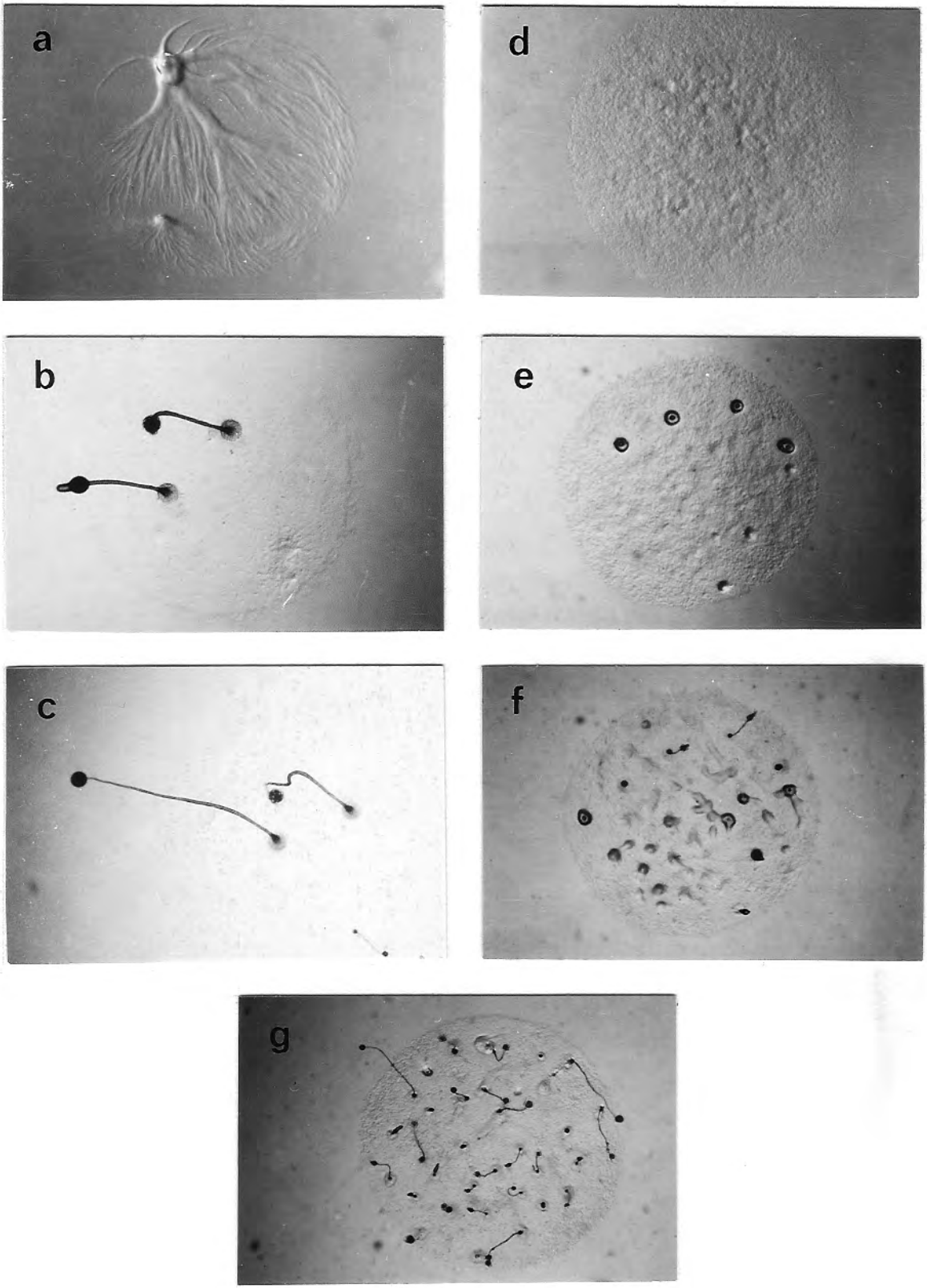
Each of these effects will be described in turn.

a. Morphology of Cells and of Aggregation on Concanavalin A Agar

Within 15 min after spreading washed vegetative amoebae on 300 µg Con A/ml, the cells round up and large vacuoles appear in the cytoplasm. They retain this appearance for several hours. Although the cells are clustered together in patches when spread, they appear to be in a monolayer. When washed or scraped from the agar, they disperse into single cells, unlike control cells which by 1.5 hr are clumped in suspension while not appearing clustered on the agar. Recovery of elongate form by the amoebae is variable, probably depending on the amount of moisture on the agar surface and therefore the number of Con A molecules in which the cells are bathed upon plating. Usually, however, the amoebae begin crawling away from clumps at about 2.5 - 4.0 hr on 300 µg Con A/ml agar.

The morphology of aggregation on Con A agar is clearly distinguishable from the characteristic pattern for D. discoideum (Fig 13). The Con A-treated cells first move into local collections of cells (7 hr) which are larger than the clusters of amoebae normally seen during the preaggregation phase. These collections slowly increase

Figure 13. Morphology of aggregation of D. discoideum on plain agar (a - c) and on 200 µg Con A/ml agar (d - g). In both sequences the cells were at 1.25 X 10^4 cells/mm^2 (a,d) 21 hr; (b, e) 30 hr; (c, f) 37 hr; (g) 50 hr of development. Notice the difference in the morphology of aggregation, the timing of aggregation, and the size of the fruiting bodies [note that (g) is at 1.3X higher magnification than (c).] (a) X16; (b) X16; (c) X12; (d - g) X16.


a
d
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e
c
f
g

in size over the period when the untreated cells aggregate (Fig. 13 a, d) and eventually become rounded mounds of cells (Fig. 13 e, f). The mounds may serve as centers for aggregation of some amoebae peripheral to them, but at 200 µg Con A/ml or more, no aggregation streams emanate from them; indeed, amoebae moving toward them do not establish intercellular contacts until they have joined the cell mass. Unlike controls these amoebae are not highly elongate as they move toward the mound of cells. At lower Con A concentrations (50 and 100 µg Con A/ml) aggregation streams may appear, but are reduced, depending on the concentration of Con A. Morphology of the aggregating cells is likewise less affected at 50 and 100 µg Con A/ml. Each mound gives rise to a slug which forms a single fruiting body (Fig. 13 e, f, g). The developmental sequence subsequent to aggregation appears completely normal.

b. Delay of Aggregation in the Presence of Concanavalin A

The time course of development in the presence of Con A was examined by inspecting cultures for completion of aggregation as heralded by the initiation of stalk formation. This stage was selected as a marker because of the difficulty in defining earlier aggregation stages with precision for cultures incubated in the presence of Con A. It was found that the time the initial aggregate appears is delayed by Con A (Fig 13, Table 6). The period of delay increases as the Con A concentration is raised. At 50 μg Con A/ml completed aggregates appear nearly concurrently with or shortly after the controls. However, when the concentration of Con A is increased to 400 μg/ml the onset of stalk formation is delayed about 14 hr under these conditions. The effect of any one concentration of Con A depends on the density of the responding cells, lower cell densities experiencing greater delays in aggregation than higher ones.

Table 6. Effect of Concanavalin A in delaying aggregation of D. discoideum amoebae*

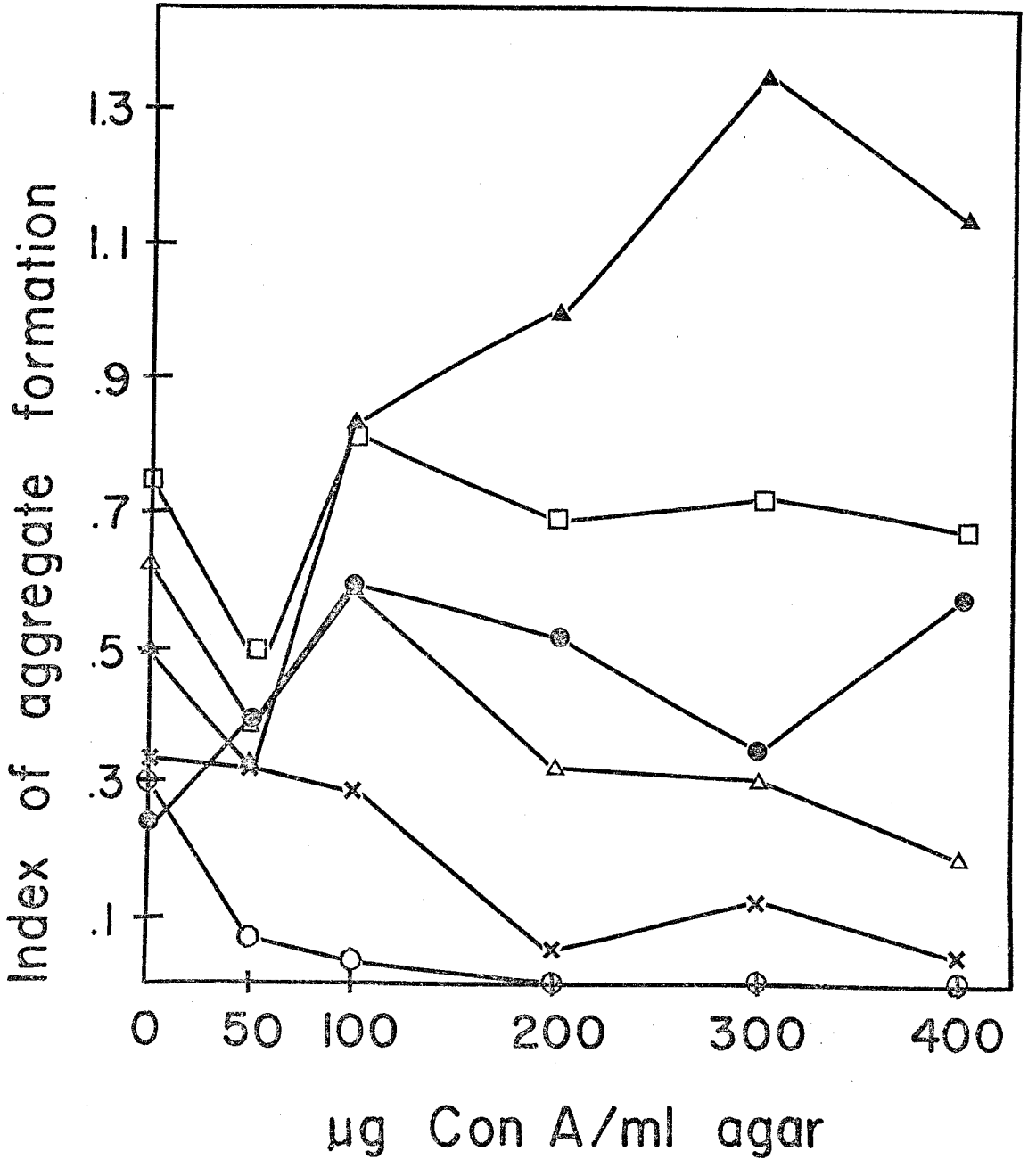
Cell treatment	Hours to completion of the first aggregate		
	Cell density (cells/mm^2)		
	2.5×10^3	7.5×10^3	1.25×10^4
0 µg Con A/ml	23	23	22
50	30	27	24
100	30	28	27
200	42	30	30
300	38	32	31
400	53	34	33
5×10^{-2} M α-MG	21	30	20.5
200 µg Con A + 2.5×10^{-2} M α-MG	21	21	22

*Vegetative amoebae were removed from plates and washed 3 - 5 times in cold saline to remove uneaten bacteria. 2 µl droplets of cell suspension were applied to hydrophobic agar containing Con A and/or α-MG to produce the test densities of cells (for details see Materials and Methods). Each cell density was prepared in duplicate and triplicate. Reported here are the average times of completion of the first aggregate for different cell densities from a single preparation of cells. The experiment was repeated four times.

c. Effect of Concanavalin A on the Index of Aggregate Formation

Although in all cases examined Con A retards the onset of aggregation, it does not similarly affect the rate at which centers are completed once aggregation has begun. This phenomenon can be assessed by examining the index of aggregate formation derived by dividing half of the total number of aggregation centers by the difference between the time half have formed less the time that the initial aggregate formed. This effect is related to the cell density of responding cultures. In control cultures increasing the cell density generally increases the index of aggregate formation (Fig. 14). This increase in the index is due to an increase in the number of centers formed as the cell density increases rather than an effect on the time it takes for centers to be completed; half of all the aggregates that will be formed are completed over a 2 - 3 hr period in controls regardless of cell density. Completion of aggregates extends over a much longer period (roughly 24 hr or more) in the presence of 50 - 100 μg Con A/ml. Nevertheless, at high cell densities (7.5×10^3 - 1.25×10^4 cells/mm^2) some Con A concentrations

Figure 14. Effect of Con A on the index of aggregate formation of D. discoideum. Washed vegetative cells were deposited in microdrops on agar containing different concentrations of Con A. Aggregation was studied for the following cell densities: ▲, 1.25 X 10^4 cells/mm^2; □, 1.0 X 10^4 cells/mm^2; ●, 7.5 X 10^3 cells/mm^2; △, 5.0 X 10^3 cells/mm^2; ✕, 2.5 X 10^3 cells/mm^2; ○, 1.0 X 10^3 cells/mm^2. Generally, at low cell densities Con A depresses the index of aggregate formation. At high cell densities (>7.5 X 10^3 cells/mm^2) some Con A concentrations increase the index. Each is the average of triplicate cultures.


Index of aggregate formation
1.3
1.1
.9
.7
.5
.3
.1
0
50
100
200
300
400
µg Con A/ml agar

increase the index of aggregate formation as compared with controls (Fig. 14). This reflects the fact that Con A greatly increases the number of centers formed by a given number of cells (see next section). A consistent exception is at 50 μg Con A/ml where the index is lower than controls. At 5.0 X 10^3 cells/mm^2 Con A generally depresses the index of aggregate formation. Below that density of cells most concentrations of Con A greatly depress the index.

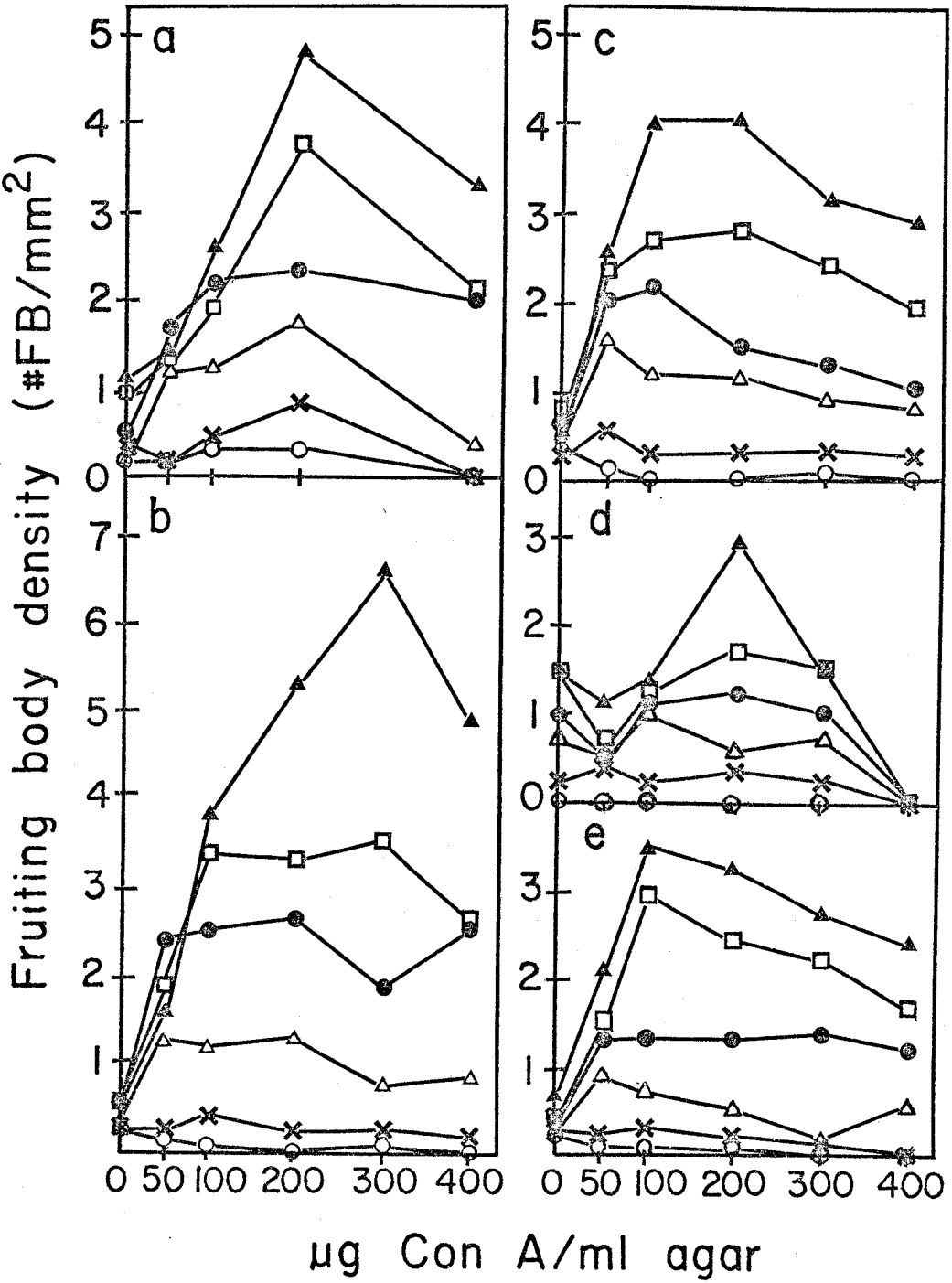
d. Effect of Concanavalin A on Fruiting Body Density

The effect of Concanavalin A on the number of fruiting bodies formed by various cell densities was examined using small populations of amoebae. Fruiting body formation is extremely sensitive to environmental factors, especially light and humidity, as well as the physiological condition of the cells. Although I attempted to carefully control the environmental and cellular conditions, the numerical results for different experiments of fruiting body density proved highly variable. Because different batches of bacterial grown slime mold amoebae may vary physiologically,

it is often not possible to meaningfully pool results from different preparations of cells (J. T. Bonner, personal communication). Graph 15a-e represents results from five different experiments; each panel, e.g. 15a, represents data obtained from triplicate or quadruplicate experiments with the same batch of cells; 15b is a different experiment but likewise each point is the average of 3 - 4 replicate plates.

Con A has the general effect of increasing the density of aggregation centers and thus the density of the resulting fruiting bodies (Fig. 13, 15). The degree to which the number of fruiting bodies/mm^2 is increased over controls depends upon the cell density as well as the concentration of Con A in the agar. With increasing cell densities there is a small increase in the number of fruiting bodies/mm^2 in the absence of Con A (Fig. 15). However, Con A greatly increases the numbers of fruiting bodies formed at cell densities greater than 1.0×10^3 cells/mm^2. At this lowest cell density, low Con A concentrations have no effect on the density of fruiting bodies formed; at higher Con A concentrations fruiting body formation is reduced. Above 1.0×10^3 cells/mm^2 each increase in cell density

Figure 15. Effect of Con A on fruiting body density in D. discoideum. Washed vegetative amoebae were deposited in microdrops on hydrophobic agar containing various concentrations of Con A. The following cell densities were studied: ▲, 1.25×10^4 cells/mm^2; □, 1.0×10^4 cells/mm^2; ●, 7.5×10^3 cells/mm^2; △, 5.0×10^3 cells/mm^2; ×, 2.5×10^3 cells/mm^2; ○, 1.0×10^3 cells/mm^2. The total number of fruiting bodies formed in each droplet was counted and from this the fruiting body density was calculated. Each panel (a - e) represents the results of an experiment.


a
b
c
d
e
Fruiting body density (#FB/mm²)
µg Con A/ml agar
0 50 100 200 300 400

generally produces an increase in fruiting body density at a given Con A concentration (Fig. 15). That is to say, as more cells in a given area interact with Con A, more fruiting bodies are formed.

For any one cell density there is a peak representing one concentration of Con A that produces the highest fruiting body density (Fig. 15). In some cases as the cell density increases, a higher concentration of Con A is required to produce the maximum fruiting body density (Fig. 15 c, e). At the highest cell density (1.25×10^4 cells/mm^2) the greatest number of fruiting bodies is usually produced by 200 - 300 µg Con A/ml (Fig. 15 a, b, c). The decline in fruiting body density at Con A concentrations beyond the peak value actually represents an increase in the number of cells which fail to undergo morphogenesis. Generally these are peripheral cells in a population which may be continuously exposed to more Con A than central cells in a population due to diffusion of Con A from uninoculated parts of the plate.

2. Dictyostelium mucoroides

The effect of continuous exposure to Con A on development was also studied in D. mucoroides. While fruiting body morphology is normal in D. mucoroides allowed to undergo morphogenesis on Con A, Con A affects development in the following ways:

a) morphology of the cells is unaltered although aggregation morphology may be affected;

b) aggregation is delayed or inhibited completely;

c) the index of aggregate formation is reduced;

d) there is a decrease in the number of fruiting bodies formed per unit area.

