THE DEVELOPMENTAL ROLE OF MEMBRANE IN THE CELLULAR SLIME MOLD, *DICTYOSTELIUM DISCOIDEUM*

Thesis by

Jessica Tuchman

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1974

(Submitted October 4, 1973)

To my parents

With much love and with gratitude

for so many kinds of good examples

ACKNOWLEDGEMENTS

Dr. Harvey Lodish comes very close to personifying my idea of the perfect graduate student advisor. He has been consistently interested and enthusiastic and unstintingly generous with both his laboratory expertise and his enormous scientific knowledge. He seems to know precisely how to tread the line between too much and too little advice. He has found ways to share with his students something of his own intuitive sense of the meaning of a bit of raw data and of what experiment to do next. On top of it all, he has been a good friend. I am and will remain deeply grateful for the chance to spend two years in his lab.

This would be the appropriate place to thank all the friends and associates whose help and friendship have enriched the last few years. Unfortunately, my list would have to include people from two schools rather than one, and therefore reaches a ludicrous length when written out. Also, my powers of decision having been sorely overworked in the past few weeks, I am unable to decide whom not to thank. So I have contented myself with simply saying that I feel I have been incredibly lucky in the people I have known and worked with both at Caltech and at M.I.T. I thank them for everything: the learning and the laughter.

I would like to thank two people by name, Elaine Lenk for performing the electron microscopy included in this thesis, and Marilyn Smith for typing it at great speed and with unabated good humor.

ABSTRACT

This thesis concerns the role of the cell surface membrane in cell-cell interactions. Specifically, the purpose of these experiments was to discover whether the aggregation of the cellular slime mold Dictyostelium discoideum is accompanied by, and perhaps dependent on, specific differentiation of the cell surface membrane, and if so, whether such components could be isolated in an active state. To this end, partially purified cell surface membranes were prepared from both vegetative (0 hour) and developing (14 hour) cells. The membranes were characterized by sucrose gradient centrifugation, SDS polyacrylamide gel electrophoresis, and electron microscopy. It was found that membranes from both 0 and 14 hour cells possessed the ability to inhibit the developmentally controlled aggregation of slime mold cells when mixed with these cells and plated under normal laboratory conditions. HeLa cell membranes, even at the highest obtainable concentrations, were inert in this respect. Aggregation phase membranes were able to prevent cell aggregation at significantly lower concentrations than was required for vegetative membranes, and were also markedly more resistant to heat degradation. It appears that aggregation phase membranes block aggregation by preventing the attainment of aggregation competence in the developing cells, whereas vegetative membranes appear to act through a direct competition for available aggregation antigen receptor sites on the cell surface.

The effect of the differentiated membranes on several developmentally controlled enzymes was tested. Membrane treatment leads to the induction of some developmentally controlled enzymes and the repression or excretion of others. In one case, alkaline phosphatase, enzyme induction occurs 12 hours earlier than in normal cells, and the enzyme reaches approximately double its normal activity. The distribution of effected and uneffected enzymes bears no resemblance to the normal sequence of enzyme induction. The only characteristic with which the membrane effect can be linked, is the intracellular localization of the effected enzyme. The results indicate that there are some difficulties in the generally accepted view of the slime mold developmental program, and point out the crucial role played by the formation and maintenance of cell-cell contacts during normal development.

A study of the changes in protein synthesis during slime mold development was also undertaken. Total cell protein was displayed on SDS polyacrylamide gels after a 2 hour pulse label. It was found that during the first few hours of development, a single major band accounts for more than 20 percent of the total protein on the gel. Actin was purified from vegetative cells by a known procedure, and was found to correspond to the major band in several respects. The discovery of a single protein being synthesized in such quantity, and its identification as actin, provide a powerful tool for the isolation of a specific messenger RNA molecule, and for an intensive study of all the factors involved in regulating protein synthesis in a eucaryotic organism.

TABLE OF CONTENTS

THE BIOLOGICAL ROLE OF PLASMA MEMBRANE AND A REVIEW OF SLIME MOLD PHYSIOLOGY

INTRODUCTION

This thesis concerns the role of the cell surface membrane of Dictyostelium discoideum during the aggregation of these cells. There has been a steadily increasing interest in the biological and physical properties of cell surface membranes in the past years. Most of this interest has been directed towards the physical and chemical characteristics of membrane: its composition, molecular architecture and surface topology. Nevertheless, the opposite approach, the investigation of those biological phenomena in which the plasma membrane is indubitably involved has also been the subject of significant and promising studies. This Introduction will attempt first to summarize the outstanding research produced by this latter approach. The development and physiology of the cellular slime mold Dictyostelium discoideum is then discussed, as are the nature and goals of the experiments presented thereafter.

A detailed presentation of what is now known of the composition and structure of cell surface membranes is beyond the scope of this discussion and is well reviewed elsewhere (Wallach, 1972; Oseroff et al., 1973). However a few general comments are

important to what follows. Conflicting theories of membrane structure which emphasize defined static architecture, have recently lost ground to the growing realization that the cell membrane is a dynamic, rapidly turning over collection of protein, glycoprotein and glycolipid molecules embedded to some extent in a lipid bilayer (Warren, 1969; Schimke and Dehlinger, 1971; Singer and Nicolson, 1972). The changing composition of the membrane is further complicated by the confusion over exactly what constitutes the membrane: where its boundaries can be drawn both on its inner and its outer surfaces (Oseroff et al., 1973). Revel and Ito (1965) proposed the term "greater membrane" to include the glycoprotein and mucopolysaccharide exudate which is thought to comprise the associated "fuzzy coat" described by electron microscopists. Bennett (1963) has proposed the term "glycocalyx" to describe roughly the same material. An extensive review of available data led Guidotti (1972) to divide membrane proteins into two classes. One class which would include the disputed substances mentioned above, can be displaced by such treatments as sonication, high salt, and mild detergent and resembles general cell protein in its amino acid composition. The second class of protein includes those which are tightly bound to lipid, contain a high proportion of nonpolar residues, and are not displaced by mild treatments. The proteins

which have a structural or transport function, are held to be in this latter class. The protein moieties of glycoprotein, which is generally found on the outer surface of the membrane and often protudes some distance from the cell, are often hydrophobic and are released only after enzymatic digestion (Kraemer, 1971; Fairbanks et al., 1971; Marchesi et al., 1972). It is clear that numerous proteins and glycoproteins are associated with plasma membrane, more or less tightly bound. Which, if any of these can be definitively termed a part of the membrane is at present an open, and not particularly enlightening, question.

These uncertainties are even further compounded by the fact that the cell surface membrane is the only subcellular organelle which must be disrupted in order to be isolated and studied. The possible artifacts lurking in the path of the researcher who attempts to isolate and study plasma membrane are truely formidable. For example, it has been shown that lipid molecules are exchanged between different membranes during membrane isolation (Tsukagoshi and Fox, 1971). Membranes may lose or gain various ligand molecules which would critically alter their activity. There are also numerous problems, caused by the need for some disruption procedure, which can cause a critical structural rearrangement in the membrane. If membranes exist naturally as a composite of many changing and constantly inter-

acting unstable states as is currently believed, the chances of isolating a membrane which is identical in biological activity to its in vivo counterpart are small indeed. The highly cooperative nature of specialized membrane functions is well demonstrated by the work of Sidman on "reeler" mice (Delong and Sidman, 1970). Reeler mice display a muscular uncoordination phenotype which has been shown to be the result of a single point mutation. The cerebellum shows an abnormal pattern of cellular associations and connections. Both normal and reeler embryonic cells can be dissociated with trypsin and will reform aggregates in vitro. The cells from reelers form abnormal cell connections exactly similar to those they make in the brain. Thus a single mutation, presumably affecting a single protein species, leads to a wholly altered region(s) of the cell surface membrane, that involved in the particular connection of cerebellar cell to cerebellar cell.

MEMBRANE FUNCTION

The most widely studied group of biological phenomena involving the cell surface membrane is unquestionably the set of activities which make up the immunological system. The clonal selection theories of Burnet (1970) and Jerne (1971), on which most recent research in this field is intellectually based, postulate that a given immunocompetent cell is predetermined to respond to only one antigen (or to a very small number of antigens). The physical basis of this commitment lies in the presence of the corresponding antigen receptor(s) (antibody) in the cell's plasma membrane. The presence of the receptors on the cell surface has been visualized in numerous experiments using radioactive-labeled or fluorescent-labeled antigens (Davie et al., 1971; Byrt and Ada, 1969). The surface localization of antigen-antibody binding is further demonstrated by the results of passing lymphocytes from non-immunized mice through columns of beads which bear adsorbed antigens of a certain type. The population of excluded cells is unable to transfer the primary immune response to the given antigen, when injected into syngeneic immunoincompetent mice (Wigzell and Anderson, 1971).

Antigenic changes which occur as a consequence of cellular

differentiation are well documented. The plasma membrane of lymphoid cells changes profoundly during differentiation, as shown by the disappearance of TL antigen, the five-fold decrease in θ, and the six-fold increase in H-2 antigens (Aoki et al., 1969). As another example, the well-studied Forssmann antigen which appears on many types of embryonic cells is not found on adult cells (Robertson and Black, 1969).

Another widely investigated field has been that of cell-cell adhesion in vitro. Numerous tissues have been disaggregated to single cells and allowed to reaggregate in vitro. Under these conditions homologous cells adhere to each other. When two cell types are mixed, the eventual result is a circular mass of cells composed of an inner circle of one cell type, surrounded by an outer circle of the other type (Roth, 1968). Further studies led to the discovery that whereas chick embryo heart cells are usually less adhesive and aggregate around retinal epithelial cells, under some circumstances the reverse can occur (Armstrong et al., 1972). The position reversal effect was attributed to changes in the cellular adhesiveness of heart cells during cell culture. These results and others have been interpreted according to a differential adhesion hypothesis of Steinberg (1970) which states that cells have a characteristic adhesiveness (which may change during development)

and that their sorting behavior in vitro occurs so as to maximize the final total of the strengths of adhesion between all the cells in an aggregate.

A parallel ability of cells to become part of isotypic tissue in vivo has been shown by experiments of Weiss and Andres (1952). They injected black pigmented feather stem cells into the blood stream of unpigmented host chicks. The injected cells lodged in feather germs, as shown by the patches of black feathers which eventually appeared. The ability of cells to form correct intercellular connections is more even dramatically demonstrated by the exquisite specificity of cell-cell connections in the central nervous system in both normal and severely altered (by an experimenter) circumstances (Sperry, 1951; Jacobson, 1970).

A closely related area of research has centered on the role of ligands in the formation of cell-cell contacts. Specific supernatant factors have been identified from chick neural retina cells (Lilien, 1968; Lilien and Moscona, 1967), sponge cells (Humphreys, 1963; Moscona, 1968) and mouse cerebrum cells (Garber and Moscona, 1972). The mouse cerebrum factor, which is typical of these, stimulates aggregation of mouse, and to a lesser extent of chick, cerebrum cells, but does not affect other types of mouse brain cells or cells from non-nervous tissue. Its re-

lease into the supernatant depends on both the age and the differentiated state of the producing cells. Cell sensitivity to the factor is also age dependent. Moscona (1968) has proposed that ligand molecules are one means by which cells communicate. It is not known whether their release from cells cultured in vitro reflects a perturbation of the normal state, or whether such molecules may be one means of communication at a distance between cells in an organism. It is of interest in this respect that the mammalian hormone glucagon which acts on the cell surface has been shown to regulate adenyl cyclase activity in the fungus Neurospora crassa (Flawia and Tores, 1972) reflecting what may be a widespread appearance of such molecules and their conservation during evolution.

A related phenomenon to cellular adhesiveness is agglutinability. Most research in this area dates from the recent discovery of a group of plant glycoproteins known as lectins. Similar results have been obtained from the study of Concanavalin A (Con A), wheat germ agglutinin, and soybean agglutinin. Con A agglutinates transformed cells in cultures, but will not agglutinate normal cells unless the cells have been previously subjected to mild trypsinization (Inbar and Sachs, 1969a & b). The binding sites for Con A are present on normal cells, but 85% are in an unexposed, or cryptic, condition. Reversion of SV40 transformed

cells to normal cells is correlated with an apparent loss of binding sites, but is not correlated with the loss of the SV40 genome or the SV-40 specific T antigen (Inbar et al., 1969; Pollack and Burger (wheat germ agglutinin), 1969). Thus, cell agglutinability depends on the number of exposed lectin binding sites, and this number is greatly increased as a consequence of viral transformation.

It has been solidly demonstrated that lectins are not taken up into cells, but exert their effect through binding to the cell surface. It is therefore of special interest that the differentiation of lymphocytes is affected by lectin binding. The changes are apparently due to changes in the internal concentrations of various cyclic nucleotides, caused by the lectin binding (Smith et al., 1971; Greaves and Vauminger, 1972).