Each of these effects is considered in turn.

a. Morphology of Cells and of Aggregation on Concanavalin A Agar

Unlike D. discoideum, the amoebae of D. mucoroides do not become rounded up when plated on Con A agar. The amoebae assume their usual elongate morphology and appear single at all Con A concentrations tested. Vacuoles appear within the Con A treated cells, but there are fewer than in D. discoideum. At 50 and 100 μg Con A/ml

aggregation morphology of D. mucoroides appears normal (Fig. 16) even though aggregation appears later than in controls. At 200 µg Con/ml amoebae form compact mounds of cells similar to those formed by D. discoideum on the same concentration of lectin. The mounds attract a few amoebae which move toward them singly; the usual aggregation streams are absent. When plated at 1.25×10^4 cells/ml on 200 µg Con A/ml or higher Con A concentrations, many cells do not aggregate; the amoebae remain single and elongate in appearance.

b. Delay and Inhibition of Aggregation in the Presence of Concanavalin A

Using the same criteria applied to D. discoideum, Con A was found to delay the appearance of the first aggregate in D. mucoroides (Table 7). Between 7.5×10^3 and 1.25×10^4 cells/mm^2, 50 µg Con A/ml delays aggregation only 2 - 3 hr, a delay comparable to that observed in D. discoideum. As the ratio of Con A to cells is increased, either by raising the Con A concentration or lowering the cell density, the delay becomes greater than that observed with D. discoideum, until aggregation is

Figure 16. Morphology of aggregation of D. mucoroides on plain agar (a - c) and on 100 µg Con A/ml agar (d - f). In both sequences cells were at 1.25 X 10^4 cells/mm^2. (a, d) 7 hr; (b, e) 10.5 hr; (c, f) 24 hr of development. Notice that at this concentration of Con A the morphology of aggregation is like controls although the timing of aggregation and the size of the fruiting bodies formed differ from controls. X16

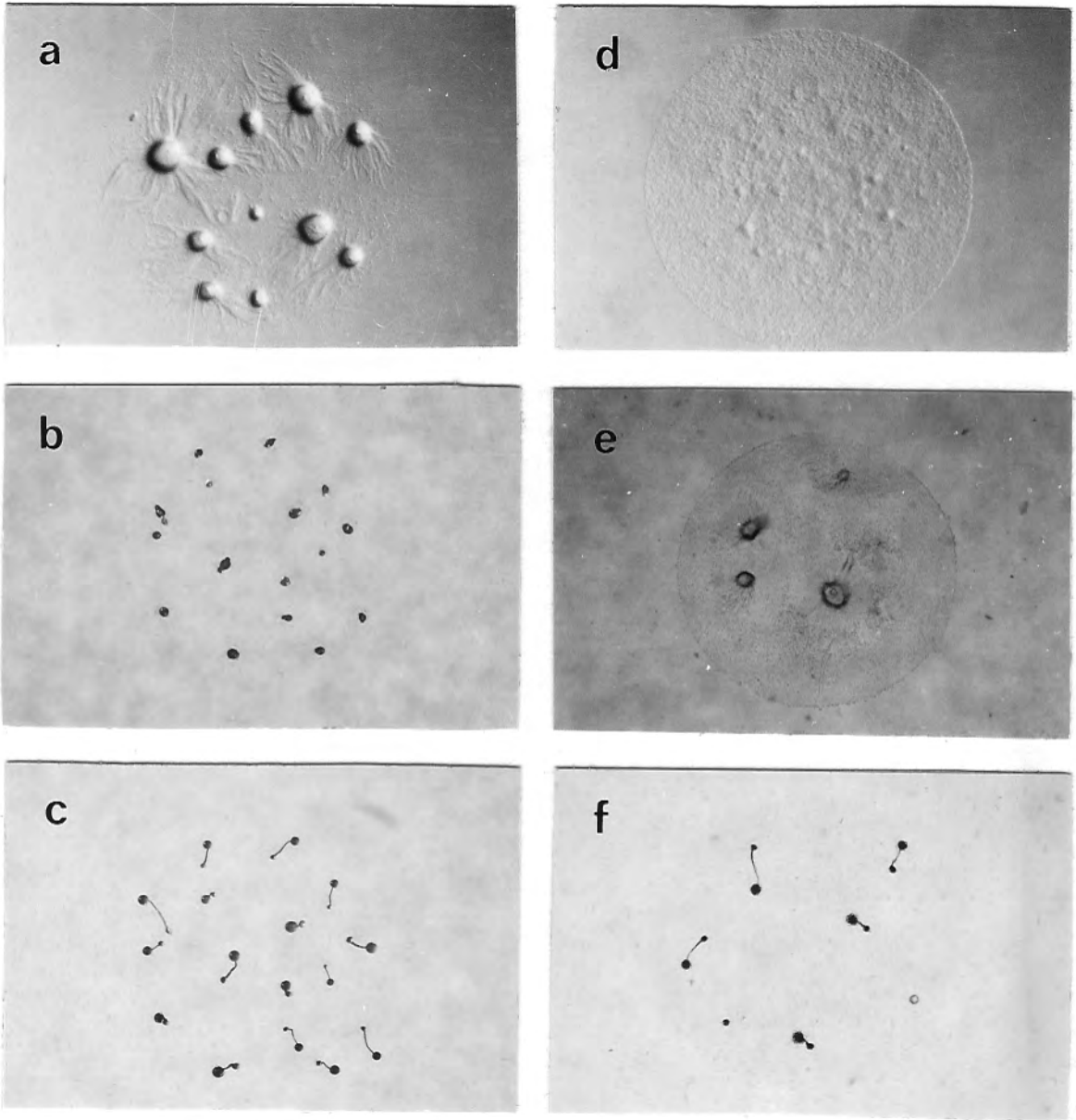

a
d
b
e
c
f

Table 7. Effect of Concanavalin A in delaying aggregation of D. mucoroides amoebae*

Cell treatment	Hours to completion of the first aggregate		
	Cell density (cells/mm^2)		
	2.5×10^3	7.5×10^3	1.25×10^4
0 µg Con A/ml	7	6	5
50	18	9	7
100	17	11	10
200	Agg. Inhib.	33	19
300	Agg. Inhib.	Agg. Inhib.	36
400	Agg. Inhib.	Agg. Inhib.	Agg. Inhib.
5×10^{-2} M α-MG	7	6	6
200 µg Con A/ml + 2.5×10^{-2} M α-MG	8	6	6

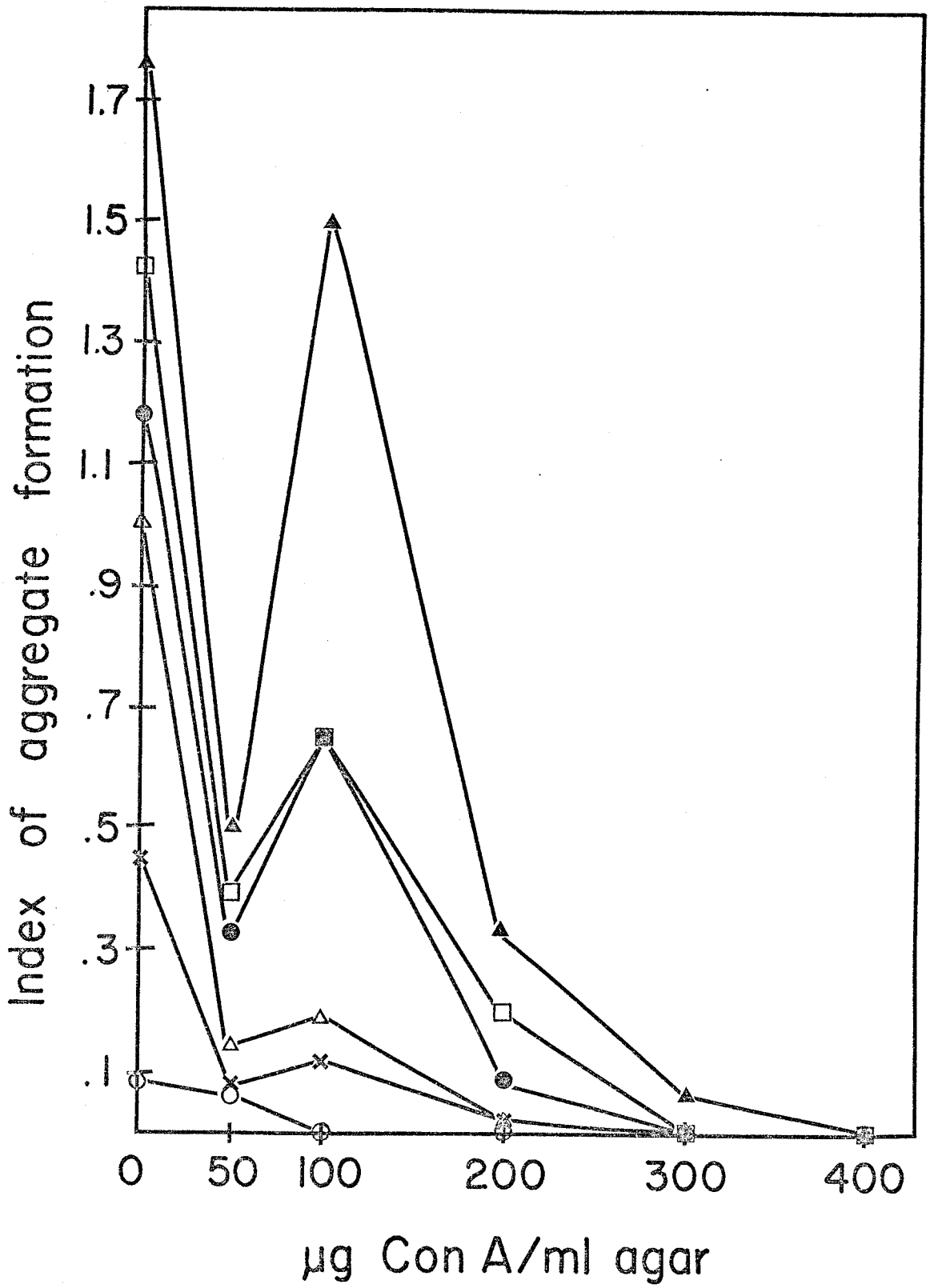
*Experimental procedure was identical to that reported in Table 6 for D. discoideum. Agg. Inhib. means aggregation of amoebae was completely inhibited under the indicated conditions.

completely inhibited. At 300 µg/ml, Con A completely inhibits center formation at all but the highest cell density tested. No signs of aggregation are observed at 400 µg Con A/ml in any experiment with D. mucoroides. This is in contrast to D. discoideum which aggregates under identical conditions.

c. Effect of Concanavalin A on the Index of Aggregate Formation

In addition to delaying the appearance of the first completed aggregate in D. mucoroides, Con A retards the rate at which centers are completed once aggregation has begun (Fig. 17). This was determined by examining the index of aggregate formation. The effect is related to the cell density of responding cultures: control cultures show an increased index of aggregate formation as the cell density increases. In control cultures half of all aggregates are completed over a 2 - 3 hr period regardless of the cell density; therefore, the increase in the index of aggregate formation is due to the fact that increasing the cell density increases the number of centers formed. At any one Con A concentration,

Figure 17. Effect of Con A on the index of aggregate formation in D. mucoroides. Washed vegetative amoebae were deposited in microdrops on agar containing different concentrations of Con A. Aggregation was studied for the following cell densities: ▲, 1.25×10^4 cells/mm^2; □, 1.0×10^4 cells/mm^2; ●, 7.5×10^3 cells/mm^2; △, 5.0×10^3 cells/mm^2; ×, 2.5×10^3 cells/mm^2; ○, 1.0×10^3 cells/mm^2. All Con A concentrations depress the index. Each point is the average of triplicate cultures.

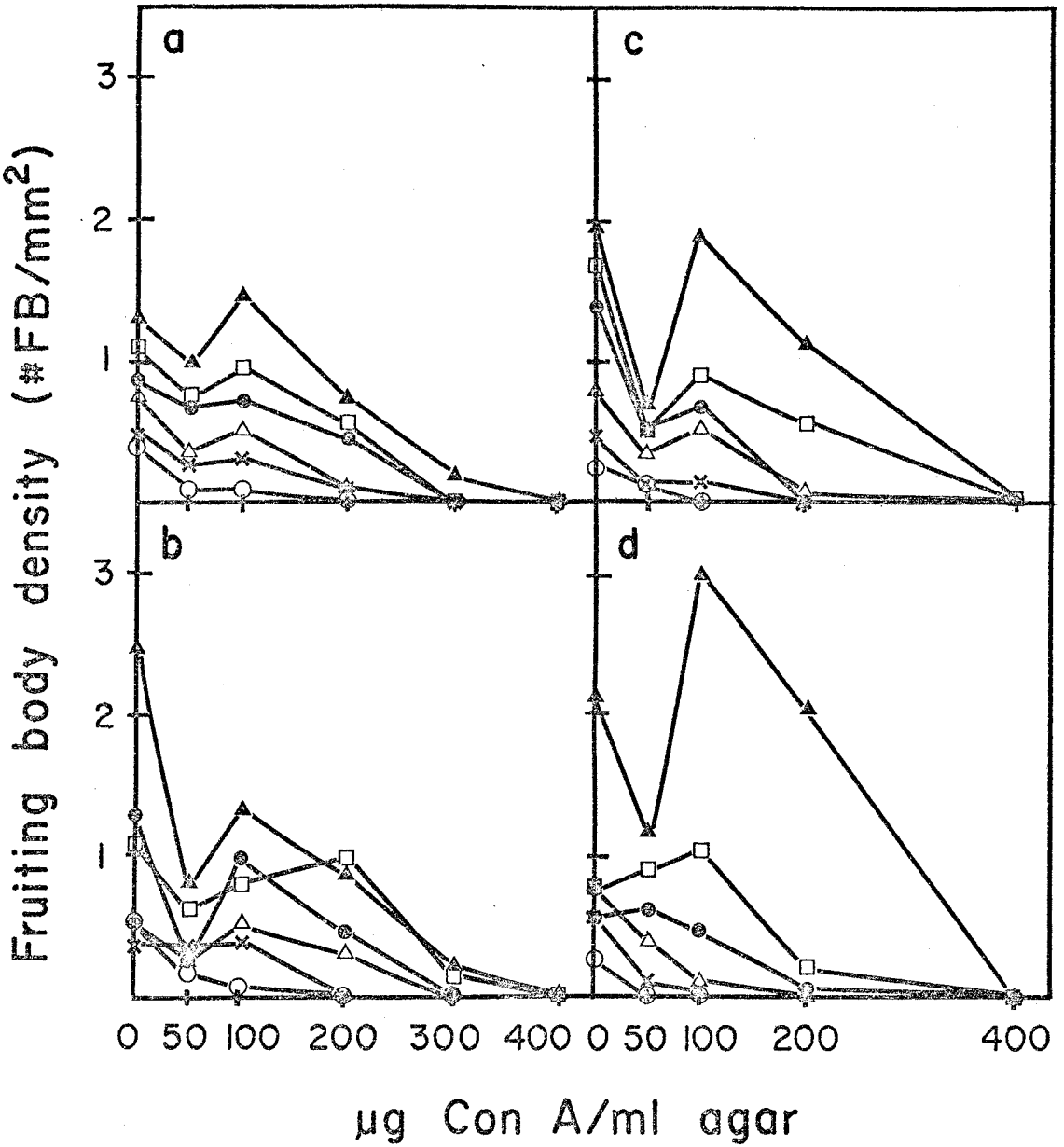

Index of aggregate formation
1.7
1.5
1.3
1.1
.9
.7
.5
.3
.1
0
50
100
200
300
400
µg Con A/ml agar

increasing the cell density usually increases the index of aggregate formation also. But when compared with controls, the index is depressed by Con A. This is a reflection of the fact that fewer centers are formed by D. mucoroides when incubated on Con A agar as well as the fact that the period for completion of aggregates is extended 2 - 7 times by Con A, depending on the concentration.

d. Effect of Concanavalin A on Fruiting Body Density

Unlike D. discoideum, D. mucoroides exhibits a decrease in fruiting body density in the presence of Con A. Generally as the concentration of Con A is increased, the density of fruiting bodies is reduced (Fig. 16, 18). There is a break in the overall trend at 100 µg Con A/ml, where the number of fruiting bodies is greater than at 50 µg or 200 µg Con A/ml, but lower than the control value. One exception was observed in some experiments at 1.25×10^4 cells/mm^2 treated with 100 µg Con A/ml; here the number of fruiting bodies may be above the control value. The other exception is with the lowest density of cells when development is essentially inhibited

Figure 18. Effect of Con A on fruiting body density in D. mucoroides. Washed vegetative amoebae were deposited in microdrops on hydrophobic agar containing various concentrations of Con A. The following cell densities were studied: ▲, 1.25×10^4 cells/mm^2; □, 1.0×10^4 cells/mm^2; ●, 7.5×10^3 cells/mm^2; △, 5.0×10^3 cells/mm^2; ×, 2.5×10^3 cells/mm^2; O, 1.0×10^3 cells/mm^2. The total number of fruiting bodies formed in each droplet was counted and from this the fruiting body density was calculated. Each panel (a - d) represents the results of an experiment using a single population of cells. Each point is the average of 3 - 4 replicate drops.


a
c
b
d
3
2
1
Fruiting body density (#FB/mm²)
0 50 100 200 300 400
0 50 100 200 400
µg Con A/ml agar

at all Con A concentrations tested. The decline in fruiting body density at the higher Con A concentrations (200 - 400 µg/ml) actually results from a decrease in the number of cells participating in aggregation.

B. Effect of Concanavalin A on Cyclic AMP Responsiveness (Konijn Test)

From the preceding results it is apparent that Con A affects aggregation of the slime mold amoebae. Since aggregation has been shown to be mediated by the chemotactic action of cyclic AMP (Bonner et al., 1969), the possibility exists that Con A might be interfering with the response of amoebae to cyclic AMP. To test for this possiblity, aggregation competent amoebae were tested for their ability to respond to exogenously supplied cyclic AMP by means of the Konijn test (Konijn, 1970).

1. Dictyostelium discoideum

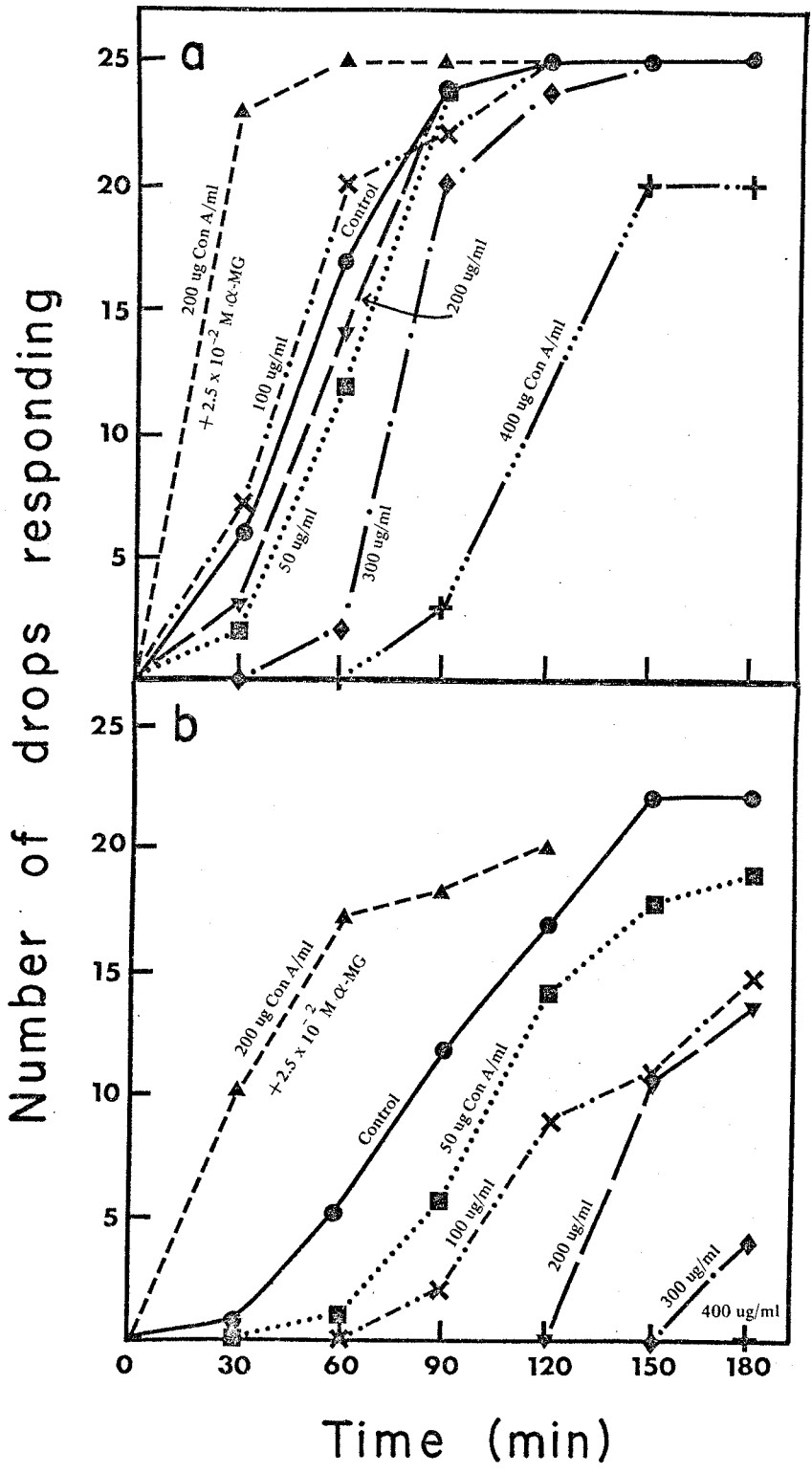
In cultures on low concentrations of Con A (50 - 200) µg/ml) exposed to 10^{-3} M cyclic AMP, the first amoebae are observed to respond by moving outside the droplet boundary at a rate very close to controls (Fig. 19a).

Figure 19. Effect of Con A on the rate of response of a population of droplets of D. discoideum amoebae to (a) 10^{-3} M and (b) 10^{-6} M cyclic AMP. Vegetative cells were deposited on hydrophobic agar in 25 droplets of saline each at a density of 3.7×10^{3} cells/mm^{2}. Cyclic AMP in saline was applied with a microsyringe in a 0.1 µl drop 0.1-0.2/mm from the edge of each droplet of sensitized amoebae. Movement of amoebae outside the perimeter of the drop was considered a response to the cyclic AMP. The rate of response was scored for cells incubated on various concentrations of Con A:

●—, 0 µg/ml; ■····, 50 µg/ml; ✕·—, 100 µg/ml

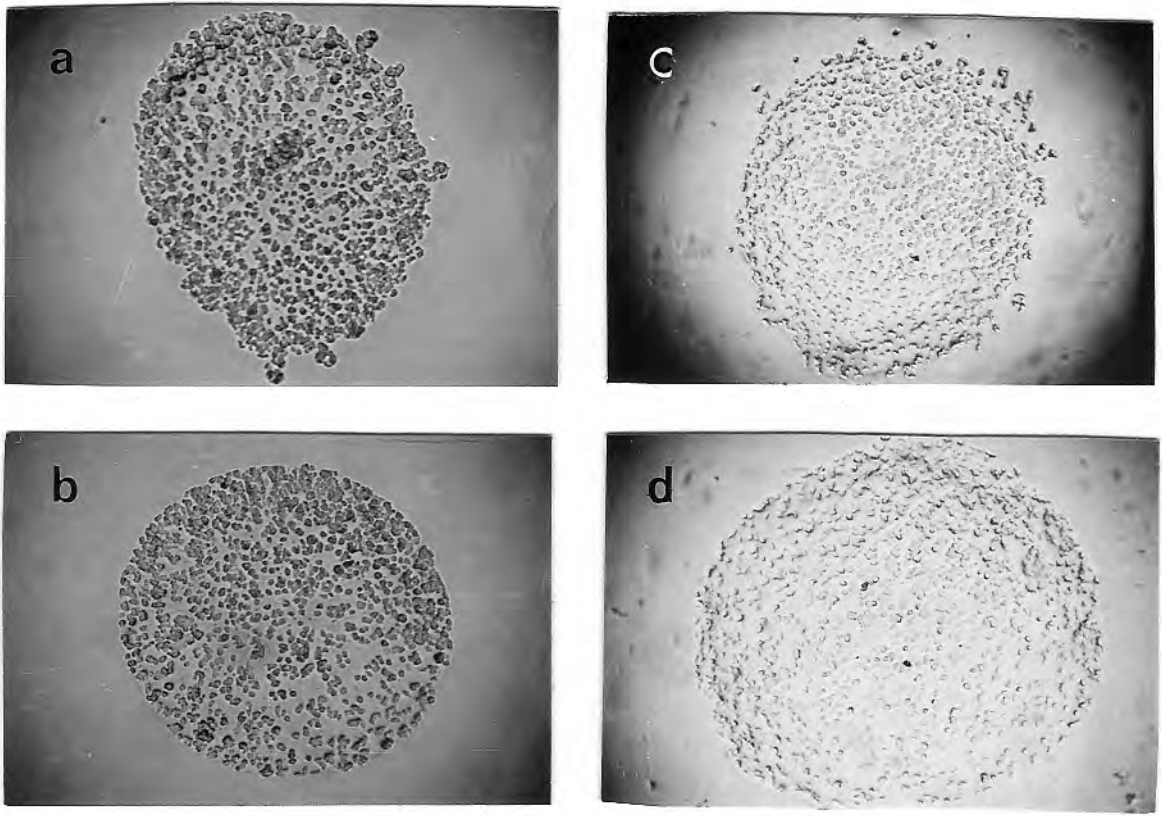
▼—, 200 µg/ml; ◆·—, 300 µg/ml; ✚····, 400 µg/ml;

▲– –, 200 µg/ml + 2.5×10^{-2} M α-MG.


a
b
Number of drops responding
Time (min)
0
30
60
90
120
150
180
5
10
15
20
25
200 ug Con A/ml
+2.5 x 10⁻² M α-MG
100 ug/ml
Control
50 ug/ml
300 ug/ml
200 ug/ml
400 ug Con A/ml
200 ug Con A/ml
+2.5 x 10⁻² M α-MG
Control
50 ug Con A/ml
100 ug/ml
200 ug/ml
300 ug/ml
400 ug/ml

Although the time for movement of the first amoebae beyond the droplet boundary, which is considered to be a positive Konijn response (Konijn, 1970), is similar to controls, the extent of movement of most of the cells in each droplet on Con A is clearly less than controls (Fig. 20). In some cases cells on low concentrations of Con A (50 - 200 µg/ml) respond to cyclic AMP by crowding away from the boundary. Above 200 µg Con A/ml there is inhibition of the initial response, the degree of inhibition increasing with Con A concentration. These results suggest that cyclic AMP overcomes the Con A-mediated inhibition when the Con A concentration is low relative to cyclic AMP concentration. If so, lowering the cyclic AMP concentration should result in reduction of the cyclic AMP response at the lower concentrations of Con A. This predicted shift in response was found. Using 10^{-6} M cyclic AMP the response of D. discoideum is depressed by all Con A concentrations (Fig. 19b). As the Con A concentration is increased, the rate of the initial response is further reduced. At 300 and 400 µg Con A/ml inhibition of the cyclic AMP response is essentially complete.

Figure 20. Response of a droplet of D. discoideum amoebae to 10^{-3} M cyclic AMP. (a) Cells on plain agar 2.5 hr after depositing cyclic AMP near the right side at the point marked by the impression in the agar. (b) Control on plain agar to which no cyclic AMP was added showing no movement of cells outside the droplet. (c) Cells on 200 μg Con A/ml agar 2.5 hr after applying cyclic AMP to the right of the droplet. (d) Control on 200 μg Con A/ml agar to which no cyclic AMP was added showing no movement of cells outside the droplet.


a
C
b
d

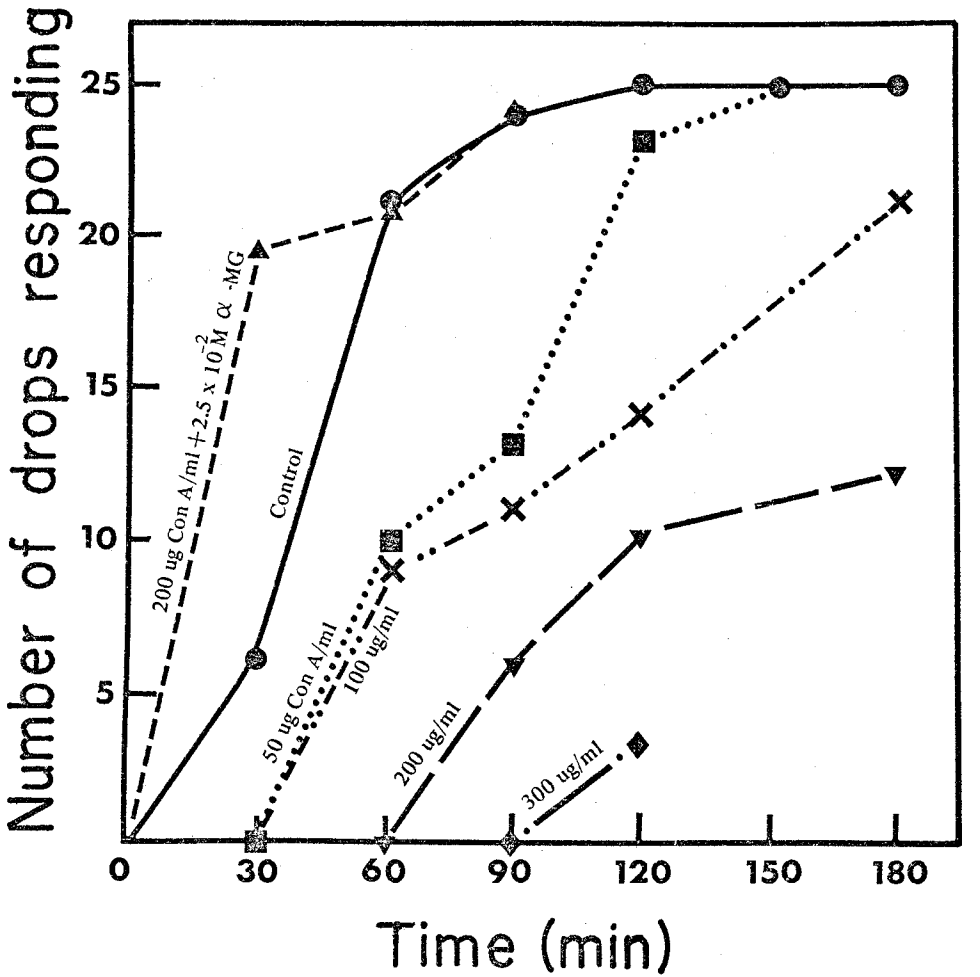
2. Dictyostelium mucoroides

In D. mucoroides the chemotactic movement of amoebae to 10^{-3} M cyclic AMP is inhibited by all concentrations of Con A tested (Fig. 21). In this species the degree to which the response rate is inhibited is directly related to the Con A concentration.

C. Effect of α-Methyl-D-Glucopyranoside on Morphogenesis and the Cyclic AMP Response

In both the morphogenetic experiments and the cyclic AMP response assays, a haptenic sugar for the Con A combining site, α-methyl-D-glucopyranoside (α-MG) was employed as a control for the specificity of Con A action. In the morphogenetic experiments on both species, cultures to which α-MG alone or both Con A and α-MG had been added showed a developmental rate near control values (Table 6, 7). When D. discoideum aggregates on α-MG or α-MG with Con A, fruiting body density is equivalent to controls. In contrast to what is seen on Con A, the aggregation centers formed on α-MG exhibit few, unusually long, thick streams indicating the sugar itself has some effect on

Figure 21. Effect of Con A on the rate of response of a population of droplets of D. mucoroides to 10^{-6} M cyclic AMP. Droplets were prepared as in Fig. 19. The rate of response was scored for cells incubated on various concentrations of Con A: ●—, 0 µg/ml; ■···, 50 µg/ml; ✕·—, 100 µg/ml; ▼—, 200 µg/ml; ◆·—, 300 µg/ml; +···, 400 µg/ml; ▲–, 200 µg/ml+2.5 X 10^{-2} M α-MG.


Number of drops responding
25
20
15
10
5
0
30
60
90
120
150
180
Time (min)
200 ug Con A/ml + 2.5 x 10⁻² M α -MG
Control
50 ug Con A/ml
100 ug/ml
200 ug/ml
300 ug/ml

the morphology of aggregation. In D. mucoroides α-MG alone reduces fruiting body density slightly from controls. With Con A it reverses somewhat the Con A mediated reduction of fruiting body density but does not raise it to control levels, possibly because of its own effect on aggregation. Aggregation morphology is similar to that of D. discoideum on α-MG alone. In the study of the cyclic AMP response α-MG not only counteracts the inhibitory effect of Con A, but produces a more rapid response than controls (Fig. 17a, b; 18). When tested alone, α-MG increases the rate of response of the amoebae to cyclic AMP. These controls indicate that because α-MG is able to reverse the action of Con A on aggregation and the cyclic AMP response, the effect of Con A on the slime mold is mediated by specific interaction of Con A with residues of one of its haptenic sugars.

D. Effect of Concanavalin A on Cell Motility

In order to examine the possiblity that the Con A mediated delay in aggregation might be due to Con A immobilizing the cells, movement of individual amoebae was studied. The average rates of movement of amoebae

plated on plain agar or agar with Con A added appear in Table 8. At 1 X 10^3 cells/mm^2, D. discoideum amoebae in droplets deposited on 50 µg Con A/ml move slightly more slowly than do untreated amoebae during the first two hours after plating. Later, cells on Con A move at rates nearly equivalent to controls. When allowed to aggregate under these conditions, the Con A-treated amoebae are severely delayed: at 28 hr, when 85% of control droplets show completed aggregates, only 14% of the Con A-treated droplets show evidence of aggregation. Thus the delay in aggregation is apparently not due to a decrease in cell motility.

When the ratio of cells to Con A concentration is increased to 2.5 X 10^3 cells/mm^2 on 50 µg Con A/ml, the Con A-treated cells move at a rate near control cells initially, but move more slowly at later times. The most pronounced difference is during the 2.25 - 2.75 hr post-plating period. Under these conditions, Con A has less effect on delay of aggregation than at the lower cell density. By 20 hr, when all of the control droplets have aggregated, 70% of the Con A-treated populations have done so.

Table 8. Rate of movement of amoebae in the presence and absence of Concanavalin A*

Cell density (cells/mm^2)	Hours after plating	Rate of movement (mm/hr)	
		Plain agar	Con A agar (50 µg/ml)
		D. discoideum	
1.0 X 10^3	0.50- 1.00	0.169	0.119
	1.75- 2.25	0.159	0.116
	4.75- 5.25	0.082	0.086
	10.00-10.75	0.069	0.087
2.5 X 10^3	0.50-1.00	0.153	1.44
	2.25-2.75	0.208	0.087
	7.50-8.00	0.153	0.101
		D. mucoroides	
1.0 X 10^3	0.50-1.00	0.096	0.163
	2.50-3.00	0.106	0.229
	4.50-5.83	0.304	0.357
	4.83-5.25	0.328	0.475

*Vegetative cells were washed from plates and applied to either plain or Con A agar in 10^{-1} µl droplets producing the test cell densities. Movement of individual amoebae was mapped using a microscope equipped with a camera lucida.

In both controls and experimental cultures there seem to be two populations of cells with respect to movement: those actively moving about and others which have rounded up and are stationary. Rounded cells were observed to elongate and commence movement during observation. It should be noted that the proportion of cells that are stationary will dramatically affect the average rate of movement of the sample population.

Washed vegetative D. mucoroides amoebae were plated at 1 X 10^3 cells/mm^2 on 50 µg Con A/ml. The amoebae incubated on Con A were clearly moving faster than the untreated cells during each period of observation (Table 8). These conditions of culture were shown to delay aggregation of Con A-treated D. mucoroides amoebae.

III. Biochemical Studies

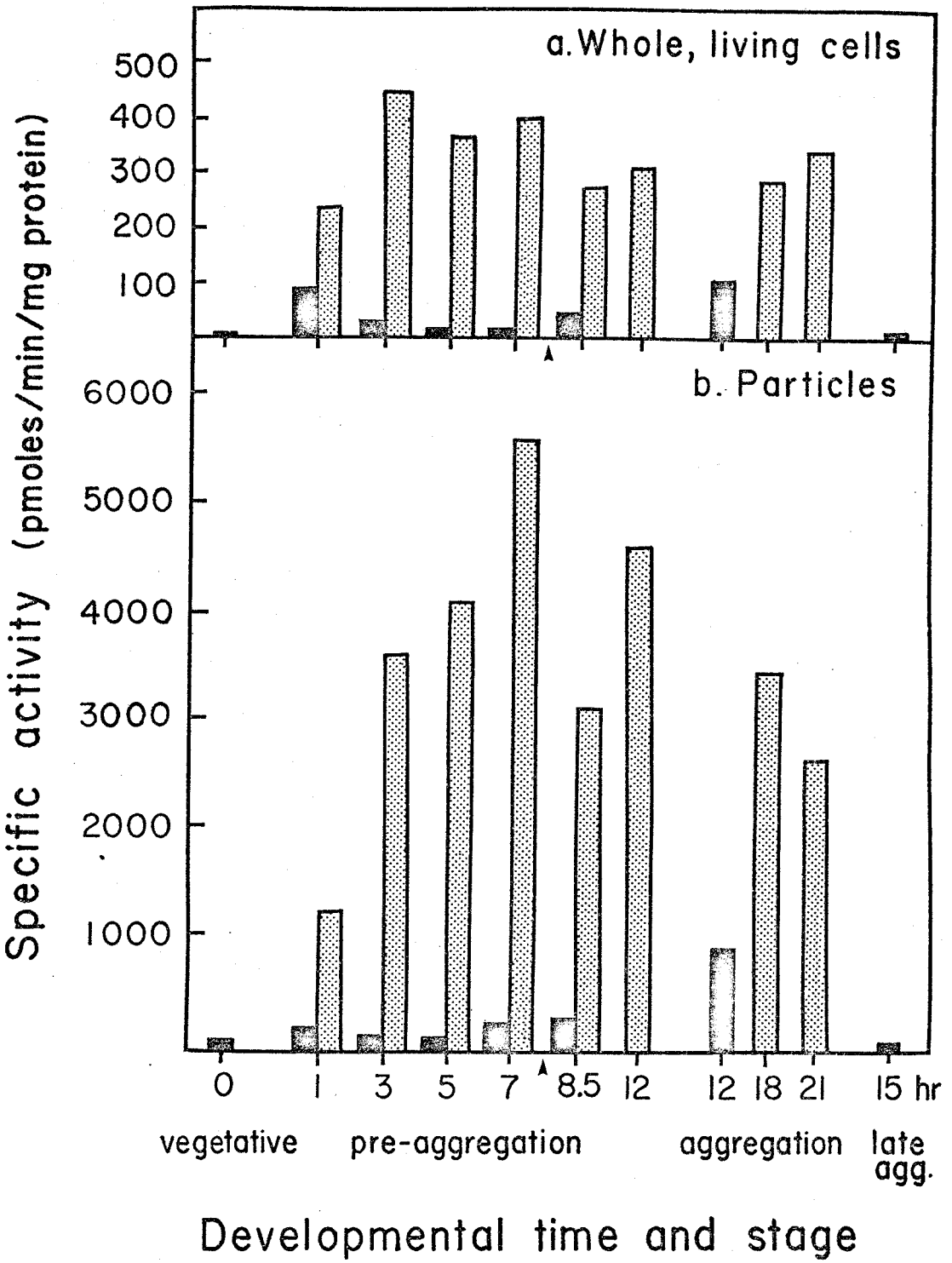
Malchow et al. (1972) described a membrane-bound cyclic AMP phosphodiesterase (mPDE) in D. discoideum. This enzyme is developmentally regulated so that it increases in specific activity at the time of aggregation; at all other periods it was found at low specific activities. The authors suggest that mPDE participates in the detection of cyclic AMP and chemotactic aggregation of the amoebae. It was therefore decided to examine the possibility that Con A affects aggregation and the cyclic AMP response by affecting the activity of this enzyme through its interaction at the cell surface.

A. Membrane-Bound Phosphodiesterase Activity of D. discoideum Incubated on Concanavalin A

1. Enzyme Activity During Development

The activity of mPDE exposed at the cell surface was determined by using whole cells in the assay. Over the course of normal development, there is a transient increase in activity about one hour after plating washed vegetative cells on agar (Fig. 22a). The activity drops

Figure 22. Membrane phosphodiesterase activity of D. discoideum amoebae during development on plain and Con A agar. (a) Activity at the surface of whole, living cells. Amoebae were plated on agar at 1 X 10^4 cells/mm^2, incubated at 22°C in the light and collected at the developmental times indicated. An arrow separates determinations on different preparations of cells. Cells were washed 3 X in Sörensen's phosphate, pH 6.5. 100 µl of cells was mixed with 200 µl of phosphate buffer and placed in a water bath at 19 ± 0.5°C. After 5 min preincubation, 10 µl of [^{3}H]cyclic AMP (150 pmoles) was added. Samples were removed at 5 and 10 min and the reaction stopped with TCA. Activity was analyzed as described in Materials and Methods. Each bar is the result of duplicate determinations. (b) Activity in particulate preparations of membranes. Cells were collected and washed in 0.05 M TRIS-HCl buffer, pH 7.4 containing 0.02 M $MgSO_4$ and frozen. The 27,000 x g sediment was assayed for PDE activity. The assay was conducted as for whole cells except that to 50 µl of particles was added 5 µl of [^{3}H]cyclic AMP (200 pmoles). ■ , plain agar ▩ , 300 µg Con A/ml agar


a. Whole, living cells
500
400
300
200
100
b. Particles
6000
5000
4000
3000
2000
1000
Specific activity (pmoles/min/mg protein)
0
1
3
5
7
8.5
12
12
18
21
15 hr
vegetative
pre-aggregation
aggregation
late agg.
Developmental time and stage

by 3 hr and does not increase again until early aggregation, about 12 hr under these conditions of culture. At late aggregation the specific activity of mPDE is very low. Changes in mPDE were also measured using particulate preparations which include membranes from internal regions of the cell as well as the surface. The specific activities for such preparations are always higher than in the case of mPDE activities determined on whole living cells (Fig. 22b). However, the changes during development in the specific activity of mPDE of particle preparations generally parallel the changes in the surface mPDE alone. This has also been shown in the work of Malchow et al. (1972).

In cells developing in the presence of 300 µg Con A/ml, the activity of mPDE exposed at the cell surface increases dramatically by one hour to a level greater than that observed in controls at the time of aggregation (Fig. 22a). The specific activity of mPDE exposed at the cell surface continues to increase until 3 hr, then remains high (2.7 - 4.5 times aggregating controls) through the pre-aggregation period. When the cells finally aggregate on Con A, surface mPDE is 2.8 - 3.4 times more active than that observed at aggregation of control cultures.