The general role of cell surface carbohydrate in cellular recognition is an intriguing one. Carbohydrates appear to be particularly well suited to this function because of the very large number of specific molecular structures which can be made from a small number of monomers. Four different monosaccharides in a chain can be arranged in many more combinations than can twenty amino acids in a polypeptide chain of comparable size (Ginsburg and Kobata, 1971). This is due to the possibility for forming bonds between several different hydroxyl groups, because

of the option for α or β linkage and because of the possibility of forming branched chains. The unusual distribution of glucose, the most abundant sugar in nature, adds further speculative strength to this possible role for carbohydrates. Glucose is found in large quantities in animals both free in the intercellular fluid and in glycogen, but it is virtually absent from mammalian glycoproteins and glycolipid (Ginsburg and Kobata, 1971). This distribution might be explained if exposed carbohydrates in glycoprotein and glycolipid molecules were actually involved in intercellular recognition reactions.

Experimental support for these suppositions has recently appeared. Carbohydrates are involved in the intercellular adhesion of embryonic chick neural retina, and mouse ascites cells (McGuire, 1972). Other work has shown that blood platelets bind to a wound by sticking to carbohydrate moieties of collagen (Basmann, 1971; Jamieson et al., 1971). Both investigators suggest that the binding occurs through a glycosyl transferase-substrate reaction. The possible widespread role of glycosyl transferases in cell-cell interactions has recently attracted a great deal of attention (Roseman, 1970; Roth and White, 1972). The theory is an attractive one, because the binding reaction is also an enzymatic reaction and therefore has the inherent ability to modify both cells by the very act of forming the inter-

cellular contact. This would give rise to a highly specific but yet flexible system.

Another recent field of research involving the cell surface membrane has been the investigation of cell fusion mediated by inactivated Sendai virus. Although the molecular mechanisms by which fusion occurs remain unknown, it has been established that the fusion promoting factor of the virus lies in the lipoprotein layer of its membrane (Barbanti-Brandano et al., 1970). When mouse melanocytes are fused with macrophages, a plasma membrane receptor site necessary for phagocytosis in macrophages disappears from the heterokaryon within twenty hours after fusion (Gordon and Cohn, 1970). However it has been shown that trypsin treatment unmasks the receptor, leading to the conclusion that it is blocked in the heterokaryon by a melanocyte cell surface factor. The implication is that after fusion a general redistribution of surface molecules occurs so that within a short time the surface of the heterokaryon resembles a composite of the two parent types. This conclusion is reinforced by the results of Frye and Edidin (1970) who fused mouse and human cells which were marked with fluorescent labeled antibodies. Ten minutes after fusion fluorescent microphotography revealed heterokaryons composed of a red half and a green half (representing the two fluoresceins). After forty minutes however the two colors

were evenly distributed over the entire cell surface. The conclusion that the cell membranes are dynamic structures in which surface antigens are free to move and redistribute seems inescapable.

A field which appears to be growing increasingly complex is that concerned with the various phenomena grouped under the title "contact inhibition". Some progress has been made through the realization that contact inhibition of growth and of movement are two distinct phenomena (Stoker and Rubin, 1967; Macieira-Coelho, 1967). The work of Schutz and Mora (1968) and of Gurney (1969) indicates that inhibition of growth does require cell-cell contact, however the situation with respect to inhibition of movement is not so clear. A recent review by Kohn and Fuchs (1970) includes an extensive discussion of the conflicting results in this area of investigation in both normal and neoplastic cells.

Neoplasia presents the investigator with a bewildering array of cellular defects and abnormalities of which no single one has yet been identified as a characteristic of every type of malignancy. However a large number of changes involving the cell surface membrane are already well known. A variety of antigenic changes, including the appearance of the embryonic Forssmann antigen on adult cells after transformation (O'Neill,

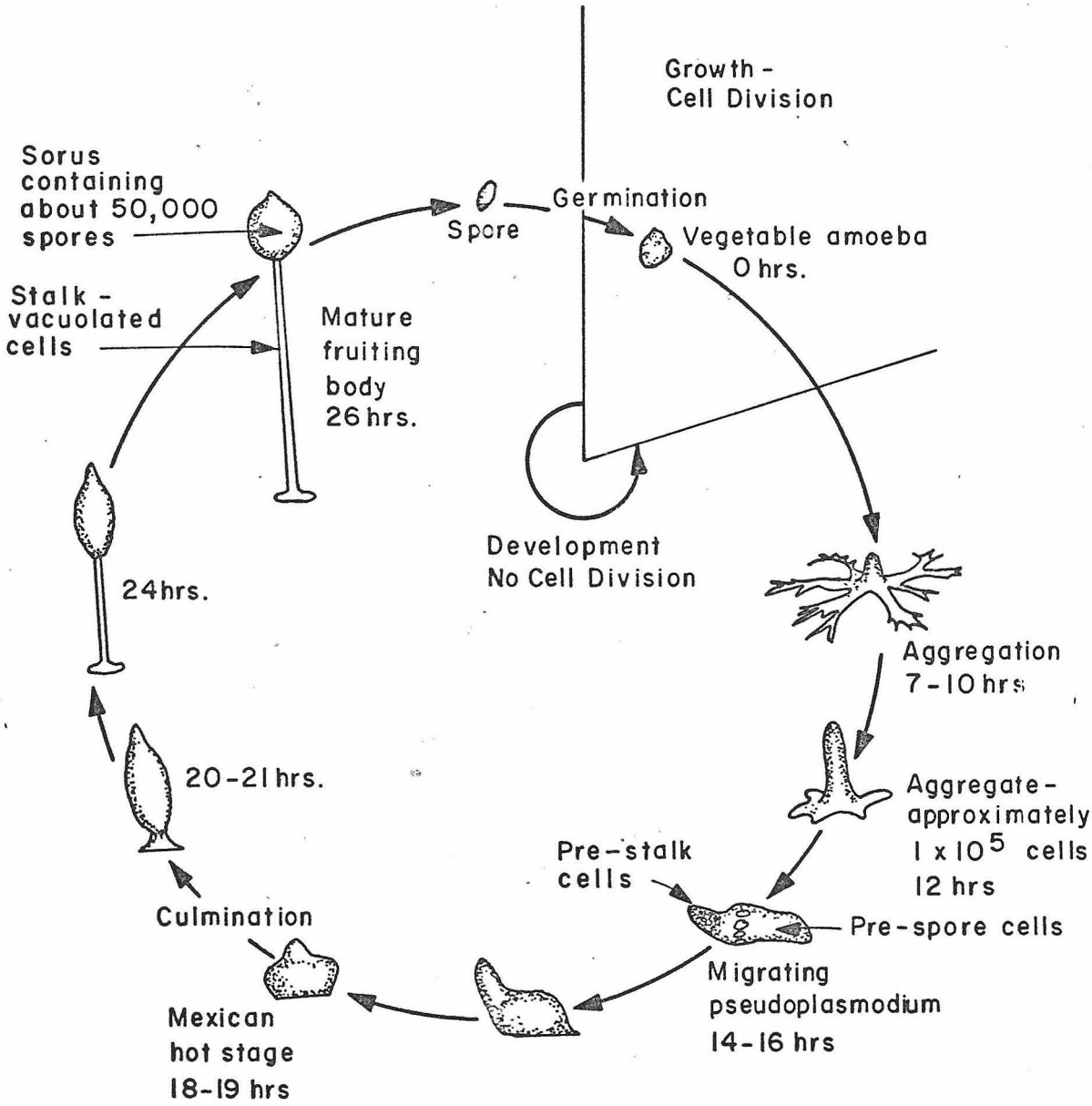
1968; Siskind and Benacerraf, 1969) have been reported. Both increases and decreases in cell agglutinability due to transformation are known (Sela et al., 1970). Neoplastic cells show an altered response to physiological regulatory factors (Holley, 1972) and a variety of changes in contact inhibition of both movement and growth (see Kohn and Fuchs, 1970 for review). The pioneering work of Loewenstein and co-workers (1967) has demonstrated that normal cells can communicate by means of electrical coupling created by exchange of ions between neighboring cells. They have found defective coupling of this type among hepatoma cells and between malignant thyroid and gastric cells (Jamakosmanovic and Loewenstein, 1968; Kanno and Matsui, 1968). Hepatoma cells also display altered intercellular junction sites. Benedetti and Emmelot (1967) have reported a significant deficiency of gap junctions in these cells as compared to the number in normal liver. A similar result has been noted in squamous cell cervical carcinomas (McNutt et al., 1970). Finally, plasma membranes of neoplastic cells have been reported to show altered permeability, notably a specific "leakness" to certain intracellular enzymes (Sylven et al., 1959) which is not due to cell damage. Considering all the available evidence, there may well come a time when one can state with certainty, as some workers have already suggested, (Wallach, 1972; Holley, 1972) that <u>all</u> neoplasias involve some abnormality of the cell surface membrane.

SLIME MOLD PHYSIOLOGY

The outline of slime mold development which follows does not intend to be a comprehensive review of what is known. Rather it includes a brief outline of the slime mold life cycle and its physiology, with an emphasis on those aspects of the life cycle which involve intercellular communication in one form or another.

The schematic outline of the life cycle of Dictyostelium discoideum, shown in Figure 1, can be divided into two mutually exclusive phases: growth and development. The development phase is triggered by the exhaustion of nutrients and is abruptly halted if nutrients reappear. The two phases are also distinguished by the absence of intercellular communication during vegetative growth, and the many forms of such interaction during development. It should be noted that there has been a report that vegetative amoebae excrete a substance which causes the amoebae to move away from each other (Samuel, 1961). With the exception of this primitive form of communication, it can be stated with some confidence that 0 hour cells (vegetative cells just removed from a food source) are a homogeneous population with respect to past and present cellular interactions.

THE LIFE CYCLE OF THE CELLULAR SLIME MOLD



The first eight hours of development involve biochemical preparations for aggregation. Dictyostelium aggregation is mediated by cyclic AMP (Konijn et al., 1967; Barkley, 1969). During the period preceeding the appearance of visible aggregates, there is a one hundred-fold increase in the production of cyclic AMP (Bonner, 1969) a concomitant increase in the sensitivity of the response to cAMP (Bonner, 1969) and changes in the production and activities of an extracellular cAMP phosphodiesterase (Malkinson and Ashworth, 1973; Chang, 1968) and an inhibitor of the phosphodiesterase (Gerisch et al., 1972). The evidence indicates that the cells aggregate along a gradient of cAMP (Bonner, 1971). However experimental proof of the existence of such a gradient has not yet been reported and the means by which it might be produced remains a subject of considerable theoretical debate (Keller and Segel, 1970 a & b; Cohen and Robertson, 1971a & b). On the basis of visual observations which show that cells aggregate in a series of pulses of movement (Arndt, 1937; Cohen and Robertson, 1971a) Shaffer (1957) proposed that the cAMP gradient passes outward from the center by a relay system from cell to cell. Gerisch (1968) has elaborated on and further developed this theory.

There is some evidence that by the time the cells begin to form aggregates the population is no longer homogeneous. Ashworth and Sackin (1969) have proposed that aggregates are initiated by aneuploid cells which carry two copies of the chromosome bearing the gene(s) responsible for cAMP production. Takeuchi (1963) has reported that an antigen found on mature spores is found in differing amounts among pre-aggregation cells. The cells containing increased amounts of the antigen were distributed randomly among the aggregating cells. Takeuchi has also reported (1969) that pre-aggregation cells can be sorted into two classes on the basis of a difference in their buoyant density. Thus there is some evidence that after eight hours of development the developing cells are no longer a homogeneous population, and that the position of a cell in the aggregate may not be due to chance, but may be the result of some intrinsic property of the cell.

It is nevertheless clear, that if such differences in the cell population do exist, they are phenotypic, not heritable. As shown in Figure 1, migrating slugs contain an anterior region composed of pre-stalk cells and a posterior region of pre-spore cells. If slugs are cut in half and kept for some time under conditions in which they continue to migrate, each half will eventually form a small but normal

fruiting body containing the correct proportions of spore and stalk cells (Bonner and Adams, 1958). Bonner (1952) followed isolated anterior ends of cut slugs through ten fruiting cycles but could detect no differences between the final population of cells and the parent population.

The nature of the cell-cell contacts formed during aggregation, are not well known. Gerisch (1968) has shown that Dictyostelium cells possess two distinct types of "stickiness". One type, which is distributed over the entire cell surface, is sensitive to 10^{-2}M EDTA, while the other, localized at the ends of the cells is resistant. Gerisch also demonstrated that aggregation phase cells (cells after 10 hours of development) will clump in the presence of EDTA whereas vegetative amoebae will not. Microphotography has revealed (Gerisch, 1968; Cohen and Robertson, 1971) that aggregating cells form chains in which the cells are connected end to end.

Sonneborn has exhaustively studied the immunological properties of developing cells (Sonneborn et al., 1964). He characterized several cell surface antigens including one which is undetectable in vegetative amoebae and rises rapidly to a peak during the first 8 hours of development. Gerisch and coworkers (Beug et al., 1970, 1973) have described a similar anti-

genic determinant using different immunological tests.