Particulate preparations also show increases in mPDE activity relative to controls (Fig. 22b). At one hour of development mPDE activity of Con A-treated cultures is roughly six times that of one hour controls, and already exceeds that observed at aggregation of the controls. Specific activity of mPDE continues to increase until 7 hr when activity on Con A is 20 times greater than controls of the same age and 6 times greater than the peak activity observed at aggregation in controls. Although the assays for later developmental stages were done on a different preparation of cells, activity seems to drop subsequent to the 7 hr peak, but remains high preceding and during aggregation on Con A agar. Activity measured in particulate preparations prepared from Con A-treated cells is at all times much greater relative to controls than that assayed for enzyme at the surface of whole cells.

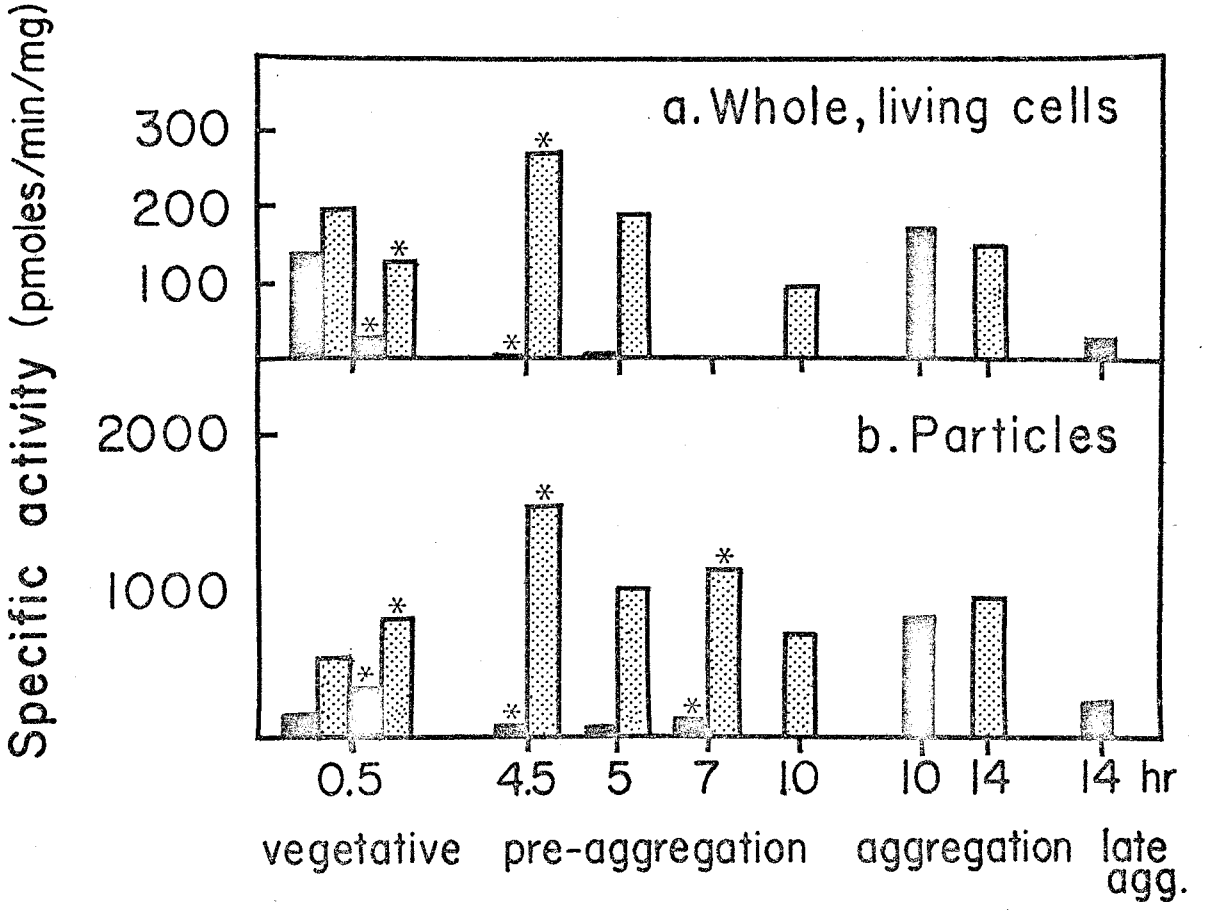
One series of experiments was conducted in which the inoculated plates were held in the cold room for 10 - 12 hr before allowing development to proceed at 22°C. This is a procedure commonly used for Dictyostelium as it facilitates handling. Results for one developmental sequence using two different preparations of amoebae are

reported in Fig. 23a, b. When plated cells are held at 4°C before being shifted to 22°C, it is difficult to say that a given developmental time is equivalent to the same time for cells not held in the cold. Nevertheless, control cultures show roughly the same patterns of mPDE activity for whole cells and particulate membranes as for cells not held at 4°C. One possible exception is the mPDE activity in particles from cells out of the cold for one half hour (Fig. 23b), which is substantially higher than those observed early in development in Fig. 22b.

Con A stimulates mPDE under these conditions of culture also, but to a lower degree than for cells not held in the cold. For surface mPDE in whole cells incubated on Con A the highest activity assayed is 61% of the highest activity observed in cells not held at 4°C. At the time control cells are aggregating, the enzyme activity of cells on Con A actually drops below control levels, and then increases when these cells aggregate (Fig. 23a). For particulate preparations the activity of Con A-treated amoebae is about 28% of the highest activity observed in cells not held at 4°C. Activity of particles from Con A cultures drops to near control levels at the time of early

Figure 23. Membrane phosphodiesterase activity of D. discoideum amoebae during development at 22°C. after 10 - 12 hr preincubation at 4°C. Cells were prepared and assayed as described in Fig. 22. A * designates determinations from one preparation of cells; all others are from one other preparation. (a) Activity at the surface of whole, living cells. (b) Activity in particulate preparations of membranes.

■ , plain agar ▨ , 300 μg Con A/ml agar


Specific activity (pmoles/min/mg)
a. Whole, living cells
300
200
100
b. Particles
2000
1000
0.5
4.5
5
7
10
10
14
14 hr
vegetative
pre-aggregation
aggregation
late agg.
Developmental time and stage

aggregation of the controls and then increases again as the Con A cultures begin to aggregate (Fig. 23b).

2. Kinetics of Appearance of Elevated Phosphodiesterase Activity

In order to examine the Con A-mediated increase in mPDE more closely, specific activity of the enzyme was assayed at 10, 30 and 60 min of development. The data from whole cells for surface mPDE (Fig. 24) shows that activity of the surface enzyme increases in controls by 30 min, Con A has yet to produce a measurable effect on surface mPDE activity. By one hour mPDE activity at the cell surface of Con A cultures is three times that of controls. α-MG, the haptenic sugar for Con A, has no effect on mPDE at one hour. When mixed with Con A, α-MG reduces the difference in mPDE activity between Con A-treated cultures and controls to 16%, indicating that the increased activity seen in the presence of Con A is due to Con A binding.

The data from particulate preparations of cells in the first hour of development indicates that by 30 min Con A has already enhanced mPDE activity to a level

Figure 24. Phosphodiesterase activity assayed in living D. discoideum amoebae during the first hour after plating on plain or Con A agar. mPDE at the cell surface (——) was determined as described in Fig. 22a. ePDE secreted per minute (·····) was determined as described in Table 9. ●, ■, plain agar; ✕, □, 300 μg Con A/ml agar; O, 3.75×10^{-2} M α-MG; ⊗, 300 μg Con A/ml + 3.75×10^{-2} M α-MG.

400
300
200
100
Specific activity (pmoles/min/mg protein)
30
20
10
Specific activity secreted (pmoles/min/mg protein/min)
0
10
30
60
Time (min)

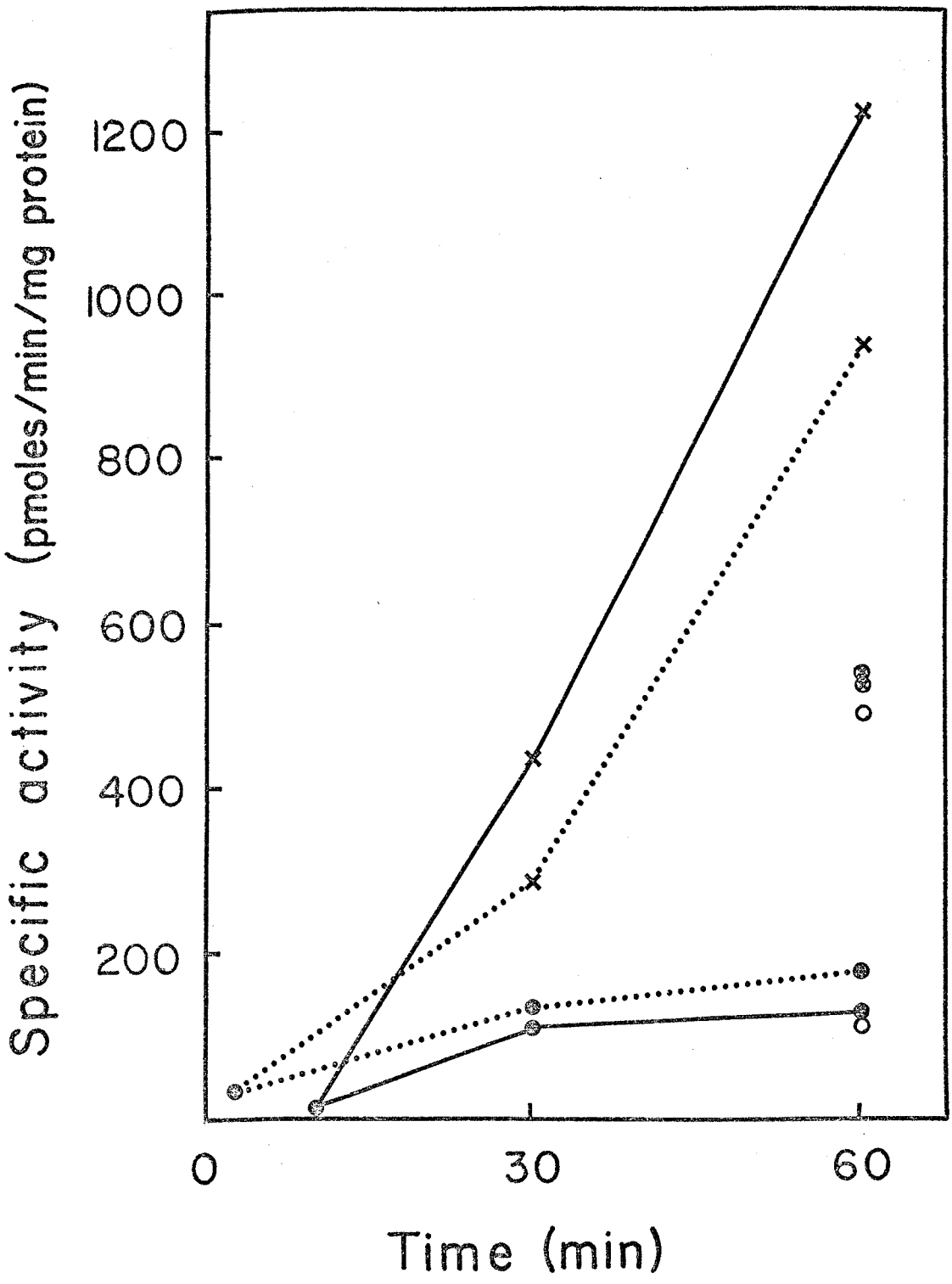
2 - 4 times that of controls (Fig. 25). By one hour, when mPDE enhancement of surface mPDE is first detected (Fig. 24), mPDE activity in particulate preparations from Con A cultures exceeds that of controls by 5.2 - 10 fold (Fig. 25). α-MG added to Con A cultures reduces by about half the enhancement effect of Con A on mPDE.

B. Studies Concerning the Mode of Action of Concanavalin A on Phosphodiesterase Activity in D. discoideum

1. Enzyme Activity in the Presence of Bovine Serum Albumin

In order to determine whether the increase in mPDE activity observed in cells incubated on Con A is a non-specific effect resulting from incubation on exogenous protein, the amoebae were allowed to develop on bovine serum albumin (BSA) at a concentration equimolar to the concentration of Con A used. It was found that the specific activity of mPDE in a particulate membrane preparation from cells cultured 3 hr in the presence of BSA is equivalent to controls.

Figure 25. Effect of Con A on phosphodiesterase activity of crude particle fraction; two separate experiments. Cells were analyzed for mPDE in particles as described in Fig. 22b. For these experiments cells were plated at a density of 1 X 10^4 cells/mm^2 on agar containing (●–, ●··) agar only; (✕–, ✕··) 300 μg Con A/ml agar; (O) 3.75 X 10^{-2} M α-MG; (⊗) 300 μg Con A/ml agar + 3.75 X 10^{-2} M α-MG.


Specific activity (pmoles/min/mg protein)
1200
1000
800
600
400
200
0
30
60
Time (min)

2. Effect of Concanavalin A on Other Enzymes

It is possible that Con A stimulates the metabolism of D. discoideum amoebae generally so that many enzymes appear at higher levels than normal. To test this possibility the activities of other enzymes were determined for cultures incubated on 300 μg Con A/ml. Extracellular phosphodiesterase (ePDE), which is secreted by the amoebae at high levels during the growth phase (Chang, 1968; Chassy, 1972; Pannbacker and Bravard, 1972), but is relatively inactive during development (Riedel et al., 1973), was assayed from initial plating to late aggregation. Con A increases the specific activity of ePDE secreted per minute by a low amount throughout development (Table 9). During the pre-aggregation period Con A appears to delay the usual fluctuations observed in ePDE activity.

Lactate dehydrogenase, a soluble enzyme of the cytoplasm, was assayed at 3 hr of development on Con A agar in two separate experiments. There is no difference in the levels of LDH activity between control cells and those incubated on Con A (Table 10).

Table 9. Extracellular phosphodiesterase activity secreted during development of *D. discoideum* on plain agar or Concanavalin A agar.*

Developmental stage	Hours after plating	Phosphodiesterase activity secreted/min (pmoles/min/mg protein/min)	
		Plain agar	Con A agar (300 µg/ml)
Washed vegetative	0.0	6.8	
Pre-aggregation	1.0	5.4	8.0
	3.0	0.5	2.9
	5.0	0.2	2.0
	7.0	0.9	2.6
	8.5	1.3	0.9
	12.0		1.3
Aggregation	12.0	0.3	
	18.5		1.1
Late aggregation	15.0	.2	
	21.0		1.5

*Cells were scraped from agar at the appropriate time and washed three times in Sörensen's phosphate, pH6.5. 100 µl of cells was mixed with 200 µl of buffer and placed in a water bath at 19±0.5°C. After 15 min, the cells were removed by centrifuging for 5 min at 2°C. 150 µl of supernatant was extracted and used to assay for ePDE. After a 5 min preincubation, 150 pmoles of [^{3}H] cyclic AMP was added. The reaction was stopped with TCA after 10 min and ePDE activity determined (see Materials and Methods).

Table 10. Lactate dehydrogenase activity in *D. discoideum* at 3 hr of development on plain agar of Concanavalin A agar*

Volume of supernatant assayed	Lactate dehydrogenase activity ($\Delta O.D._{340nm}$/min/mg protein)	
	Plain agar	Con A agar (300 µg/ml)
Exp. 1: 50 µl	17.3×10^{-3}	18.7×10^{-3}
25 µl	19.0×10^{-3}	17.6×10^{-3}
Exp. 2: 50 µl	12.2×10^{-3}	10.2×10^{-3}
25 µl	12.8×10^{-3}	14.9×10^{-3}

*After 3 hr of development on agar, cells were collected in 0.5M phosphate buffer, pH 7.4, and frozen overnight. Each sample was thawed, centrifuged at 10,000xg for 20 min and the supernatant assayed for breakdown of pyruvate in the presence of NADH by monitoring the change in optical density at 340 nm.

3. Metabolic Inhibitor Studies

Cycloheximide, at concentrations which have been found to inhibit [^{3}H]-leucine incorporation in slime molds (Sussman, 1965), lyses amoebae incubated on Con A but not those on agar containing cycloheximide alone. It was therefore not possible to determine whether inhibition of protein synthesis alone would inhibit Con A-mediated enzyme enhancement.

When actinomycin D is added to Con A agar at concentrations which inhibit 75% of the [^{3}H]-uridine incorporation (Sussman and Sussman, 1965), the amoebae show lower levels of mPDE activity in particulate membrane preparations than in the presence of Con A alone after 3 hr of development. In two separate experiments, actinomycin D with Con A reduced to 45% and 18% the mPDE activity measured in the presence of Con A alone.

4. Enzyme Activity Assayed in the Presence of Concanavalin A

When Con A is added to the mPDE assay mixture of cells that have undergone 5 hr of development in the absence of Con A, cells assayed for 10 min show mPDE

activity in the cell surface close to controls. Particulate preparations from control cultures at 0.5 , 5 and 7 hr were assayed for mPDE activity in the presence of Con A; they also have activities equivalent to controls (Table 11).

5. Inhibitors of Membrane Phosphodiesterase Activity

a. Cyclic Inosine Monophosphate

In order to determine whether the mPDE affected by Con A is similar to that which normally increases in activity at aggregation, inhibitors of slime mold PDE (Malchow et al., 1972) were employed. 5mM cyclic 3',5'-inosine monophosphate (cyclic IMP) reduces activity of particulate mPDE from aggregating amoebae (12 hr) to 12 pmoles/min/mg compared with 1043 pmoles/min/mg in untreated particles. Thus mPDE activity is reduced to 1.1% by controls of cyclic IMP. Particulate preparations from cells cultured on Con A agar for 12 hr have a specific activity of 5072 pmoles/min/mg compared with 288 pmoles/min/mg for those assayed in the presence of 5mM cyclic IMP. This represents 5.7% of the activity of particles in the absence of cyclic IMP.

Table 11. Membrane phosphodiesterase activity in *D. discoideum* assayed in the presence of Concanavalin A*

Sample	Hours after plating	Phosphodiesterase activity (pmoles/min/mg protein)	
		Enzyme assayed in:	
		buffer only	buffer+ 500 µg Con A/ml
Surface mPDE	5.0	2.7	6.5
Particule mPDE	0.5	344.0	302.0
	4.5	121.0	121.0
	7.0	142.0	166.0

*Cells were removed from plain agar after various periods of development and assayed for membrane phosphodiesterase as described in Materials and Methods. Con A in buffer was added to the assay mixture of one of identical preparations producing a final concentration of 500 µg Con A/ml reaction mixture. The total time of exposure to Con A in treated samples was 15 min. Reported are the means of duplicate determinations.

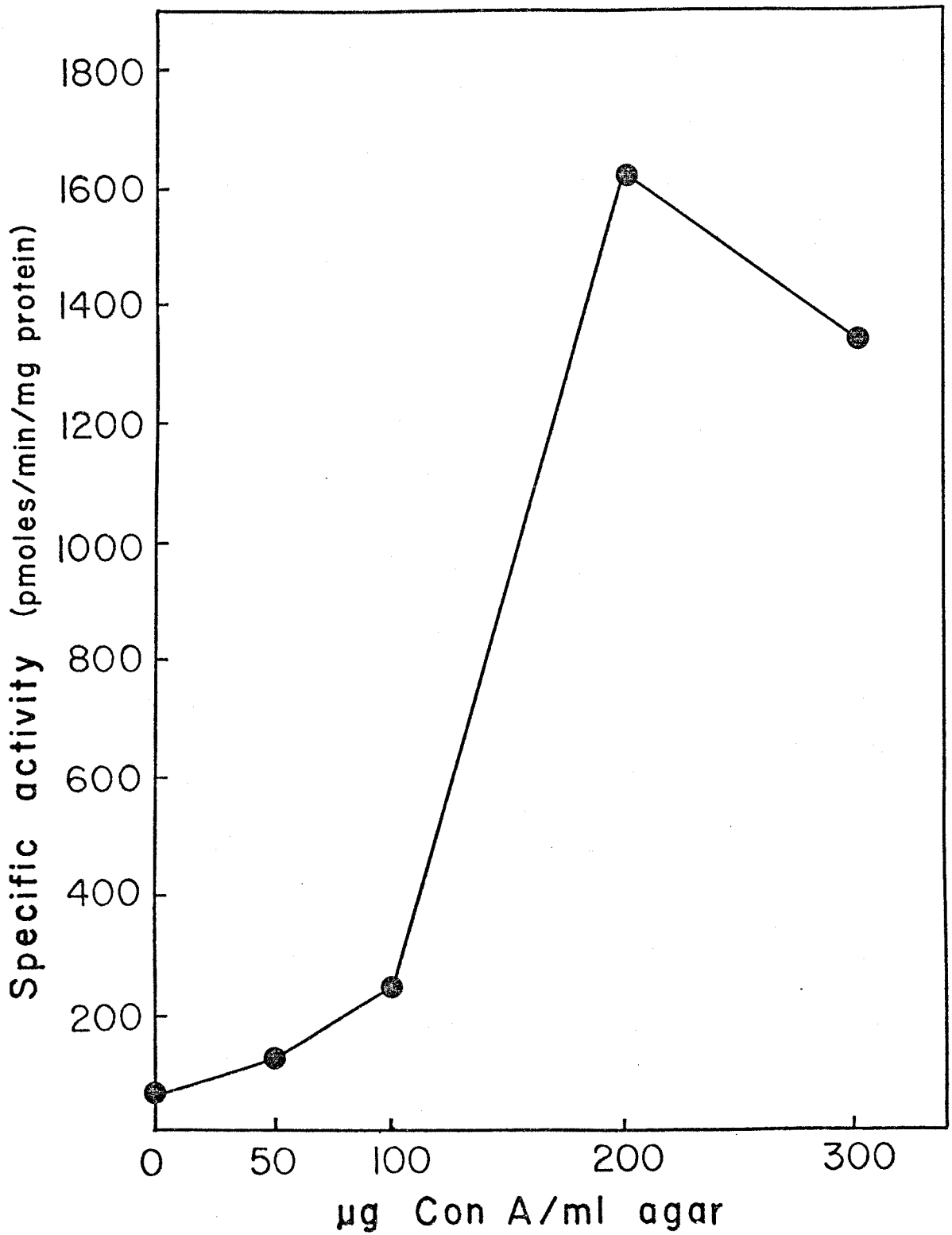
b. Glutathione

Aggregation phase particles (12 hr) from control cultures assayed in the presence of 10 mM glutathione have a specific activity of 634 pmoles/min/mg, 61% of untreated samples. Particulate preparations from amoebae incubated 12 hr on Con A have a specific activity of 2291 pmoles/min/mg in the presence of 10 mM glutathione, which is 45% of untreated membranes.

6. Effect of Concanavalin A Concentration on Membrane Phosphodiesterase Activity at an Early Developmental Stage

The mPDE enhancing ability of 50 to 300 µg Con A/ml agar was determined using particle preparations from 3 hr cultures. Below 200 µg Con A/ml there is a small increase in mPDE activity in 3 hr time; 200 and 300 µg Con A/ml dramatically increase mPDE activity during this period (Fig. 26).

Figure 26. Effect of Con A concentration on phosphodiesterase activity in D. discoideum. Cells were collected at 3 hr 20 min of development on agar containing Con A. Particles were prepared and mPDE analyzed as described in Fig. 22b.


1800
1600
1400
1200
1000
800
600
400
200
Specific activity (pmoles/min/mg protein)
0
50
100
200
300
µg Con A/ml agar

7. Effect of Cyclic AMP on the Concanavalin A-Mediated Increase in Membrane Phosphodiesterase Activity

Because cyclic AMP was found to reverse the Con A-mediated inhibition of chemotaxis, the effect of cyclic AMP on mPDE activity was examined. This enzyme is greatly stimulated in particles prepared after 3 hr of exposure to 10^{-3}M cyclic AMP (Table 12). Lower concentrations of cyclic AMP have little affect on the activity of the enzyme. When mixed with 300 µg Con A/ml in agar, 10^{-3} M cyclic AMP prevents the large increase in specific activity seen with Con A alone. In the presence of 300 µg Con A/ml the lower concentrations of cyclic AMP do not reverse Con A-mediated enzyme enhancement.

C. Receptivity of D. discoideum Amoebae to Phosphodiesterase Enhancement by Concanavalin A at a Later Developmental Stage

To determine whether D. discoideum remains receptive to Con A-mediated enhancement of mPDE during later stages of development, mPDE activity was assayed in cells removed from plain agar after 5 hr of development and transferred

Table 12. Effect of cyclic AMP on particle-bound phosphodiesterase activity in D. discoideum at 3 hr of development.*

Cyclic AMP concentration	Phosphodiesterase activity (pmoles/min/mg protein)	
	Cyclic AMP agar	Cyclic AMP + 300 µg Con A per ml agar
0 (control)	33	1800
10^{-3} M	298	380
10^{-5} M	55	1870
10^{-7} M	69	2400

*Cells were removed from agar containing either cyclic AMP or cyclic AMP + Con A. Particles were prepared and mPDE assayed as described in Materials and Methods. Reported are the means of duplicate determinations.

to Con A agar for 1 hr. Such cells have 36% higher mPDE activity than controls handled in the same way but transferred to plain agar, and 20% higher mPDE activity than cells kept on plain agar for 6 hr of development (Table 13). This stimulation of activity is low when compared with the 600-1000% increase in activity observed after 1 hr on Con A commencing at the initiation of development.

Morphologically, cells at 5 hr of development which are transferred to 300 μg Con A/ml for 1 hr resemble those incubated on Con A agar between 0 and 1 hr of development. The cells appear separate, round and vacuolated. Control cells which were replated on plain agar at 5 hr quickly become extended, have few vacuoles and within an hour have collected into mounds of cells surrounded by areas free of cells; these cultures resemble the undisturbed controls at 6 hr.

Table 13. Effect of Concanavalin A on phosphodiesterase activity in D. discoideum at different stages of development*

Conditions of culture	Phosphodiesterase activity (pmoles/min/mg protein)					
	Whole cells				Particles	
	mPDE		ePDE		mPDE	
	Plain agar	Con A agar	Plain agar	Con A agar	Plain agar	Con A agar
1 hr on plain agar or Con A agar	126	377	7.6	11.1	128	1228
	59	481	4.4	30.8	180	940
	86	228	5.4	8.0	205	1257
5 hr on plain agar replated to plain or Con A agar for 1 hr	45	88	0.7	4.5	109	152
6 hr on plain agar or Con A agar	47	630	2.0	6.1	128	1476

*Vegetative cells from liquid culture flasks were washed and plated on either plain agar or agar containing 300 µg Con A/ml. Cells which had been allowed to develop for 5 hr were rubbed from the agar with a glass rod and saline, centrifuged 5 min, resuspended at the original cell density and replated on either plain or Con A agar. After 1 hr these replated cells were collected as well as those which had remained undistributed for 6 hr. Whole cells were analyzed for mPDE and ePDE; particles were prepared and analyzed for mPDE described in Materials and Methods. The PDE activities reported for 1 hr are the results of three separated experiments. Each value is the average of duplicate determinations.

D. Membrane Phosphodiesterase Activity of *D. mucoroides* Incubated on Concanavalin A

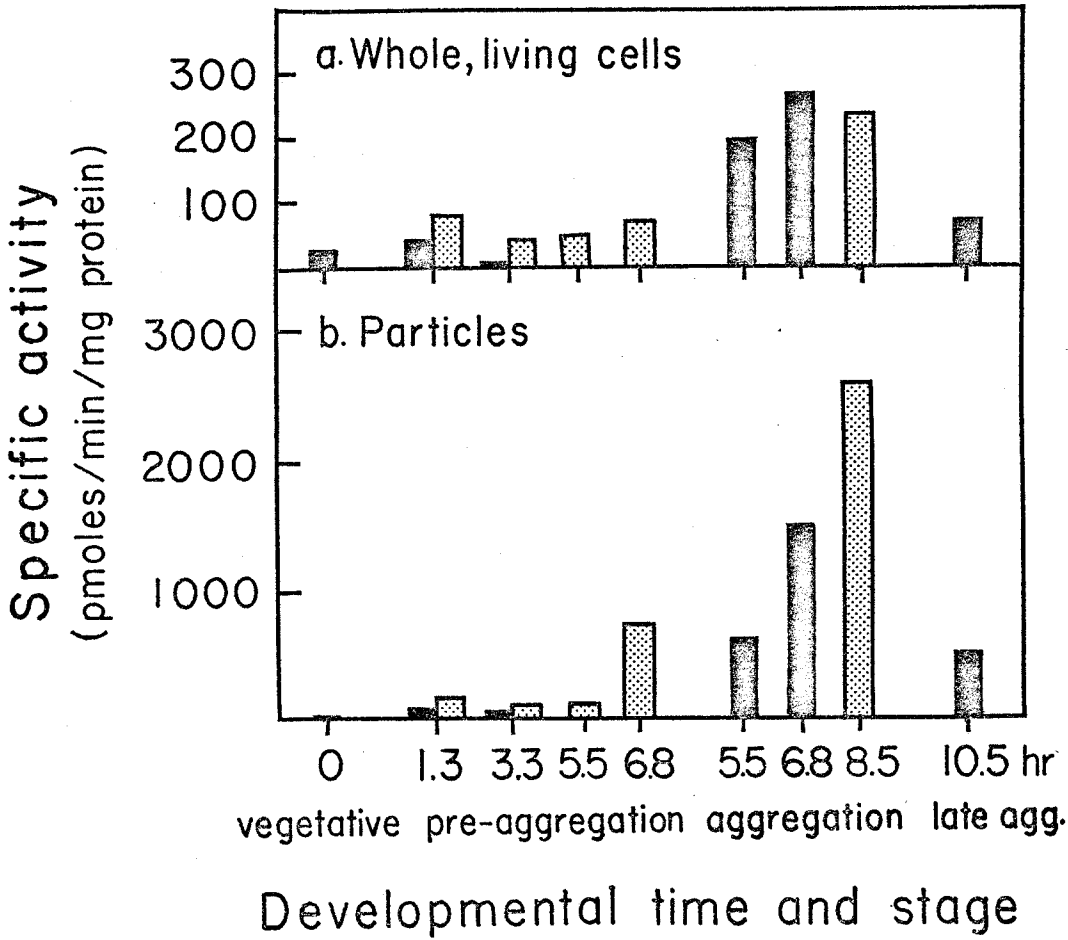
1. Membrane Phosphodiesterase Activity during Development

The data for surface mPDE assayed during development in *D. mucoroides* appears in Fig. 27a. Surface mPDE in control cultures of *D. mucoroides* is initially present at a specific activity near that observed initially for *D. discoideum* (Fig. 22a). Activity drops prior to increasing during the aggregation period. Specific activity of mPDE at the surface of aggregating *D. mucoroides* is more than twice that observed in aggregating *D. discoideum*. mPDE activity decreases again at late aggregation.

In *D. mucoroides* the effect of Con A on mPDE activity was studied at 100 μg Con A/ml, the highest concentration which allows most of the cells to develop. There is a slight increment in mPDE (40 pmoles/min/mg) over controls at 1 hr 20 min on Con A. The specific activity of mPDE at the cell surface remains elevated above controls at roughly this increment throughout the pre-aggregation period, then increases as the amoebae approach aggregation

Figure 27. Membrane phosphodiesterase activity of D. mucoroides amoebae during development on plain or Con A agar. (a) Activity at the surface of whole, living cells. Amoebae were plated on agar at 1 X 10^4 cells/ml agar, incubated at 22°C in the light and collected at the developmental times indicated. This graph was derived from a single preparation of cells. Cells were assayed PDE as described in Fig. 22a for D. discoideum and Materials and Methods. Each bar is the result of duplicate determinations. (b) Activity in particulate preparations of membranes was analyzed in the same way as for D. discoideum (see Fig. 22b).

■ , plain agar ▩ , 300 µg Con A/ml agar.


a. Whole, living cells
300
200
100
b. Particles
3000
2000
1000
Specific activity
(pmoles/min/mg protein)
0
1.3
3.3
5.5
6.8
5.5
6.8
8.5
10.5 hr
vegetative
pre-aggregation
aggregation
late agg.
Developmental time and stage

(Fig. 27a). This increase in surface mPDE is delayed in association with the morphological delay in aggregation observed in the presence of Con A. Extracellular PDE is secreted at similar specific activity levels in control and Con A-treated cultures during development (Table 14).

In particulate membrane preparations the mPDE activity of control cells remains low until early aggregation when it increase (Fig. 27b). This activity drops by late aggregation. Cultures incubated on 100 µg Con A/ml agar for 1 hr 20 min have 2.5 times the specific activity of mPDE in controls; mPDE activity remains slightly higher than controls through most of the pre-aggregation period (Fig. 27b). At 6 hr 45 min, when controls are aggregating, activity of particulate mPDE from Con A cultures is rising, although it is only half of control values. By early aggregation on Con A specific activity has risen to a level somewhat higher than control cultures at the same developmental stage.

Table 14. Extracellular phosphodiesterase activity secreted during development of *D. mucoroides* on plain agar or Concanavalin A agar.*

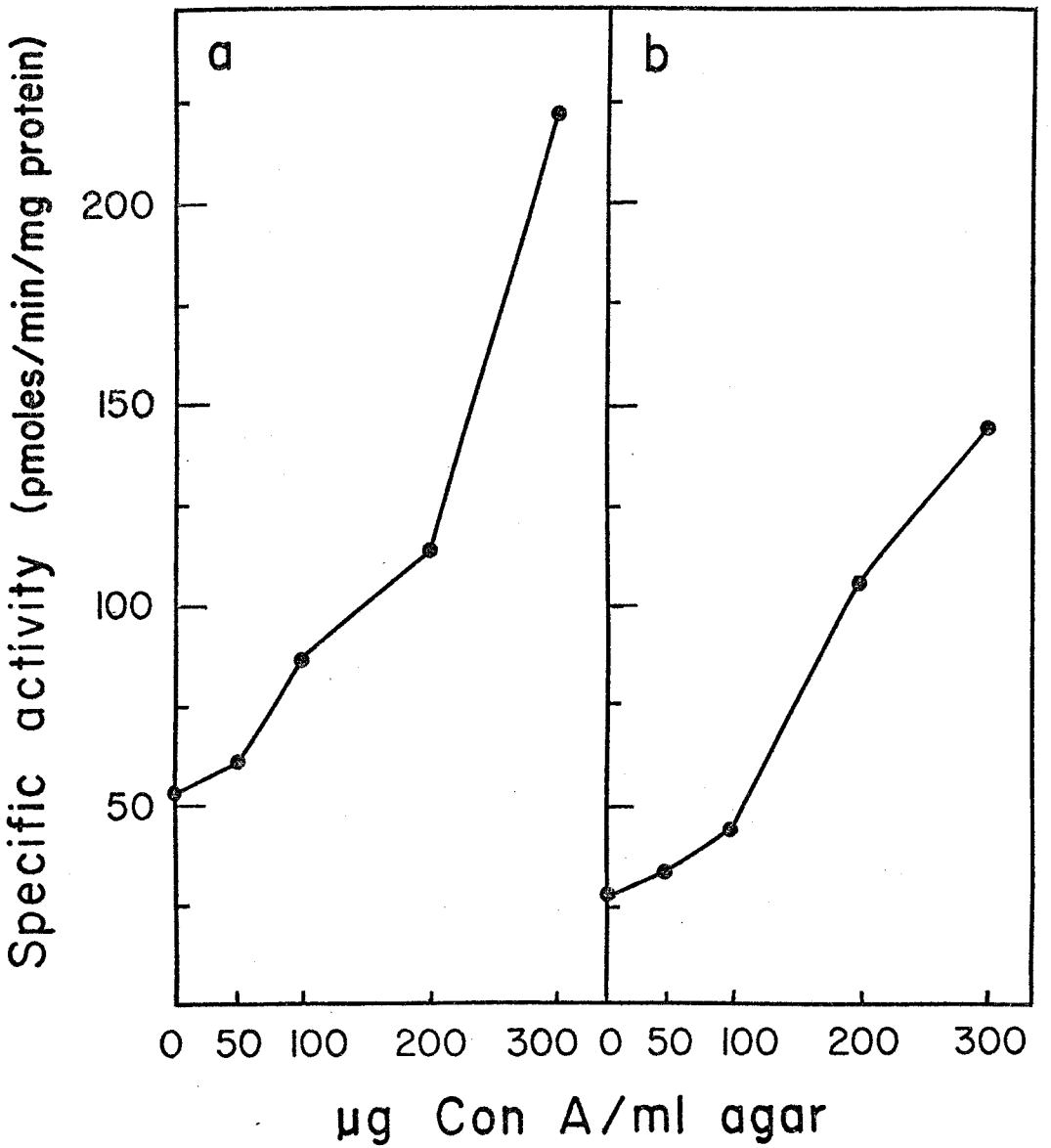
Developmental stage	Hours after plating	Phosphodiesterase activity secreted/min (pmoles/min/mg protein/min)	
		Plain agar	Con A agar (300 µg/ml)
Washed vegetative	0.0	7.5	
Pre-aggregation	1.3	4.8	6.8
	3.3	1.0	1.8
	6.8		0.9
Aggregation	6.8	2.2	
	8.5		3.0
Late aggregation	10.5	1.3	

*The procedure was identical to that used for ePDE assays in *D. discoideum* as reported in Table 9.

2. Effect of Concanavalin A Concentration on Membrane Phosphodiesterase Activity

The ability of 50 - 300 µg Con A/ml to stimulate mPDE was examined in particulate membrane preparations from D. mucoroides. In order that results could be compared with those obtained for D. discoideum at 3 hr of development, membranes were prepared both at 1 hr 20 min, which represents an equivalent developmental period for D. mucoroides, and 3 hr 20 min, which represents an equivalent period of exposure to Con A. The degree of stimulation of mPDE is directly related to the Con A concentration at both developmental times with 300 µg Con A/ml producing by far the greatest increase (Fig. 28). The mPDE activity at all concentrations of Con A is greater at 1 hr 20 min than at 3 hr 20 min. During this period enzyme activity of cells on 0, 50, and 100 µg Con A/ml drops by 50%. Activity on 200 µg Con A/ml remains constant while on 300 µg Con A/ml it drops by 40%.

Figure 28. Effect of Con A concentration on phosphodiesterase activity in D. mucoroides. Cells were collected at (a) 1 hr 20 min and (b) 3 hr 20 min of development on agar containing Con A. Particles were prepared and mPDE assayed as described in Fig. 22b.


a
b
Specific activity (pmoles/min/mg protein)
200
150
100
50
0 50 100 200 300 0 50 100 200 300
µg Con A/ml agar

DISCUSSION

The importance of this work arises from the finding that the activity of a developmentally regulated enzyme can be influenced by molecular interactions at the cell surface. In the general sense, this contributes to our knowledge of the role the cell surface can play in controlling development. In the specific case of cellular slime mold aggregation, it supports the hypothesis that events at the cell surface are important for aggregation as well as the notion that membrane phosphodiesterase (mPDE) is important in regulating aggregation. Furthermore, the results strongly suggest that the mPDE enzyme can be controlled by events at the level of the cell surface. The work points up important differences between two species of Dictyostelium which aggregate in response to cyclic AMP. The data which support these assertions will be discussed in turn.

I. Interaction of Concanavalin A with the Cell Surface

A. Pattern of Binding and Redistribution of Receptors

Con A binds to the cellular slime mold surface by interaction with receptor molecules, most likely a variety of glycoproteins and/or glycolipids all of which are receptors for the lectin by virtue of the appropriate specific sugars present in the carbohydrate moiety. Normally the Con A receptor molecules are randomly dispersed on the cell surface, as shown when washed vegetative amoebae are fixed prior to exposure to Con A and stained to localize bound Con A. However, incubation of Con A with the living cell brings about a redistribution of its surface receptors so that they coalesce as a cap at one end of the cell. Subsequently, the Fl-Con A may be found in spots as well as the cap. Bound Con A and its receptors are then shed by the cell. The Con A-receptor complex, as detected by using either Fl-Con A or the diaminobenzidine method, does not appear in the cytoplasm of these cells (Gillette et al., 1974). Beug et al. (1973) found that specific antibody directed against cell surface components likewise is not endocytosed but is sloughed by aggregation-competent D. discoideum amoebae. Weeks (1975) using ^{125}I-Con A has failed to find evidence of endocytosis while Darmon and Klein (1976) using a similar method suggest that some lectin is internalized. The preponderance of evidence indicates that Con A produces its effect on the

slime mold solely by its interaction with specific receptors at the cell surface.

This situation bears many similarities to the interaction of tetravalent Con A with lymphocytes and fibroblasts. Both of these cell types possess randomly distributed Con A receptors (Inbar et al., 1973b); Rosenblith et al., 1973) which are rearranged into caps when incubated with Con A (Smith and Hollers, 1970; Rosenblith et al., 1973). Although these cells can endocytose the Con A-receptor complex, internalization of the lectin apparently is not a prerequiste for the biological effects of Con A on lymphocytes (see Introduction; Greaves and Bauminger, 1972). Cap formation in these cells depends upon the generation of energy by the cell and on functional microfilaments (Taylor et al., 1971; de Petris, 1975); receptor-microtubule protein interactions have also been implicated (Edelman et al., 1973; de Petris, 1975; Hoffstein et al., 1976). These findings have given rise to the theory that those surface receptors which cluster or cap are those which extend through the membrane and attach to anchor molecules in the cytoplasm (de Petris and Raff, 1973). Further, Yahara and Edelman (1975) suggest that mitogenic stimulation of lymphocytes by multivalent ligands may rely on metabolic changes initiated by rearrangements of the cytoplasmic

anchors as a result of ligand-induced rearrangement of cell surface receptors.

Con A causes a clustering of surface receptors in both lymphocytes and slime mold amoebae, and produces biological effects in both by its actions at the cell surface. Like lymphocytes, D. discoideum does not cap Fl-Con A in the presence of sodium azide therefore energy is required for Con A to cap its receptors (Gillette, unpublished observations; Weeks and Weeks, 1975). It is therefore possible that a common mechanism may transduce surface changes into biological effects. In the case of the slime mold this suggests that Con A activates mPDE by interaction with surface sites which may be linked to the machinery responsible for activation of a pre-existing molecule or de novo synthesis of the enzyme and its incorporation into the membrane.