Cells which have been incubated in the absence of a food source for eight hours whether on plates, in roller cultures, or in suspension on a rotary shaker, will form normal aggregates within 2-3 hours after plating (or replating in the case of cells incubated on plates) (Gerisch, 1968; Chapter I this thesis). Such cells have been termed "aggregation competent" cells. Experiments described in Chapter I show that the only requirement for acquisition of this state appears to be the series of biochemical events which are induced by the absence of an available food source. There is no necessity for cell-cell contact during this process, or for the formation of gradients of any chemical substance. The immunological results described above demonstrate that cell surface changes do occur during this period, however they do not shed light on the crucial question of whether such changes are necessary for aggregation competence. In this respect, it is of interest that Sonneborn et al. (1964) tested thirteen morphological mutants which are unable to aggregate, for the presence of the so-called aggregation antigen. Twelve of the thirteen strains were found to contain the antigen. It is also not yet known whether the appearance of new antigens is the result of a con-

figurational change in the cell membrane which exposes antigens that were previously blocked, or whether synthesis of new proteins is involved. Thus the changes in the cell cytoplasm and on its plasma membrane which are necessary for the attainment of aggregation competence remain largely unknown, although various changes have been documented and evidence exists which indicates that the critical regions will be found to be localized at the ends of the cell.

The period immediately following aggregation, between 12 and 16 hours of development, is one of the most intriguing but least understood phases of slime mold development. A variety of experiments, using vitally stained, chemically stained, and radioactively labeled cells, as well as numerous cutting, grafting and cell mixing experiments, all lead to the conclusion that this is a period of violent cell reassortment within the aggregate and the developing slug (Raper, 1940; Bonner, 1952, 1959; Bonner and Adams, 1958; Takeuchi, 1969; Bonner et al., 1971). However the cell properties which determine these events and the mechanisms which regulate them remain a mystery.

At the end of this period, the slug contains two cell types distinguishable on histological, cytological, and biochemical grounds. The anterior 1/3 of the slug contains pre-stalk cells, while the remainder is composed of pre-spore cells containing

characteristic vacuoles. These vacuoles eventually fuse with the cell membrane to form the spore cell wall (Hohl and Hamamoto, 1969). Although both cell types contain many evidences of differentiation and commitment to one developmental fate, both the individual cells and the organism as a whole remain extremely flexible in their ability to respond to environmental change. As described above, if the slugs are cut along the pre-spore-pre-stalk line, and the separated ends are kept in conditions which prevent culmination, each end will redifferentiate so that it contains the correct ratio of pre-spore to pre-stalk cells (Raper, 1940; Gregg, 1965). If conditions are such that culmination is immediately induced, the anterior end will produce a long skinny stalk supporting a tiny sorus while the posterior end forms a large sorus on a barely visible stalk. The clear implication of such experiments is that the developmental fate of a cell can be changed by artifically changing its position in the slug. Therefore individual cells within the organism must be capable of sensing their cellular surroundings and communicating this (and possibly other) information from cell to cell.

The work of Raper (1940) and Gregg (1971) implies that such developmental flexibility not only involves intercellular communication, but actually requires cell-cell contact. Gregg

(1971) isolated both individual cells and groups of cells from organisms at various stages of development. In every case, individual cells were incapable of further differentiation or redifferentiation to a different cell type. Isolates containing small numbers of cells displayed both these capabilities. Both single cell and group isolates retain the ability to dedifferentiate to vegetative amoebae and resume cell division, when placed in the presence of a food source (Raper, 1940).

Another property of slime mold cells which apparently requires cell-cell contact is that of synergistic development. Various morphological mutants, blocked at different stages of development, can complete development and form normal fruiting bodies when mixed and plated with wild type cells (Sussman, 1954; Ennis and Sussman, 1958; Weber and Raper, 1971). The proportion of wild type cells necessary for this effect varies depending on the particular mutant involved, but proportions as low as 10 percent are sufficient in some cases. In all successful mixtures the spores produced through synergy reflect the two parent populations. Thus there is no evidence that heterokaryons are formed or that any other type of genetic exchange occurs. A thin strip of large pore dialysis membrane inserted between the mutant and wild type cells prevents synergy, indicating that either an extremely labile molecule is involved or that actual cell-cell con-

tact is required.

Returning to some slightly less mysterious events in slime mold development, the period between 16-18 hours is the period of slug migration. The coordinated tropisms of the migrating pseudoplasmodium are further clear evidence of the sophisticated cell interactions in this organism. The pseudoplasmodium migrates towards higher heat, greater humidity and light (Bonner et al., 1950; Bonner and Shaw, 1957; Francis, 1964). The duration as well as the direction, of slug migration depends on environmental conditions. Slugs incubated in the dark, or on a substratum of low ionic strength, will continue to migrate for as long as they are able (Bonner et al., 1952; Newell et al., 1969) If transferred to a buffered substratum or briefly illuminated, the slugs will cease migration and culminate. The sensitivity of the migrating slug to these environmental conditions is particularly remarkable because the individual cells within it display none of these properties prior to the beginning of development.

Perhaps the most thoroughly investigated aspect of slime mold development is the study of the so-called "developmentally controlled" enzymes. Approximately twenty enzymes have been shown to increase in specific activity at a specific time in development which is correlated with a particular morphological

stage (see Sussman and Sussman, 1969 and Newell, 1971 for review). The need for prior RNA, and concomitant protein synthesis has been well demonstrated. In most cases, enzyme induction does not occur in morphological mutants whose development is blocked prior to the stage at which induction normally occurs. The specific activity of these enzymes can often by used as a biological marker to indicate the developmental state of the cells (Newell et al., 1971; Newell, 1971). However, there are many differences in behavior among the group of enzymes considered to be developmentally controlled, and recent evidence exists which implies that some of them may not be, as originally supposed, an integral or necessary part of the slime mold developmental program (Quance and Ashworth, 1972; Tuchman, Chapter II this thesis and unpublished observations). A more complete discussion of developmental enzymes is presented in Chapter II of this thesis.

Dictyostelium discoideum is an organism particularly well suited to the study of cell-cell interactions for a variety of reasons. These include the fact that the life cycle is short, can be accomplished synchronously by large numbers of cells under laboratory conditions, and can be easily manipulated and monitored by the experimenter. More particularly, as the dis-

cussion above indicates, the cells are capable of several types of complex cellular interaction, and the time at which such interactions begin can be precisely pinpointed.