B. Effect of Continuous Exposure to Concanavalin A on Receptor Availability and Redistribution

The effect of continuous exposure to Con A on Con A receptor availability can be assessed by comparing binding of Fl-Con A by cells incubated on Con A agar with binding by control cells scraped from plain agar at the same developmental time. In the case of cells scraped from Con A

agar, Fl-Con A should bind only to Con A binding sites not already occupied by Con A from the medium and so Fl-Con A is in a sense a 'negative' stain. The results of this study demonstrate that at all developmental stages cells incubated on Con A agar as well as those briefly suspended in Con A bind Fl-Con A. The fact that cells which have been on Con A agar for 45 min or longer bind Fl-Con A at all is evidence that Con A receptors on the amoebae are not saturated by Con A from the medium. However, during the pre-aggregation period, especially at 0.75,2 and 5 hr, staining is less intense than in control cells indicating that a substantial number of Con A receptors have been occupied by unlabeled Con A from the medium.

The Con A-binding sites to which Fl-Con A binds may actually be on new receptors made in response to turnover of Con A receptors which redistributed when cross-linked by Con A from the agar. Lymphocytes which are incubated in a saturating concentration of antibody against surface immunoglobulin and washed, cap the immunoglobulin molecules and within one hour remove them from the surface into cytoplasmic vesicles; new immunoglobulin molecules begin to appear on the cell

surface by 2 hr and by 4 - 6 hr are completely regenerated (Loor et al., 1972). After a brief exposure (15 min) to Con A in suspension, the Con A-receptor complex is shed from the slime mold surface by 3 - 5 hr (Gillette et al., 1974); that study did not investigate the timing of reappearance of receptors but because all stages of slime mold cells continuously incubated in the presence of Con A do bind some Fl-Con A, it is probable that the slime mold also regenerates Con A receptors. Although saturating concentrations of Con A may exist in the agar, Con A-binding sites on new receptors may remain unbound for some time after synthesis because only the lower side of the cell is in direct contact with the substrate containing Con A.

After the first hour of development, the patterns of redistribution of Fl-Con A bound to the surface of cells continuously exposed to Con A differ from cells incubated on plain agar before staining. After 2 hr and 5 hr on Con A agar, the cells show the same initial staining pattern in redistributing Fl-Con A as controls but after the fluorescent cap and spots appear simultaneously, the cap disappears completely and only spots of fluorescence are seen (Table 3). At 7 hr and 9.5 hr, cells incubated on

Con A agar bind Fl-Con A only in spots within 10 min after staining; this localization of stain is more rapid than is found earlier on Con A agar and completely unlike control cells at any stage.

These changes in the pattern of Fl-Con A binding may be a result of the way the cell is processing unlabeled Con A already bound to it from the medium at the time the stain is applied. Receptors which are in the process of being capped or forming spots due to interaction with Con A from the medium may have free Con A-binding sites; Fl-Con A binding to these unsaturated sites could be expected to progress rapidly to a terminal stage in the shedding process. The processing of these bound receptors could also directly influence the rate at which newly bound receptors are prepared for shedding.

The unusual patterns of Fl-Con A staining observed in cells continuously incubated on Con A agar also may reflect regeneration of receptors. The spotted pattern of staining with Fl-Con A by D. discoideum amoebae which have been incubated on Con A agar several hours is similar to the pattern of binding of fluorescent anti-surface immunoglobulin antibody by splenic lymphocytes which have

been incubated in saturating concentrations of unlabeled anti-surface immunoglobulin antiserum. If the lymphocytes are continuously exposed to anti-surface immunoglobulin antiserum, they begin within 1 hr to lose immunoglobulin receptors. Subsequently, about 30% of the cells show only one or two fluorescent spots if stained with fluorescently labeled antiserum (Loor et al., 1972); cells with typical staining are almost absent. Loor et al.(1972) suggest that the staining in spots only, without ring or cap formation, is the result of repeated rounds of surface immunoglobulin regeneration due to rapid and continuous turnover as a consequence of the interaction with the corresponding antibodies. It is possible that the regenerated receptors are synthesized faster than the cytoplasmic anchors (possibly microfilaments) to which they are normally connected. This may mean that many are not hooked up to the redistribution machinery of the cytoplasm and would account for the absence of capping.

The change in the redistribution pattern of cells continuously exposed to Con A is observed only during the pre-aggregation period of development. By early aggregation cells which have undergone development on Con A agar have

open Con A receptors which bind Fl-Con A and redistribute it in a pattern similar to controls of the same developmental stage. This indicates that unlabeled Con A from the agar has less effect on redistribution of the open receptors which bind Fl-Con A at aggregation than earlier samples from Con A agar. It may also mean that there is no more Con A available in the medium.

C. Membrane Fluidity and Cell Motility

These results demonstrating redistribution of Fl-Con A are evidence that cellular slime mold amoebae have fluid membranes. This finding agrees with current theory which views the cell membrane as a fluid mosaic with a lipid matrix in whose plane embedded proteins are able to float freely and independently (Singer and Nicholson, 1972). It also suggests that the slime mold membrane, like that of the lymphocyte, is subject to modulation of surface constituents. Among unicellular organisms, Paramecium, Tetrahymena and Colpoda also can modulate their surfaces, that is, redistribute and turnover surface components in response to antibody made against surface molecules (Beale, 1957). Entamoeba redistributes Fl-Con A into a posterior cap (Martínez-Palomo et al., 1974).

The significance of surface modulation lies in the fact that the membrane is sensitive to differential stimulation and in response can segregate its components and/or trigger cytoplasmic events. Lymphocytes possess many different surface molecules which can be distinguished by their ability to bind antigens and lectins with different specificities (Loor, 1974). Binding at only certain of these sites, presumably those which are attached to cytoplasmic structures, stimulates mitogenesis (Edelman et al., 1973). Other sites, when bound, do not cause extensive membrane rearrangements (Taylor et al., 1971; Karnovsky and Unanue, 1973), do not increase membrane fluidity, and do not mediate mitosis (Toyoshima and Osawa, 1975). These sites stay exposed and may actually redistribute forward in a countercurrent fashion when a group of specific receptors flow backward while being capped and stripped from the membrane (de Petris and Raff, 1973). Thus capping and shedding of surface receptors by Con A is an event limited to those receptors which bind Con A; it does not signify turnover of the membrane as a whole.

Capping is an active phenomenon of the fluid membrane rather than a passive response to cross-linking part of the surface of the membrane during cell movement (Unanue et al., 1974). Capping does not require adhesion to a substrate and actual cell movement since caps form on lymphocytes kept in suspension by agitation and in drops of cells floating on oil as well as on cells in contact with solid surfaces (de Petris and Raff, 1973). D. discoideum amoebae also cap Con A both when moving on a solid substrate and when suspended in liquid (Gillette et al., 1974). The observation that drugs which completely inhibit lymphocyte movement do not inhibit capping (Unanue et al., 1974) unequivocally demonstrates that this phenomenon does not have its basis in cell movement. Rather, the ability of a cell to cap certain receptors when treated with specific multivalent ligand is related to receptor density and distribution on the cell surface (Karnovsky and Unanue, 1973) as well as the nature of the surface-cytoplasmic interrelationships (Yahara and Edelman, 1975).

However, capping itself seems to affect cell movement. de Petris and Raff (1973) as well as Unanue et al. (1974) report that cross-linking lymphocytes at their surfaces with

bivalent antibody triggers them into moving; there is no indication that when the surface is covered with antibody the lymphocytes are immobilized. Fibroblasts treated with Con A are also highly motile and rapidly clear Con A from the leading edge of the cell (Ambercrombie et al., 1972). In both lymphocytes and fibroblasts the cross-linked receptors flow to the trailing end of the moving cell where they cap. These results are comparable to those found in amoebae of the cellular slime mold. Con A receptors are capped in both species without immobilizing the cells.

II. Effects of Continuous Exposure to Concanavalin A on Development

A. Delay of Aggregation

The results of this study show that when washed vegetative amoebae are allowed to develop on Con A agar, aggregation is delayed in a dose-dependent manner. As the cell density is reduced, it takes longer for cells to aggregate on a given concentration of Con A (Table 6).

Several elements of the cell surface have been implicated in aggregation (Malchow and Gerisch, 1974). Membrane phosphodiesterase, cyclic AMP-binding sites, and end-to-end contact sites are constituents of the cell surface which appear just prior to aggregation. Together with a macromolecular inhibitor of extracellular phosphodiesterase they appear to be under common genetic control (Malchow and Gerisch, 1974). Because Con A acts at the cell surface, it may affect aggregation through affecting the activity of one or all of the elements. I examined mPDE activity in the presence of Con A and found it dramatically changed.

The effect of Con A on mPDE activity, like the effect on aggregation, is dose-dependent (Fig. 26), that is, enzyme activity is increased by incubation on higher

Con A concentrations. This is not a proportional relationship, however; under the conditions tested the enzyme is not greatly enhanced until Con A concentrations exceed 100 µg/ml agar. This suggests that there is a threshold concentration beyond which Con A is highly effective in increasing enzyme activity.

The immediate cause for the delay in aggregation of D. discoideum amoebae incubated on Con A may be prolonged high levels of mPDE activity. High concentrations of mPDE would mean low concentrations of exogenous cyclic AMP, if other components of the cyclic AMP regulatory loop remain unaffected in the presence of Con A. If that is the case, aggregation would be delayed simply because cyclic AMP does not accumulate sufficiently to initiate aggregation until many hours have passed. Malkinson and Ashworth (1973) have demonstrated that slime mold amoebae allowed to differentiate in the absence of salts accomulate much less extracellular cyclic AMP than normal and are delayed several hours in aggregation.

Normally cyclic AMP accumulates prior to aggregation (Bonner et al., 1969; Malkinson and Ashworth, 1973) and it is thought that this accumulation is a necessary antecedent of aggregation (Bonner et al., 1969; Gerisch et al., 1972). Any accumulation of cyclic AMP by cells on Con A must take place in the presence of high mPDE levels. Cyclic AMP production

may in fact be stimulated through accumulation of 5'-AMP, the breakdown product produced by mPDE acting on cyclic AMP. 5'-AMP has been shown to be the activator of adenyl cyclase (Rossomondo and Sussman, 1973), the enzyme which synthesizes cyclic AMP. Any slowly increasing amount of 5'-AMP in the medium might therefore have a cumulative effect, stimulating cyclic AMP production over a period of time so that the system eventually adjusts to the continuous high mPDE levels in a way that allows aggregation to proceed.

Alternatively, the delay in aggregation may be due to a change in sensitivity of amoebae to cyclic AMP. This is suggested by the observation that in the Konijn test cells on Con A agar have a lower rate of response to low concentrations of cyclic AMP than controls (Fig. 19b). This would mean they would aggregate more slowly than controls in the presence of normal levels of cyclic AMP. If at the same time they have high mPDE levels, thus retarding the rate of accumulation of cyclic AMP, they would certainly exhibit delayed aggregation. The reduced sensitivity to cyclic AMP may be a reflection of the time required for the surface to recover from the effects of capping of Con A receptors and to regenerate new ones, especially if these receptors are involved in chemotaxis.

B. Increase in Membrane Phosphodiesterase Activity

Another question raised by this study is with regard to the premature, excessive and prolonged increase of mPDE activity brought about by Con A, that is, why soon after plating on Con A agar the level of activity is higher than is normally ever observed in D. discoideum and why the high level is maintained.

From the staining data Con A appears to interact only with the cell surface of D. discoideum, so the increase in mPDE activity effected by Con A must be due to binding of Con A to a surface site. Since mPDE increase is a normal event during development, Con A may be mimicking a factor produced during pre-aggregation that triggers mPDE synthesis or activation. If this is the case, the surface receptors for this factor should be present in interphase cells in some form capable of binding Con A. This may explain why the enzyme appears prematurely.

It might be supposed that under the conditions of these experiments, a greater proportion of mPDE stimulating receptors is bound by the available Con A than is bound by the factor which normally stimulates mPDE as the cells

approach aggregation. This might produce an early increase in mPDE above aggregation levels. The magnitude of the Con A effect may be further inhanced by the turnover of Con A receptors in response to being cross-linked and shed. Synthesis of additional rounds of Con A receptors would allow many more stimulatory interactions between Con A and its receptor and may produce the excessively high levels of enzyme observed on Con A.

The continued synthesis of Con A receptors, stimulated by shedding of the same from the surface, may also be responsible for the prolonged increase in mPDE. The inability of cells which have been allowed to develop 5 hr on plain agar to respond to Con A with mPDE enhancement indicates that under normal conditions the Con A receptors are themselves changing (or their hook-up in the cytoplasm is changing) so that Con A no longer elicits a response. The change in the receptors may involve only a change in the oligosaccharide moiety of glycoprotein. Except for this, the same receptor may still be present at pre-aggregation when the normal stimulator of mPDE binds and elicits the usual mPDE increase. Such a minor modification of the Con A receptor as D. discoideum approaches aggregation is in fact indicated by the recent work of Weeks (1975). He found that as amoebae of an axenic strain approach aggregation there is a reduction in the affinity of a portion of Con A-binding sites which he attributes to a

in the structural complexity of certain of the Con A receptors. On this basis Con A would not only induce continued synthesis of its receptors but also would delay or prevent their differentiation (modification). This might further contribute to the delay in aggregation.

C. Effects on Morphogenesis

In addition to delaying aggregation and increasing mPDE activity, Con A affects morphogenesis, altering aggregation morphology and fruiting body density. Several characteristic features distinguish the morphology of aggregation of D. discoideum on Con A from normal aggregation. The individual amoebae seem to slowly pile into mounds which are the equivalent of aggregation centers since each becomes the unit of morphogenesis, producing a fruiting body. Missing are the characteristic elongate amoebae, aggregation streams and intercellular contacts that always appear during normal aggregation (Fig. 13). Those peripheral cells that move to the center do so singly, not in streams. The extent to which cultures express altered aggregation morphology depends on the concentration of Con A on which they develop.

Con A also affects the density of fruiting bodies formed by a given density of cells (Fig. 15). In modifying the density of fruiting bodies Con A has actually affected the distance over which cells are drawn into each aggregation center. This area is termed the aggregation territory. The size of the territory determines the number of cells which will form the morphogenetic mass, and since one aggregate forms one fruiting body, the territory size fixes the size of the fruiting body (Bonner and Dodd, 1962). In a cell population as a whole, the size of territories established at aggregation determines the density of resulting fruiting bodies, territory size being inversely related to the density of fruiting bodies. Therefore if Con A influences fruiting body density, it has actually produced its effect when the territories were being established during aggregation.

It is probably that low cyclic AMP levels due to high mPDE activity are responsible in part for some of the altered pattern of aggregation observed in the presence of Con A. Early in normal aggregation, when little cyclic AMP has accumulated and cells have low sensitivity to it,

a few cells may be observed to move toward a nascent center in a manner similar to cells aggregating on Con A. In mutants defective or delayed in production of the inhibitor of ePDE and/or mPDE regulation, aggregation takes place in the absence of streams and results in small aggregation territories and, subsequently, small fruiting bodies (Malchow et al., 1972; Riedel et al., 1973). Such mutants with reduced ePDE inhibitor would be expected to accumulate cyclic AMP at a lower rate than normal, as would cells with early high mPDE activity, supporting the notion that low cyclic AMP may be the basis for the altered aggregation pattern on Con A. The absence of elongated cell morphology also is very likely a reflection of low cyclic AMP levels since single cells incubated on cyclic AMP agar assume the elongate morphology (Gillette, unpublished observations).

Another possibility is that the low but constant elevation of ePDE secretion in D. discoideum on Con A (Table 9) is responsible for some of the aberrant aggregation morphology observed. Data for certain classes of aggregation mutants suggest that ePDE is responsible for establishing territory size. Mutants defective or

delayed in production of ePDE inhibitor (which therefore would have abnormally high ePDE levels during pre-aggregation) and those with excessive ePDE production show no aggregation streams and small territories (Riedel et al., 1973). Another class of aggregation mutants which produce virtually no ePDE have extremely large territories with marked streams of aggregating cells. However, mutants without a detectable defect in ePDE regulation still have reduced or deficient stream formation; some of them also have reduced mPDE levels (Malchow et al., 1972; Riedel et al., 1973); therefore it appears that mPDE or the co-regulated elements of chemotaxis are involved to some extent in stream production. The data are consistent with the hypothesis that ePDE has a role in setting up the gradient along which streams form as well as establishing the limits of the aggregation territory.

In the case of cells incubated on Con A it can be assumed that even at the low increment in the rate at which ePDE is secreted, 1 - 2 pmoles/min-mg/min, ePDE will accumulate to a greater extent in the agar than is normal before aggregation because aggregation is delayed by several hours on Con A. In light of the information regarding ePDE mutants this accumulation of ePDE may be responsible for the alteration in territory size produced by Con A.

III. Activation of Membrane Phosphodiesterase by Concanavalin A in Dictyostelium discoideum

The data clearly show that in the presence of Con A mPDE appears prematurely and at levels higher than those prevailing normally at aggregation in D. discoideum. The activation is a specific effect of Con A in this system since it is prevented by adding the haptenic sugar α-MG to the Con A medium, and incubation of the cells on non-specific protein (BSA) does not produce the effect. Con A does not bring about a general metabolic stimulation of the cells since lactate dehydrogenase, a soluble enzyme of the cytoplasm, is unaffected. Nor does Con A effect an increase in the specific activity of 5'-nucleotidase, which is also membrane bound with most of its catalytic sites exposed intracellularly (Filosa, unpublished observations). The amount of ePDE activity secreted per minute is increased by 1-2 pmoles/min/mg/min during the developmental phase on Con A. Therefore there appears to be no general metabolic stimulation by Con A.

This raises a question as to whether Con A stimulates increased synthesis of the enzyme, or whether it in some way activates enzyme which is already present. Actinomycin D studies indicate that RNA synthesis, possibly mRNA, is required to sustain the high levels of mPDE activity at 3 hr of development; because cycloheximide kills the

cells in the presence of Con A, the role of protein synthesis cannot be estimated. Killick and Wright (1974) caution against actinomycin D studies pointing out that the primary effect of the drug may be inhibition of differentiation by a variety of mechanisms, with only secondary consequences to those changes in enzyme specific activity which accompany normal differentiation. Determination of whether Con A brings about true induction of mPDE would require a more definitive study in which the appearance of new enzyme molecules as measured by radioimmune assay is blocked by inhibition of mRNA synthesis.

Con A apparently does not work directly on the enzyme itself for when Con A is added to the assay mixture of particles or whole cells not previously exposed to Con A, it has no affect on the rate of substrate breakdown in the 15 min assay period. This does not exclude the possibility that Con A activates a pre-existing form of the enzyme in a more indirect manner. Mammalian PDE exists in multiple molecular forms, some of which are regulated by association with a specific protein activator (Cheung, 1971). It is possible that a similar form of control exists for slime mold mPDE and that Con A promotes premature association of the enzyme with a regulator molecule and in this way prematurely activates mPDE.

The normal developmental program involves an increase in a membrane-bound form of PDE at the time of aggregation and secretion of an ePDE with high substrate specificity (Malkinson and Ashworth, 1973). Prior to aggregation the only PDE which appears is a PDE with low substrate affinity which is released extracellularly during the growth phase (Pannbacker and Bravard, 1972; Chassy, 1972). Therefore regulation of PDE during development includes the deposition of the enzyme in the membrane as well as extracellular accumulation of enzyme with high substrate specificity. Con A appears to stimulate prematurely an event which normally occurs at aggregation, namely, increase in PDE activity and its deposition in the membrane.

This raises a question as to whether Con A is mimicking a factor normally produced during development which enhances mPDE activity at aggregation. This would imply that such a factor produces its effect by binding to surface receptors which are linked to internal machinery involved in activation of pre-existing enzyme or synthesis of the enzyme and its incorporation into the membrane (Gillette and Filosa, 1973). As Con A brings about a redistribution of receptors and it is not internalized, rearrangement of receptors or their removal from the surface may be the basis for activating mPDE. This

suggests that the usual activator may be a multivalent ligand. Two different carbohydrate-binding proteins accumulate extracellularly prior to the increase in mPDE (Rosen et al., 1973; Klein and Darmon, 1976); it is not known whether they affect mPDE activity.

Cyclic AMP itself can activate PDE although it is unknown whether the effect is direct or secondary. I found that incubation of washed vegetative D. discoideum amoebae on 10^{-3} M cyclic AMP causes a sizeable, premature increase in mPDE by 3 hr of development. Klein (1975) has recently shown that both ePDE and mPDE are activated by cyclic AMP in differentiating D. discoideum amoebae, possibly by induction. Cyclic AMP has a similar affect on soluble and membrane-bound PDE in mammalian cells (D'Armiento et al., 1972; Manganiello and Vaughan, 1972; Schwartz and Passonneau, 1974).

Cyclic AMP is able to prevent two effects that Con A produces in D. discoideum. At high concentrations cyclic AMP overcomes the Con A-mediated inhibition of chemotaxis as well as the Con A-mediated enhancement of mPDE activity. In the experiments where cyclic AMP reverses mPDE activation the cells are incubated initially on agar containing both Con A and cyclic AMP so the two molecules may be competing for primary action. Cyclic AMP alone at 10^{-3} M produces a significant increase in mPDE (Table

12). However, cyclic AMP (10^{-3} M) plus Con A does not give an additive effect. The mPDE activity is about 21% of that obtained with Con A alone and about 27% higher than that obtained with cyclic AMP alone. Thus in the presence of 300 μg Con A/ml, 10^{-3} M cyclic AMP reduces the high level of mPDE activity seen with Con A alone; at the lower cyclic AMP concentration, the effect of Con A prevails, producing high levels of mPDE activity. This result suggests that the two molecules may compete in their action on mPDE activation so that above a threshold the molecule in greater excess has most effect on mPDE activity and at the same time prevents the other molecule from producing the effect it would have alone.