It is critically important that studies of cellular interactions begin with a homogeneous cell population and be carried out in conditions as close to the natural state as possible. The difficulties in extrapolating from the results gained from the study of artificial cell societies (whether assayed by adhesiveness, agglutinability, immunological activity, etc.) to actual in vivo function are awesome. We have attempted to avoid these difficulties by studying a biological phenomenon, the process of developmentally programmed aggregation in Dictyostelium. We have asked the question are there molecules on the cell surface necessary for aggregation, and if so, can they be isolated in a biologically active membrane preparation? Further, when do these molecules appear during normal development, and does the interaction between two neighboring cell surfaces signal the cell or affect its subsequent development in any way? We have been able to isolate a partially purified plasma membrane fraction which is capable of specifically blocking the aggregation of other cells. We have investigated the nature of this interaction by monitoring previously characterized developmentally controlled events; particularly morphogenesis and the specific activities of develop-

mentally controlled enzymes.

This system affords the opportunity to eventually purify the active component(s) from the membrane preparation and study its interaction directly with cells which are in their normal condition, and are carrying out a well characterized biochemical program. Such a system would constitute a tool for the study of the role of the cell surface membrane in cellular communication which has not been previously available.

Chapter III of this thesis turns to a different subject. In it we describe the major changes in the pattern of slime mold protein synthesis during development. We have discovered that during the first few hours of development a single protein species accounts for 20 percent of the total protein synthesis. The protein was found to be a component of the membrane fraction and was purified and identified as slime mold actin. Although we have no explanation of why the cells might need such greatly enhanced synthesis of actin at this stage of development, it may provide the means for isolating a pure mRNA, and studying its regulation during translation.

REFERENCES

Aoki, T., Hammerling, C., de Harven, E., Boyse, E.A., & Old, L.J. (1969). J. Exp. Med. 130, 979.

Armstrong, P.B. & Niederman, R. (1972). Devel. Biol. 28, 519.

Arndt, A. (1937). Roux' Arch. Enwicklungsmech Organ. 136, 681.

Ashworth, J.M. & Sackin, M.J. (1969). Nature 224, 817.

Barbanti-Brodano, G., Oyagani, S., Katz, M. & Koprowski, H. (1970). Proc. Soc. Exp. Biol. Med. 134, 230.

Barkley, D.S. (1969). Sci. 165, 1133.

Basmann, H. B. (1971). Biochem. Biophys. Res. Comm. 43, 1118.

Benedetti, E.L. & Emmelot, P. (1967). J. Cell Sci. 2, 449.

Bennett, H.S. (1963). J. Histochem. Cytochem. 11, 2.

Beug, H., Gerisch, G., Kempff, S., Riedel, V. & Cremer, G. (1970). Exp. Cell Res. 63, 147.

Beug, H., Katz, F.E. & Gerisch, G. (1973). J. Cell Biol. 56, 647.

Bonner, J. T. (1952). Am. Nat. 86, 79.

Bonner, J. T. (1959). Proc. Nat. Acad. Sci. U.S. 45, 379.

Bonner, J. T. (1969). Scient. Am. 220, 78.

Bonner, J. T. (1971). Ann. Rev. Microbiol. , p. 75.

Bonner, J. T. & Adams, M. S. (1958). J. Embryol. Exp. Morphol. 6, 346.

Bonner, J.T., Clarke, W.W., Jr., Neely, C.L., Jr., & Slifkin, M.K. (1950). J. Cell Comp. Physiol. 36, 149.

Bonner, J. T., Sieja, T.W. & Hall, E.M. (1971). J. Emryol. Exp. Morphol.

Bonner, J. T. & Shaw, M.J. (1957). J. Cell Comp. Physiol. 50, 145.

Burnet, F.M. (1970). Nature 226, 124.

Byrt, P. & Ada, G.L. (1969). Immunology 17, 503.

Chang, Y. Y. (1968). Sci. 160, 57.

Cohen, M.H. & Robertson, A.D. (1971a). J. Theoret. Biol. 31, 119.

Cohen, M.H. & Robertson, A.D. (1971b). J. Theoret. Biol. 31, 131.

Davie, J.M., Rosenthal, A.S. & Paul, W.E. (1971). J. Exp. Med. 134, 517.

Delong, G.R. & Sidman, R.L. (1970). Devel. Biol. 22, 584.

Ennis, H.L. & Sussman, M. (1958). J. Gen. Microbiol. 18, 433.

Fairbanks, G., Steck, T.L. & Wallach, D.F.H. (1971). Biochem. 10, 2606.

Flawia, M.M. & Tores, H.N. (1972). Proc. Nat. Acad. Sci. U.S. 69, 2870.

Francis, D.W. (1964). J. Cell Comp. Physiol. 64, 131.

Frye, D.L. & Edidin, M. (1970). J. Cell Sci. 7, 319.

Garber, B.B. & Moscona, A.A. (1972). Devel. Biol. 27, 235.

Gerisch, G. (1968). Curr. Topics Devel. Biol. 3, 157.

Gerisch, G., Malchow, D., Riedel, V., Muller, E. & Every, M. (1972). Nat. New Biol. 235, 90.

Ginsburg, V. & Kobata, A. (1971). Structure and Function of Biological Membrane. L. Rothfield (ed.) Academic Press, New York, p. 439.

Gordon, S.J. & Cohn, X. (1970). J. Cell. Biol. 47, 75a.

Greaves, M.F. & Vauminger, S. (1972). Nat. New Biol. 235, 67.

Gregg, J.H. (1971). Devel. Biol. 26, 478.

Gregg, J.H. (1965). Devel. Biol. 12, 377.

Guidotti, G. (1972). Ann. Rev. Biochem., p. 731.

Gurney, T. (1969). Proc. Nat. Acad. Sci. U.S. 62, 906.

Hohl, H.R. & Hamamoto, S.T. (1969). J. Ultrastruct. Res. 26, 442.

Holley, R.W. (1972). Proc. Nat. Acad. Sci. U.S. 69, 2840.

Humphreys, S. (1963). Devel. Biol. 8, 27.

Inbar, M., Rabinowitz, Z. & Sachs, L. (1969). Int. J. Cancer 4, 690.

Inbar, M. & Sachs, L. (1969). Nature 223, 710.

Jacobson, M. (1970). in The Neurosciences, vol. 2, F.O. Schmitt (ed.), Rockefeller Univ. Press, New York, p. 116.

Jamakosmanovic, A. & Loewenstein, W.R. (1968). Nature 218, 775.

Jamieson, G.A., Urban, C.L. & Barber, A.J. (1971). Nature New Biol. 234, 5.

Jerne, N.K. (1971). Europ. J. Immunol. 1, 1.

Kanno, Y. & Matsui, Y. (1968). Nature 218, 775.

Keller, E.F. & Segel, L. (1970a). Nature 227, 1365.

Keller, E.F. & Segel, L. (1970b). J. Theoret. Biol. 26, 399.

Kohn, A. & Fuchs, P. (1970). Curr. Topics in Microbiol. & Immunol. 52, 95.