One significant difference between Con A and cyclic AMP is the way in which they interact with the cell membrane. Con A causes cross-linking of surface molecules followed by capping, shedding and possibly regeneration of receptors. Cyclic AMP binds to only one site at a time and therefore cannot produce these effects. Cross-linking and/or receptor turnover may be the basis of the excessive increase in mPDE caused by Con A as compared with the modest increase mediated by cyclic AMP.

In the Konijn test the cells presumably have produced high levels of mPDE during their stay on Con A agar prior to application of cyclic AMP. High mPDE brought about by Con A is probably the reason for the reduced rate of response to low levels of cyclic AMP. However, at high concentrations cyclic AMP may be able to compete in some way with Con A for effector action. This may be by direct competition through the same pathway, possibly by acting at a different site(s) on the same receptor molecule or via indirect competition by acting through a separate pathway which is antagonistic to the Con A effect. This need not be an effect on enzyme synthesis, although that is certainly a possibility. It may be at the level of altering activities or interactions of existing molecules.

IV. Recent Papers Concerning the Effect of Concanavalin A on *Dictyostelium discoideum*

After I completed the experiments reported here, two articles appeared (Weeks and Weeks, 1975; Darmon and Klein, 1976) which examined the effect of Con A on aggregation and PDE activity in *D. discoideum*. A discussion relating these papers to my own work follows.

Weeks and Weeks (1975) attempted to repeat some experiments reported here and previously published (Gillette and Filosa, 1973). They found that wild type *D. discoideum* cells plated on agar containing Con A produced delays in aggregation similar to those reported in the present work. Under their conditions of culture, the wild type strain was delayed 8 hr on 20 μg Con A/ml and 30 hr on 800 μg Con A/ml. When Weeks and Weeks used an axenic strain of *D. discoideum* they found that the delay of aggregation was only 6-8 hr on agar with 500 μg Con A/ml; 200 μg Con A/ml produced no delay.

Since they found that Con A had a much less pronounced effect on aggregation of the axenic strain, Weeks and Weeks expected that the rise in mPDE reported for axenic cells treated with Con A would also be less than had been reported for the wild type strain (Gillette and Filosa, 1973). Unlike my experiments reported here in which mPDE activity was measured in cells on Con A agar, Weeks and Weeks (1975)

measured the enzyme in cells exposed to Con A in shake cultures. Activity of particle-bound PDE was found to increase dramatically during the first hour of incubation in Con A but to drop by one-third at 2 hr. It continued to fall so that around 6 hr it was less than controls. They made no measurements of the mPDE activity on axenic or wild type cells differentiating on agar.

I found that in the wild type strain incubated on Con A agar mPDE activity increases for 3 hr and then remains high throughout the pre-aggregation period (Fig. 22). Thus, the results for the two strains are consistent with the notion that continuous high levels of mPDE are responsible for delaying aggregation since the delay in the axenic strain is only as long as the transitory increase in mPDE.

Darmon and Klein (1976) examined the ability of amoebae of the axenic strain to form intercellular contacts after removal from shake cultures containing Con A. The ability of the cells to form these contacts is a commonly used assay for the "aggregation competence" of amoebae aged in liquid culture. They found a delay in acquisition of aggregation competence in Con A-treated cultures; the extent of the delay depended upon Con A concentration, cell concentration and the length of time cells were incubated in the lectin. The first two observations confirm the findings of the

present study although different methods were used in the experiments.

They then examined PDE activity periodically after a 10 min pulse with 100 μg Con A/ml followed by incubation in buffer and during continuous incubation of cells with Con A in shake cultures. After the 10 min pulse with Con A a substantial peak in ePDE appears in the first 4 hr in Con A-treated cells. No difference is seen in Con A cultures and controls with respect to total cellular PDE (cytoplasmic plus membrane bound) until after 4 hr when Con A-treated cells show enhanced enzyme activity.

Continuous exposure to Con A in shake cultures, conditions that totally inhibit cell differentiation (Darmon and Klein, 1976), results in a decrease in levels of three products normally secreted at this time: ePDE, ePDE inhibitor, and N-acetylglucosaminidase. By 2 hr of incubation with Con A there is an increase in total cellular PDE which starts to decline at 5 hr but is less than the peak activity achieved by controls. Although their method of incubation and the strain of cells are the same as those used by Weeks and Weeks (1975), the shape of the activity curves differs. This may be due in part to the fact that Darmon and Klein report total cellular PDE rather than mPDE alone.

Darmon and Klein (1976) regard ePDE and cellular PDE as forms of the same enzyme and attribute the increase in cellular PDE in the presence of Con A to a decrease in secretion of ePDE and its inhibitor. However, I found that ePDE activity secreted was increased in the presence of Con A. In addition I found that mPDE activity rapidly increased to a much higher level than is normally achieved at aggregation, which is different from their results for cellular PDE.

Darmon and Klein (1976) also added Con A to shake cultures after they had been incubated for several hours in buffer. They found at 1.5 hr and 8.5 hr the cells were more responsive to Con A-mediated stimulation of mPDE than cells treated at the time they were removed from the food source. This result is very different from my experiments with the wild type organism on agar. I found that the responsiveness of the cells to mPDE stimulation decreases as they approach aggregation.

The basis for the differences in my results compared with Darmon and Klein (1976) as well as Weeks and Weeks (1975) may lie in differences in the strains of D. discoideum used in the studies and/or the methods by which the cells were cultured.

Although the axenic strain used by Weeks and Weeks (1975) and Darmon and Klein (1976) was derived from the wild type D. discoideum, it has been reported to differ from NC-4 in chromosome number (Muroyama et al., 1976) and enzymic composition (Quance and Ashworth, 1972). Of significance for the comparison of my results with those of other investigators mentioned above, is the observation that the size and density of fruiting bodies formed by axenic amoebae is dependent on the growth medium used (Quance and Ashworth, 1972). When grown in the absence of glucose, the amoebae form fruiting bodies which are smaller and more numerous than those produced by medium with glucose strongly suggesting a change in their aggregation behaviour. Weeks (1975) has shown that the axenic strain also differs from wild type in having more Con A receptors by which it can be agglutinated. It is therefore possible that differences between the two strains may account for differences in their response to Con A.

Another important variable which can affect enzymic composition as well as the rate of differentiation is the method by which the cells are incubated after removal from growth conditions. I allowed the amoebae to differentiate on agar. Weeks and Weeks (1975)

allowed the cells to differentiate on agar during their study of delay of aggregation but performed mPDE assays on cells from shake cultures. Darmon and Klein (1976) incubated cells in shake cultures for all experiments. To assess any effect on aggregation they looked at the ability of cells to form end-to-end contacts (aggregation competence); aggregation itself was not monitored.

Malkinson and Ashworth (1973) point out that when slime mold amoebae are shaken in buffer the time of appearance of ePDE is altered so that peak activity is found several hours earlier than when the amoebae are incubated on solid substrate. The ePDE is also produced in much greater amounts than when development proceeds on solid medium. These experiments indicate that liquid culture can affect both the time and amount of enzyme production and/or secretion. Indeed, the amoebae do not differentiate in liquid culture; the cells must be removed to solid substrate to differentiate and after being shaken for longer than 6 hr in Sorensen's phosphate buffer, they seem to suffer damage, their ability to form fruiting bodies deteriorates, and the time taken to form fruiting bodies also increases (Lee, 1972; Malkinson, and Ashworth, 1973). For these reasons direct

comparisons between cells incubated in liquid culture and on solid substrate cannot be made.

Significantly, all three papers (Gillette and Filosa, 1973; Weeks and Weeks, 1975; Darmon and Klein, 1976) show that mPDE increases upon incubation of D. discoideum with Con A. The crucial question is why the premature increase in enzyme activity appears in the membrane.

V. A Comparison of Aggregation in Dictyostelium discoideum and D. mucoroides

A surprising finding to emerge from this study is that D. discoideum and D. mucoroides respond so differently to Con A, especially as they are thought to be closely related species (Bonner, 1967) and possess many common elements of aggregation (as outlined in the Introduction). In comparing the effect of Con A on each species (Table 15) it is clear that the two species differ with respect to Con A binding and redistribution, the functional nature of their Con A receptors, and their morphogenetic response to Con A. A discussion of these results follows.

A. Binding of Concanavalin A to the Cell Surface

Based on the Con A binding studies, the cell surface of D. mucoroides differs from D. discoideum with respect to Con A receptors. Both vegetative phase and interphase D. mucoroides amoebae show low intensity staining with Fl-Con A as compared with D. discoideum. This may reflect fewer Con A-binding sugar residues on the surface or it may be the result of fewer or the residues

Table 15. A comparison of the effects of Concanavalin A on Dictyostelium discoideum and D. mucoroides

	D. discoideum	D. mucoroides
Staining of washed vegetative and pre-aggregation amoebae with Fl-Con A	Intense	Faint
	Cap disperses into spots	Cap shed from tail; no spots
	Staining little modified after 2 hr on Con A agar	Staining much reduced after 2 hr on Con A agar
Agglutination titre	12.5 - 50 µg Con A/ml	25 - 50 µg Con A/ml
Morphology on Con A agar	Rounded with vacuoles	Elongate without vacuoles
Effect of Con A on aggregation	Dose-dependent delay of aggregation at all Con A concentrations	Aggregation delayed at low Con A concentrations; inhibited at high
	Increased rate of completion of half of the total aggregation centers	Reduced rate of completion of half of the total aggregation centers
	Aggregation territory greatly reduced	Aggregation territory increased
Motility on Con A agar	Slightly reduced for 2 hr	Greatly increased for 5 hr
Cyclic AMP response		
10^{-3} M cyclic AMP	High Con A concentrations inhibit; low do not	All Con A concentrations inhibit
10^{-6} M cyclic AMP	All Con A concentrations inhibit	
ePDE activity on Con A	Slight increase in secretion	No effect on secretion
mPDE activity on Con A	Increases in 1 hr to level greatly exceeding normal peak activity	Increases in 1 hr to small fraction of normal peak activity
	Level increases for 3 hr and remains very high during pre-aggregation	Level remains near 1 hr increment during pre-aggregation
	Level at aggregation nearly 3 X normal	Level at aggregation near normal
	All Con A concentrations more effective in enhancing mPDE than in D. m.	

being available to bind the Fl-Con A. D. mucoroides has a higher agglutination end-point than D. discoideum as well as being less completely agglutinated by higher concentrations of Con A. These findings suggest that less Con A would be bound by vegetative D. mucoroides amoebae plated on Con A agar than by D. discoideum.

Both species cap Fl-Con A. Unlike D. discoideum, early stages of D. mucoroides exhibit diffuse staining prior to capping and they cap more slowly. The caps of D. mucoroides are less well defined, indicating the receptors may be less densely packed at the uropod or fewer in number than for D. discoideum. Since redistribution and capping is not a characteristic of all surface macromolecules (de Petris and Raff, 1973), the fact that both slime mold species have receptors which can be redistributed and capped by Con A indicates some degree of similarity if not in the receptors themselves, then in their mobility in the membrane and/or the anchorage of the receptors to subsurface elements.

A consistent difference in the way the two species redistribute Fl-Con A is the lack of formation of fluorescent spots by D. mucoroides after capping. These

spots are characteristic of pre-aggregation amoebae of D. discoideum incubated in Fl-Con A.

Staining of D. mucoroides incubated on Con A agar with Fl-Con A suggests that these cells also are not saturated by Con A in the medium. After 2 hr on Con A agar they still pick up Fl-Con A in a diffuse pattern but appear to lose it rapidly for after 15 min in Fl-Con A the only fluorescence apparent is in particulate debris. Samples from cultures which have been on Con A agar longer show little staining. This contrasts with D. discoideum where cells on Con A agar for 2 hr redistribute Fl-Con A as they would have without prior exposure to Con A. In this case Fl-Con A is localized in spots in all pre-aggregation stages removed from Con A agar. Thus pre-incubation on Con A has less effect on the way D. discoideum amoebae redistribute Fl-Con A than in D. mucoroides, where staining is much reduced by prior interaction with Con A. This may be due to the lower number of Con A receptors available in D. mucoroides being more nearly saturated by Con A from the agar throughout the pre-aggregation period. It may also reflect the ability of D. discoideum to regenerate receptors which have been capped by Con A and shed while D. mucoroides may be unable to do so.

B. Aggregation on Concanavalin A Agar

The two species react very differently to being plated on Con A agar. D. discoideum amoebae round up and large vacuoles appear in the cytoplasm within 15 min, whereas D. mucoroides amoebae do not round up and possess no unusual vacuoles at all Con A concentrations tested. When motility of the cells on Con A was tested, D. discoideum was found to move at a slightly slower rate than controls in the first 2 hr after plating. D. mucoroides amoebae on the other hand show nearly a two fold increase in the rate of movement in the first 3 hr after plating and the rate continues to be high until at least 5 hr.

Both species, however, show delayed aggregation in the presence of Con A. At 50 and 100 µg Con A/ml agar, the delays are of comparable lengths for both species at a given cell density (Tables 6 and 7). On 200 µg Con A/ml, D. mucoroides is delayed 2 - 3 times longer than D. discoideum at cell densities where aggregation takes place. Below 7.5 X 10^3 cells/mm^2 D. mucoroides does not aggregate at all (Table 7). At higher Con A concentrations higher cell densities are also inhibited from aggregating.

In contrast D. discoideum is able to aggregate at all Con A concentrations tested, although aggregation is more delayed as the Con A:cell ratio increases (Table 6).

Above a threshold cell density D. mucoroides is apparently more susceptible to Con A interference with aggregation than is D. discoideum. In other words, D. discoideum is more able to overcome the effect of Con A in delaying aggregation. The higher susceptibility of D. mucoroides may be related to its lower number of available Con A receptors and the shorter interphase period. If cells overcome the delay by inactivating Con A bound to the cell surface, D. mucoroides would be less effective in inactivating the Con A avaliable in the medium because it would bind less Con A at any given round of receptor turnover and have less time to do so before normal aggregation. This type of recovery mechanism would suggest that D. mucoroides should also experience greater delays at lower Con A concentrations than D. discoideum. As this is not the case, some alternative mechanism must be involved.

The susceptibility of D. mucoroides to Con A may be a reflection of the difference in responsiveness of the two cell types to cyclic AMP when incubated on Con A. At high

levels of cyclic AMP (10^{-3} M), low Con A concentrations have little effect on the rate of response of D. discoideum amoebae. Only at the higher Con A concentrations ($\bar{>}$300 μg/ml) is the response inhibited. However all concentrations of Con A decrease the response of D. mucoroides to 10^{-3} M cyclic AMP such that successively higher levels of Con A inhibit chemotaxis to a greater extent. It appears that sufficient cyclic AMP can overcome the effect of Con A on D. discoideum, but not on D. mucoroides.

C. Effect of Concanavalin A on Membrane Phosphodiesterase

Membrane PDE activity is increased in both species by incubation on Con A agar. In order to compare the effect of Con A on D. mucoroides with that in D. discoideum, particulate mPDE was assayed at both 1 hr and 20 min, an equivalent developmental time for D. mucoroides, and 3 hr 20 min, an incubation period equivalent to that used for D. discoideum on Con A agar. All concentrations of Con A cause greater elevation of mPDE in D. discoideum, whether compared with D. mucoroides at 1 hr 20 min or 3 hr 20 min

(Table 16). The ratio of activities is greater for 3 hr 20 min incubation than at 1 hr 20 min because the activity has already declined in D. mucoroides (Fig. 28).

At no Con A concentration tested does the mPDE activity of D. mucoroides even approach the normal peak value obtained for particles from aggregating D. mucoroides amoebae. The greatest increase, observed at 1 hr 20 min on 300 µg Con A/ml, is only 15% of the aggregation value for D. mucoroides. In contrast the lowest increase observed for D. discoideum on Con A is 15% of that species' aggregation value and the increase(s) in mPDE induced by 200 and 300 µg Con A/ml after 3 hr 20 min are roughly 200 - 400 % of the normal peak at aggregation. Part of this difference is a reflection of the fact that at aggregation D. mucoroides has twice the particulate mPDE of D. discoideum (Fig. 22b, 27b). Nevertheless, the values for D. discoideum on Con A are clearly much greater than for D. mucoroides in addition to surpassing the maximum in mPDE activity which is normally attained by D. discoideum at aggregation.

Table 16. Relative increase in particle-bound phosphodiesterase activity by Concanavalin A on *D. discoideum* vs *D. mucoroides**

Con A concentration (µg/ml agar)	mPDE specific activity in D.d. at 3 hr 20 min / mPDE specific activity in D.m. at time x	
	x = 1 hr 20 min	x = 3 hr 20 min
0	1.2	2.2
50	2.0	3.6
100	2.8	5.4
200	14.3	14.1
300	6.0	9.2

*Particles were prepared from cells incubated on various concentrations of Con A agar for 1 hr 20 min or 3 hr 20 min and assayed for mPDE (see Materials and Methods). The ratio of mPDE activities in the two species was derived from this data.

D. Effect of Concanavalin A on Aggregation Territories

The size of aggregation territories of both D. mucoroides and D. discoideum is affected by Con A, although in different ways. In D. discoideum territory size (the inverse of fruiting body density) is dramatically decreased, especially between 200 and 300 μg Con A/ml. In contrast, Con A increases the territory size of D. mucoroides. At high Con A concentrations and low cell densities the increase actually reflects the inhibitory effect of Con A on aggregation per se.

As pointed out earlier, there is some evidence that mPDE is responsible for establishing aggregation territories (Riedel et al., 1973). The secretion of ePDE activity is slightly enhanced in D. discoideum by Con A (Table 9) but is apparently unaffected in D. mucoroides (Table 14). Because territory size is altered in both species it would appear that factors in addition to ePDE are involved in setting up the territories. The amount of cyclic AMP in the chemotactic gradient is an obvious candidate, and its levels would be influenced by the mPDE

as well as ePDE. Therefore, the differential enhancement by Con A of mPDE in the two species may be partially responsible for the effect on aggregation territories.

The rate of cell movement may also be responsible for modifying territory size. According to Cohen and Robertson's (1971) "Doppler-shift" model for aggregation, increasing the rate of movement of the amoebae alone would be sufficient to increase the territory size. However, it may be that the cyclic AMP gradient is important in setting up the territory (Bonner et al., 1969). Alterations in the rate of movement would change the kinetic characteristics of the cyclic AMP gradient with probably effects on the aggregation territory. For D. mucoroides the doubling of the rate of movement may have the net effect of causing an amoeba to move farther in response to each pulse of cyclic AMP. This may mean the gradient of cyclic AMP in the area over which a cell moves would be less completely broken down than is normally the case. This would be expected to alter any cyclic AMP gradient making it shallower and could result in a larger territory (Bonner et al., 1969).

E. Differences in Normal Membrane Phosphodiesterase Levels

Previous to this study mPDE had not been examined in D. mucoroides. I found that as in D. discoideum the enzyme exists at low levels in this species until a few hours before aggregation when it undergoes a dramatic increase in specific activity; after aggregation it falls to low levels (Figs. 27a, b). As aggregation is several hours earlier in D. mucoroides than D. discoideum, the increase in mPDE is observed earlier. Although the activity at the peak of aggregation was assayed only twice, the level of mPDE at the surface of D. mucoroides at aggregation appears to exceed that of D. discoideum by nearly 2.0 - 2.7 fold. Activity of mPDE in the particulate membrane fraction of aggregating D. mucoroides exceeds that of aggregating D. discoideum by a factor of 1.6. Although more extensive studies are needed for conclusive evidence, it appears that at aggregation D. mucoroides has higher mPDE activity than D. discoideum.

An early sensitivity of D. mucoroides to cyclic AMP (Bonner et al., 1969) and formation of locally dense collections of amoebae (Shaffer, 1962) have been correlated

with an early rise in cyclic AMP binding by this species (Mato and Konijn, 1975). Since Mato and Konijn (1975) measured total cyclic AMP binding, their study does not demonstrate whether the increase is due to an increase in non-catalytic cyclic AMP-binding sites or increased binding at the catalytic site(s) of mPDE or both. My work shows that there is in fact an increase in mPDE activity several hours before aggregation (Figs. 27a, b) and this presumably contributes at least in part to the increased cyclic AMP binding as well as the early chemotactic sensitivity to cyclic AMP of D. mucoroides. This early increase in mPDE is possibly linked to the rapid accumulation of cyclic AMP by D. mucoroides during the first six hours after plating (Bonner et al., 1972).

F. Differences in the Response to Concanavalin A

The species differences in the response of amoebae to Con A may have a basis in differences in the number of receptors bound in the two species, in the functional nature of the receptors, and/or in differences in the way the species redistribute the bound Con A. As demonstrated by agglutination studies and Fl-Con A binding, fewer

receptors on D. mucoroides amoebae bind exogenous Con A than in D. discoideum. Many of these may actually be functionally different molecules. This is suggested by the different pattern and intensity of staining with Fl-Con A, increased cell motility on Con A, low mPDE enhancement and the sensitivity of aggregation and chemotaxis to Con A in D. mucoroides as compared with D. discoideum. The receptors could differ in one or more ways: they could be different molecules with different activities, they could be essentially the same molecule which when "stimulated" behaves differently,and/or the receptors when "stimulated" could behave the same but have different cytoplasmic relationships in the two species. Such functional differences as well as differences in the numbers of receptors bound would be expected to produce different cellular responses upon binding of Con A to the receptors.