Konijn, T.M., Van de Meene, J.G.C., Bonner, J.T. & Barkley, D.S.

(1967). Proc. Nat. Acad. Sci. U.S. 58, 1152.

Kraemer, P.M. (1971). In Biochemistry and Physiology of the Cell Periphery, vol. I. L. Manson (ed.) Plenum Press, New York, p. 67.

Lilien, J.E. (1968). Devel. Biol. 17, 657.

Lilien, J.E. & Moscona, A.A. (1967). Science 157, 70.

Loewenstein, W.R. & Kanno, Y. (1967). J. Cell Biol. 33, 225.

Loewenstein, W.R. & Penn, R.D. (1967). J. Cell Biol. 33, 235.

Macieira-Coelho, A. (1967). Proc. Soc. Exp. Biol. N.Y. 125, 548.

Malkinson, A.M. & Ashworth, J.M. (1973). Biochem. J. 134, 311.

Marchesi, J.T., Tillack, T.W., Jackson, R.L., Segrest, J.P. & Scott, R.E. (1972). Proc. Nat. Acad. Sci. U.S. 69, 1445.

McGuire, E.J. (1972). In Membrane Research, C.F. Fox (ed.) Academic Press, New York, p. 347.

McNutt, J.S., Hershberg, B.H. & Weinstein, R.S. (1970). J. Cell Sci. 51, 805.

Moscona, A.A. (1968). Devel. Biol. 18, 250.

Newell, P.C., Longlands, M. & Sussman, M. (1971). J. Mol. Biol. 58, 541.

Newell, P.C. (1971). Essays in Biochemistry 7, 87.

Newell, P.C., Telser, A. & Sussman, M. (1969). J. Bact. 100, 763.

O'Neill, C.O. (1968). J. Cell Sci. 3, 405.

Oseroff, A.R., Robbins, P.W. & Burger, M.M. (1973). Ann. Rev. Biochem., p. 647.

Pollack, R.E. & Burger, M.M. (1969). Proc. Nat. Acad. Sci. U.S. 62, 1047.

Quance, J. & Ashworth, J.M. (1972). Biochem. J. 126, 609.

Raper, K. B. (1940). J. Elisha Mitchell Soc. 56, 241.

Revel, J.P. & Ito, S. (1965). In The Specificity of Cell Surfaces, David & Warren (eds.), p. 213.

Robertson, H.T. & Black, P.H. (1969). Proc. Soc. Exp. Biol. Med. 130, 363.

Roseman, S. (1970). In Chemistry and Physics of Lipids 5, 270.

Roth, S. & White, D. (1972). Proc. Nat. Acad. Sci. U.S. 69, 485.

Roth, S. (1969). Devel. Biol. 18, 602.

Samuel, E.W. (1961). Devel. Biol. 3, 317.

Schimke, R.T. & Dehlinger, P.J. (1971). J. Biol. Chem. 246, 2574.

Schutz, L. & Mora, P.T. (1968). J. Cell Physiol. 71, 1.

Sela, B.A., Lis, H., Sharon, N. & Sachs, L. (1970). J. Memb. Biol. 3, 267.

Shaffer, B.M. (1957). Am. Nat. 91, 19.

Singer, S.J. & Nicolson, G.L. (1972). Science 175, 720.

Siskind, G.W. & Benacerraf, B. (1969). Adv. Immunol. 10, 1.

Slifkin, M.K. & Bonner, J.T. (1952). Biol. Bull. Mar. Biol. Lab Woods Hole 102, 273.

Smith, J.W., Steiner, A.L., Newberry, W.M. & Parker, C.W. (1971). J. Clin. Invest. 50, 432.

Sonneborn, D.R., Sussman, M. & Levine, L. (1964). J. Bact. 87, 1321.

Sperry, R.W. (1951). Growth Symposia 15, 63.

Steinberg, M.S. (1970). J. Exp. Zool. 173, 395.

Stoker, M.G.P. & Rubin, H. (1967). Nature 215, 171.

Sussman, M. (1954). J. Gen. Microbiol. 10, 110.

Sussman, M. & Sussman, R. (1969). Symp. Soc. Gen. Microbiol. XIX, p. 403.

Sylven, B., Ottoson, R. & Reves, Z. (1959). Brit. J. Cancer 13, 551.

Takeuchi, E. (1963). Devel. Biol. 8, 1.

Takeuchi, E. (1969). In Nucleic Acid Metabolism, Cell Differentiation and Cancer Growth. Cowdry, E.V. & Senoed, S. (eds.), Pergamon Press, Oxford, p. 297.

Tsukagoshi, N. & Fox, C.F. (1971). Biochem. 10, 3309.

Wallach, D.F.H. (1972). The Plasma Membrane: Dynamic Perspectives, Genetics and Pathology, Springer Verlag, New York.

Warren, L. (1969). Curr. Topics Devel. Biol. 4, 197.

Weber, A.T. & Raper, K.B. (1971). Devel. Biol. 26, 606.

Weiss, P. & Andres, G.M. (1952). J. Exp. Zool. 121, 449.

Wigzell, H. & Anderson, B. (1971). Ann. Rev. Microbiol. 25, 291.

I. INHIBITION OF THE DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM* BY ISOLATED PLASMA MEMBRANES

INTRODUCTION

The cellular slime mold Dictyostelium discoideum offers a uniquely well adapted system for the study of cellular interactions in morphogenesis, and the role of the cell membrane in these interactions.

The life cycle of this organism includes a unicellular vegetative growth phase, and after exhaustion of the food supply, a nondividing developmental phase. During development, the amoebae aggregate, forming discrete organisms composed of approximately 5×10^5 cells. This phase of the developmental program requires twelve hours under laboratory conditions. After aggregating, the cells undergo a period of major cell sorting events, after which each organism differentiates into two distinct cell types,spore and stalk, and forms an erect fruiting body. The entire program is completed in 24-26 hours.

The developing organisms possess properties not found in the individual amoebae. They are capable of coordinated movement, are phototactic (Francis, 1964) attracted towards higher humidity (Bonner and Shaw, 1957) and exhibit complex cell sorting phenomena (Bonner, 1932; Bonner, 1959; Bonner and Adams, 1958). Further evidence of the continuous exchange of information between these cells during morphogenesis is provided by

the delicate regulation of the proportions of the two developing cell types: pre-spore and pre-stalk. The ratio of pre-spore to pre-stalk cells is maintained at 2:1 and is readjusted within a single organism, through differentiation and subsequent redifferentiation, even if all of one cell type is artificially excised from the migrating slug (Bonner, 1967; Gregg, 1965).