The absence of fluorescent spots in Fl-Con A-treated D. mucoroides amoebae demonstrates that D. mucoroides redistributes Con A differently from D. discoideum. Further, preincubation of D. mucoroides amoebae on Con A agar markedly changes the pattern of redistribution of Fl-Con A

whereas in D. discoideum it does not. These differences in redistribution patterns may actually reflect a basic difference in the ability of the two species to regenerate the shed Con A receptors. D. mucoroides may lack the ability to regenerate the shed receptors so that it experiences only one round of Con A-receptor interactions which results in limited enhancement of mPDE only during the first hour on Con A. The staining patterns of D. discoideum suggest that it may be able to regenerate shed receptors so that it could be exposed to several rounds of Con A-receptor interactions over a period of time. This may be the basis for the great increase in mPDE activity throughout the first three hours it is incubated on Con A.

Mato and Konijn (1975) found that in both species there is a correlation between cyclic AMP binding and chemotactic sensitivity to cyclic AMP, both of which reach maxima at aggregation. From my study as well as that of Malchow et al. (1972), increased mPDE activity normally appears to be correlated with aggregation also. These three events are related in function as well as timing. This raises a question as to whether they all result from action at a single site. If so, does Con A

stimulate mPDE synthesis while at the same time inhibiting cyclic AMP binding or the appearance of cyclic AMP-binding sites? Once Con A binds to the receptors, it could prevent cyclic AMP binding thus reducing chemotactic sensitivity. The fact that D. mucoroides binds a maximum of four times less cyclic AMP than D. discoideum (Mato and Konijn, 1975) would explain the greater sensitivity to Con A that this species demonstrates in the presence of 10^{-3} M cyclic AMP; Con A might be expected to more nearly saturate binding sites in D. mucoroides than in D. discoideum where there are more sites.

Alternatively, perhaps the Con A-binding sites are on receptors which are normally modified during development to become receptors for cyclic AMP. Since Con A binding results in capping and sloughing of the Con A-receptor complex, cyclic AMP binding would be reduced because the receptors are removed from the surface. From the staining data D. discoideum appears to be more able to regenerate shed Con A receptors than D. mucoroides so that D. discoideum would be expected to be less sensitive to incubation on high concentrations of Con A, which is the case. Thus delay in aggregation may be due to loss

of cyclic AMP receptors (which would mean reduced sensitivity to any cyclic AMP present) as well as lowered extracellular concentrations of cyclic AMP brought about by elevated mPDE activity. Elevated mPDE might be expected to contribute more to the delay in a species like D. discoideum which may repeatedly regenerate the receptors. The loss of receptors should contribute more to delaying aggregation in a species such as D. mucoroides which experiences only a low stimulation of mPDE and appears to have poor regenerative abilities with regard to the Con A receptor.

VI. The Role of the Cell Surface in Aggregation

In order to respond chemotactically to cyclic AMP during aggregation an amoeba must be able to sense concentration differences of cyclic AMP and derive a direction of movement from this information. This implies that cyclic AMP may act at the cell surface during chemotaxis. This discovery of a cyclic AMP-binding receptor (Malchow and Gerisch, 1973) and of cyclic AMP PDE (Malchow et al., 1972) at the cell surface of aggregating amoebae mean that the chemotactic signal can be received as well as destroyed without entering the cell. Indeed, it has been shown that an inconsequential amount of labeled cyclic AMP or its breakdown product enter aggregating amoebae (Malchow et al., 1972; Moens and Konijn, 1974), so cyclic AMP must be processed at the cell surface. These findings support the notion that the primary events in chemotaxis take place at the cell surface.

The present study demonstrates that altering the level of mPDE interferes with aggregation. Increased mPDE levels appear correlated with an increase in the

time necessary to complete an aggregate. The morphology of aggregation is also affected, so that at high mPDE levels the cells do not form streams or intercellular contacts. Thus, mPDE must have an important function in regulating chemotaxis during aggregation.

I have also shown that the activity of mPDE can be regulated at the cell surface. Con A prematurely activates mPDE. Con A binds to sugar-containing macromolecules on the slime mold surface and brings about a redistribution of these receptors. As the bound Con A is not internalized by the slime mold, it must mediate its effect on the enzyme through interaction with the cell surface. This suggests that normally mPDE is activated by molecular interactions at the cell surface and that an extracellular factor would serve as an activator.

Although this would represent an unusual form of developmental control, it seems very reasonable in this system. The interphase amoebae rely on extracellular cues to direct chemotaxis of the dispersed cells. A cell in this situation must remain sensitive to changes in the environment and these can be most readily monitored at

the cell's surface. The cell could rapidly respond to environmental changes if the activity of the chemotactic machinery were itself sensitive to extracellular cues, as appears to be the case for mPDE. The activation system would be particularly efficient if the activator of the aggregation machinery were the same molecule that mediates chemotaxis, cyclic AMP. This molecule could also act via a second effector released by amoebae upon cyclic AMP accumulation. Both of these situations appear as possibilities in the case of D. discoideum for cyclic AMP as well as two slime mold carbohydrate-binding proteins accumulate extracellularly prior to the increase in mPDE (Malkinson and Ashworth, 1973; Rosen et al., 1973; Klein and Darmon, 1976). I have shown that cyclic AMP itself activates mPDE but further experiments are necessary to determine whether this is a direct or secondary effect.

SUMMARY

1. Concanavalin A (Con A), a lectin that binds to α-D-glucose- and α-D-mannose-like residues, was found to bind to the cell surfaces of Dictyostelium discoideum and D. mucoroides at various stages of development.
   a. Binding of Con A was demonstrated both at the light and electron microscope levels.
   b. The lectin is localized as a cap, then shed from the cell surface.
2. In order to investigate the functional nature of the Con A receptors, vegetative amoebae were allowed to undergo morphogenesis on Con A agar.
   a. While fruiting body morphology remained unaffected, aggregation was delayed in both species. D. mucoroides appears to be more sensitive to high Con A concentrations.
   b. Density of fruiting bodies formed in the presence of increasing concentrations of Con A was dramatically increased for D. discoideum, but decreased for D. mucoroides.

3. Both species show a reduced chemotactic response to cyclic AMP in the presence of Con A.
4. Because membrane-bound cyclic AMP phosphodiesterase (mPDE) has been implicated in the chemotactic response of aggregating amoebae, the possibility that Con A affects aggregation by changing the activity of this enzyme though its interaction at the cell surface was examined.
   a. Within one hour of exposure of vegetative D. discoideum to Con A, mPDE is found prematurely at the surface of whole cells and in particulate membrane preparations at specific activity higher than that found in untreated cells during aggregation. mPDE activity remains high during the pre-aggregation period on Con A.
   b. The enhancement of mPDE activity appears to be a specific effect on that enzyme mediated by events at the cell surface.
   c. D. mucoroides shows neither long term nor high level mPDE stimulation.
5. Cyclic AMP, at high concentration, reverses the effect of Con A on inhibition of chemotaxis and mPDE activation in D. discoideum.

RESUME

1. Concanavaline A (Con A), une lectine qui se lie à des résidus du type α-D-glucose et α-D-mannose, se lie aussi, selon ces expériences, à la surface cellulaire de Dictyostelium discoideum et D. mucoroides à diverses étapes de son développement.

a. La liaison de Con A á été démontrée au niveau du microscope optique aussi bien qu'à celui du microscope éléctronique.

b. Cette lectine se localise d'abord comme une coiffe, pour se détacher ensuite de la surface cellulaire.

2. Afin d'examiner le caractère fonctionnel des récepteurs Con A, nous avons fait subir á des amibes végétatives une morphogénie sur l'agar Con A.

a. Tandis que la morphologie sorocarpe est restée inaltérée, l'agrégation a été retardée dans les deux espèces. D. mucoroides semble plus sensible à de hautes concentrations de Con A.

b. La densité des corps fructifiants formés en présence de concentrations croissantes de Con A a été augmentée de facon dramatique dans le cas du D. discoideum. mais diminuée dans le cas du D. mucoroides.

3. Les deux espèces font preuve d'une réponse chemotactique réduite à l'AMP cyclique en présence de Con A.

4. Puisque la phosphodiesterase cyclique AMP membranée (mPDE) a déjà été impliquée dans la reponse chemotactique des amibes agrégatives, la possibilite a ete examinee que Con A influence cette agrégation en modifiant l'activité de cette enzyme par action réciproque à la surface cellulaire.

   a. Après une heure d'exposition du D. discoideum végétatif à Con A, de la mPDE se trouve prématurément à la surface des cellules intégrales et dans des préparations particulaires membranées, à un niveau d'activité spécifique plus élevé que celui remarqué dans des cellules non-traitées au cours de l'agrégation. L'activité mPDE reste haute pendant la période pré-agrégative sur Con A.

   b. Cette hausse d'activité mPDE semble être un effet spécifique sur cette enzyme, accompagnant ces événements ayant lieu à la surface cellulaire.

   c. D. mucoriodes ne fait preuve de stimulation mPDE ni à long terme ni à haut niveau.

5. L'AMP cyclique, à forte concentration, renverse l'effet de Con A sur l'inhibition de la chemotaxe et sur l'activation mPDE dans D. discoideum.

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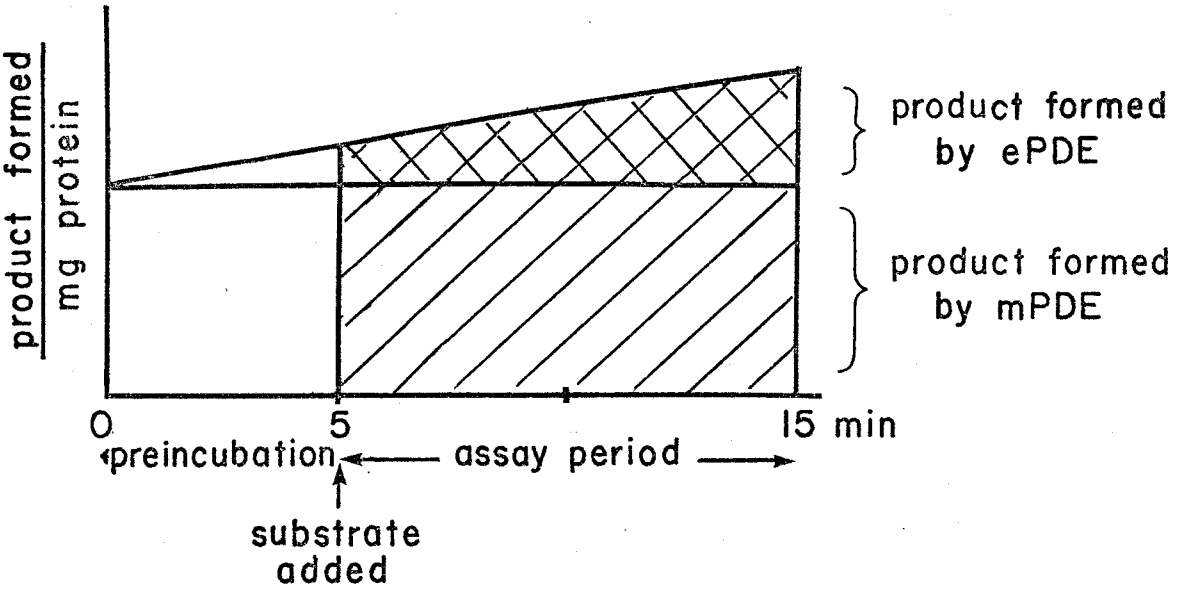
APPENDIX. Method of Calculating Membrane Phosphodiesterase Activity at the Surface of Living Cells

The total PDE activity assayed in intact living cells consists of two components: membrane PDE (mPDE) exposed at the cell surface and extracellular PDE (ePDE) which is being secreted into the medium. The specific activity of the membrane-bound enzyme remains constant over the period during which the cells are assayed. Specific activity of mPDE assayed at 5 min is not significantly different from that at 10 min when analyzed with the 2 tailed t test or Wilcoxon order rank. However, the absolute amount of ePDE is increasing over the course of the incubation; it is assumed to be secreted at a constant rate. The total PDE activity, both mPDE and ePDE, present during the assay period can be described graphically (Fig. 29). Several values described in this graph are known, including the total amount of product formed between 5 - 15 min, the product formed by ePDE at 15 min and the rate of secretion of ePDE. The amount of product formed by mPDE is the unknown to be determined.

Figure 29. Graphic representation of a phosphodiesterase assay performed on whole, living cells. The assay was conducted as described in Fig. 22a. After a 5 min preincubation substrate was added and the assay carred out for 5 hr 10 min. Product produced during this assay (shaded area) is due to the activity of both membrane-bound phosphodiesterase (single hatch) and extracellular phosphodiesterase (cross hatch).


product formed
mg protein
product formed
by ePDE
product formed
by mPDE
0
5
15 min
preincubation
assay period
substrate
added

Assays performed on living cells provide a value for the total amount of substrate converted to product between 5 and 15 min, during which [^{3}H] cyclic AMP is incubated with the system. This value, P_t, is represented by the total shaded area in the graph and is in terms of picomoles of product.

In order to determine what part of the total product formed during the assay is due to mPDE it is necessary to correct the total, P_t, for product formed by ePDE (the cross-hatched area in Fig. 29). Let us consider the upper part of the graph (Fig. 29) representing ePDE alone; this appears redrawn in Fig. 30. The product formed by ePDE between 5 and 15 min (A_{sub} = area with substrate) is the difference between the total area of the triangle describing the potential of ePDE to form product from 0 - 15 min (A_{tot}) less the area of the triangle from 0 - 5 min, which represents the preincubation period when no substrate was present (A_{pre}).

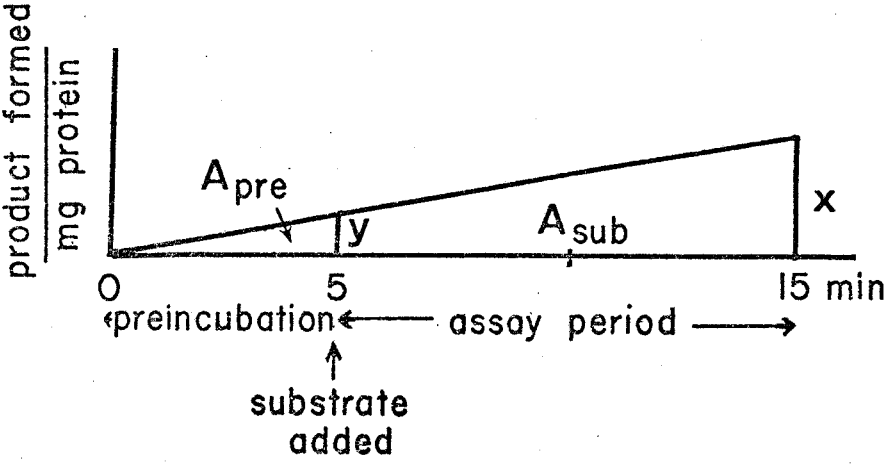
That is,

$$A_{tot} = A_{pre} + A_{sub}$$

rearranging,

$$A_{sub} = A_{tot} - A_{pre}$$

Figure 30. Graphic representation of ePDE extracellular phosphodiesterase accumulated during a whole cell assay. The enzyme accumulates during the preincubation period (A_{pre}) as well as during the assay period when substrate is present (A_{sub}).


product formed / mg protein
A pre
y
A sub
x
0
5
15 min
preincubation
assay period
substrate added

The area of a right triangle is defined as half the length times the height

$$A = \frac{1}{2} l \cdot h$$

therefore,

$$A_{tot} = \frac{1}{2} \cdot 15\text{min} \cdot x$$

$$= 7.5 \text{ min} \cdot x$$

$$A_{pre} = \frac{1}{2} \cdot 5 \text{ min} \cdot y$$

$$= 2.5 \text{ min} \cdot y$$

In both cases the enzyme units, that is product formed per min, are the same, therefore,

$$\frac{y}{5 \text{ min}} = \frac{x}{15 \text{ min}}$$

$$15 \text{ min} \cdot y = 5 \text{ min} \cdot x$$

$$y = \frac{5 \text{ min} \cdot x}{15 \text{ min}}$$

$$y = \frac{x}{3}$$

Substituting,

$$A_{pre} = \frac{1}{2} \cdot 5 \text{ min} \cdot \frac{x}{3}$$

$$= \frac{5}{6} \text{ min} \cdot x$$

$$= 0.83 \text{ min} \cdot x$$

Substituting,

$$A_{sub} = A_{tot} - A_{pre}$$
$$= 7.5 \text{ min}\cdot x - 0.83 \text{ min}\cdot x$$
$$= 6.67 \text{ min}\cdot x$$

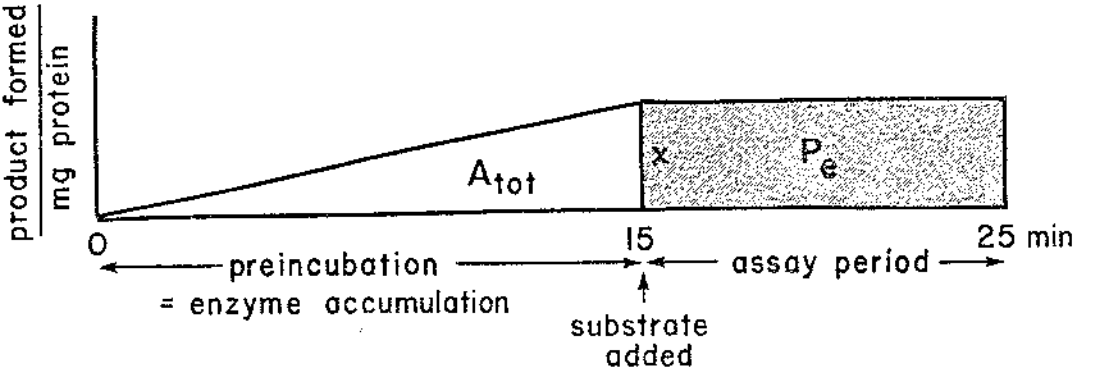
The value of x can be determined from a second experiment during which cells are incubated for 15 min under the same conditions used to determine the total activity except substrate is omitted. The cells are then removed and the cell-free supernatant containing the amount of ePDE secreted in 15 min is assayed for 10 min. This determination is expressed graphically in Fig. 31. The amount of product formed in this assay is equivalent to the amount of enzyme accumulated in 15 min times its rate constant times the total amount of time during which the reaction takes place; it is represented by the stipled region whose are is 10 min·x.

$$P_e = 10 \text{ min}\cdot x$$

$$x = \frac{P_e}{10 \text{ min}}$$

This value of x can be substituted to solve for A_{sub}.

Figure 31. Graphic representation of product produced by the cell-free supernatant containing ePDE accumulated over 15 min. Experimental determination of P_t using the method described in Table 9 allows the value of x to be calculated.


product formed / mg protein
A_{tot}
x
P_e
0
15
25 min
preincubation
= enzyme accumulation
assay period
substrate added

$$A_{sub} = 6.67 \text{ min} \cdot x$$

$$= 6.67 \text{ min} \cdot \frac{P_e}{10 \text{ min}}$$

$$= 0.67 \, P_e$$

This represents the amount of product formed by ePDE in the original whole cell assay between 5 - 15 min.

Now the product formed by mPDE (P_m) between 5 - 15 min can be determined by subtracting the product formed by ePDE ($A_{sub} = 0.67 \, P_e$) from the total (P_t). Both P_t and P_e are known and can be substituted in the equation

$$P_m = P_t - 0.67 \, P_e$$

Using a similar procedure, P_m for mPDE can be determined for assays performed between 5 - 10 min also.

Sample Calculation

Supernatant collected for 15 min from control cells (16.5 µg protein) at 8.5 hr of development converted 8.7% of the substrate which was at 37.5 pmoles to product during a 10 min assay. From this data that part of total product due to ePDE (P_e) can be caluculated:

$$P_e = 8.7\% \text{ X } 37.5 \text{ pmoles}$$

$$= 3.26 \text{ pmoles.}$$

Total product formed in a 10 min whole cell assay made between 5 -15 min was 8.48 pmoles.

Since

$$P_m = P_t - 0.67\ P_e,$$

that part of the total product formed due to mPDE (P_m) can be calculated:

$$\begin{aligned} P_m &= 8.48 \text{ pmoles} - 0.67\ (3.26 \text{ pmoles}) \\ &= (8.48 - 2.18) \text{ pmoles} \\ &= 6.30 \text{ pmoles} \end{aligned}$$

This represents the amount of substrate converted to product by mPDE over the 10 min assay, therefore

$$\begin{aligned} \text{enzyme units} &= \text{mole product formed/min} \\ &= 6.30 \text{ pmoles/10 min} \\ &= 0.63 \text{ pmoles/min} \end{aligned}$$

This can be standardized for the amount of protein used in the assay:

$$\begin{aligned} \text{specific enzymatic acitivty} &= \text{moles product/min/mg protein} \\ &= 0.63 \text{ pmoles/min/.0165 mg} \\ &= 38 \text{ pmoles/min/mg} \end{aligned}$$

Because this value is constant over the time the assay is performed, the amount of protein present is constant, and the amount of substrate is saturating throughout the assay, the reaction can be assumed to have zero-order kinetics. Therefore, the specific activity of mPDE at the cell surface at the time of this assay is 38 pmoles/min/mg.