The normal life cycle thus offers the following advantages for the study of cellular interactions: (1) Vegetative cells harvested during logarithmic growth constitute an entirely homogeneous population with respect to past and present cellular interactions (Gerisch, 1968) and (2) There exists abundant evidence for sophisticated cellular interactions in an extremely simple and easily cultivated eucaryotic organism.

We have attempted to answer the question: are there molecules on the cell surface responsible for the formation of specific cell contacts during aggregation and if so, can these be isolated in a biologically active condition? In so doing we have devised an assay which measures the ability of a partially purified membrane preparation to specifically block aggregation of Dictyostelium cells. The membranes appear to act by preventing the cells from becoming competent to aggregate, rather than by blocking the actual formation of the intercellular bonds. Insight into the nature of the interaction between the developing cells

and the membrane fragments can be gained from a comparison of subsequent biochemical events in the membrane treated cells with those observed in cells undergoing uninterrupted development. The effects of membrane treatment on the induction of enzymes known to be developmentally controlled, are described in the accompanying paper.

MATERIALS AND METHODS

Organism:

Strain AX-3, an axenic haploid strain derived from the wild-type NC-4 isolated by Raper (1935) was used throughout these experiments.

Growth and harvest of vegetative cells:

Cells were grown in HL-5 medium containing per liter: 10g proteose peptone, 5g yeast extract, 10g glucose, 0.34g KH_2PO_4, 0.67g $Na_2HPO_4 \cdot 7H_2O$ final pH 6.5. Cells were grown at 22°C on a rotary shaker at 150 rpm, and were harvested in mid-log phase at a concentration of 5×10^6/ml. The cells were harvested by centrifugation at 2500 rpm and were washed twice in cold PDF (pad diluting fluid) containing per liter: 0.111g $CaCl_2$, 4.6g $NaH_2PO_4 \cdot H_2O$, 1.51g Na_2HPO_4, 1.5g KCl, and 0.61g $MgSO_4$, final pH 6.4. The final pellet was resuspended to the desired concentration in PDF and used immediately or frozen in liquid N_2 for later use in the preparation of vegetative membranes.

Plating and harvest of developing cells:

4.5 ml of vegetative cells at 2×10^8 cells/ml in PDF were deposited on a 12.5cm Whatman No. 50 filter paper supported by two 12.5cm Whatman No. 3 filter papers saturated in PDF. Development was allowed to proceed for 14 hours, at 22°C, after which

the cells were harvested and the aggregates disrupted by washing the cells off the paper with a large bore pipette. The cells were then centrifuged at 4000 rpm, washed twice in cold PDF, re-suspended at the desired concentration, and used immediately or frozen in liquid N_2.

Cell lysis:

Cells to be used for membrane isolation were lysed by freeze-thaw in liquid N_2. This procedure resulted in approximately 95% lysis and was the most gentle method found to be effective.

Membrane preparation:

Dictyostelium discoideum cells (vegetative or aggregation phase) which had been frozen in liquid N_2 were thawed and layered on a linear gradient, 15% to 45% sucrose, 0.02M Tris pH 8.0. The gradients were centrifuged in a Spinco SW 25.2 rotor at 25,000 rpm for at least 8 hours at $4^{\circ}C$. Membranes were defined as the turbid band which appears at a density of ca. 1.16-1.17g/ml. In later experiments a step gradient was substituted: 15 ml 1.5M sucrose, 0.02M Tris; 15 ml 0.2M sucrose, 0.02M Tris overlayered with 8 ml of thawed cells in PDF. These were spun for at least 8 hours in an SW 27 rotor. In this system the membranes band at the 0.2-1.5M sucrose interface. The turbid membrane band was removed by pipetting and was diluted with at least 10 volumes of cold PDF. This solution was then centrifuged at 19,000 rpm for

30 minutes. The pellet was washed once with PDF and then resuspended in PDF with a tightly fitting Dounce homogenizer. In some cases the resuspended membranes were then autoclaved for 12-15 minutes in tightly sealed glass tubes.

Aggregation inhibition assay:

0.15 ml of vegetative or developing cells at a concentration of 3.3×10^8 cells/ml was added to 0.15 ml of the membrane preparation being tested, vortexed quickly and immediately distributed evenly by pouring onto Whatman No. 50 filters (4.25 cm) supported by an absorbent pad saturated with PDF. For photographic purposes black Millipore filters which had been previously boiled in three changes of distilled H_2O were substituted for the Whatman filter. The cells were allowed to develop in covered dishes at 22^oC in the light. Development was monitored visually at intervals for 36 hours.

Electron microscopy:

Membranes were prepared for microscopy as described above but were not homogenized or autoclaved. The cell pellet was washed in 0.01M Tris pH 7 and then fixed in gluteraldehyde and osmium tetroxide. The fixed pellet was then stained with uranyl acetate, and thin sectioned.

Additional membrane treatments:

RNase and DNase (Worthington) were incubated with membrane

preparations at a concentration of 50 µg/ml of enzyme for 30 minutes at 37°C in PDF. Trypsin and pronase (Sigma) were incubated at 2 mg/ml for 45 minutes at 37°C in 0.01M Tris pH 7.5. α-amylase (Nutritional Biochemicals Co.) was dissolved in 20mM sodium glycerophosphate, 0.01M Tris pH 7.0 and incubated with membranes for 10 minutes at 37°C. Neuraminidase (sialidase) obtained from Sigma Chemicals was dissolved at a concentration of 25 units/ml in 20mM sodium phosphate (pH 6.4), 1mM $CaCl_2$. This was incubated for 40 minutes at 37°C. All enzyme treatments were carried out in 0.5 ml in glass tubes. After incubation, the tubes were tightly sealed and the enzyme-membrane mixture was autoclaved for 15 minutes to inactivate the enzyme and sterilize the membranes. Immediately after cooling the membranes were appropriately diluted, mixed with cells and plated as described above. Control incubations of identical membrane preparations in the enzyme buffer were performed in each case.

Gel electrophoresis:

SDS polyacrylamide gels were prepared and run according to the methods of Fairbanks et al. (1971) except that samples were prepared for electrophoresis by boiling for 5 minutes in a buffer containing, in final concentration: 2.5% SDS, 10% sucrose, 10mM Tris-HCl (pH 8), 1mM EDTA (pH 8), 40mM Dithio-

threitol, and 10 µg/ml of Pyronin Y (tracking dye, obtained from Allied Chemical). Gels were run for 3-4 hours at a constant 8 mamps/gel. After destaining, the gels were scanned in a Gilford spectrophotometer equipped with a linear transport accessory, at 550 nm for Coomassie Blue and 560 nm for PAS stain.

RESULTS

Membrane preparation and characterization:

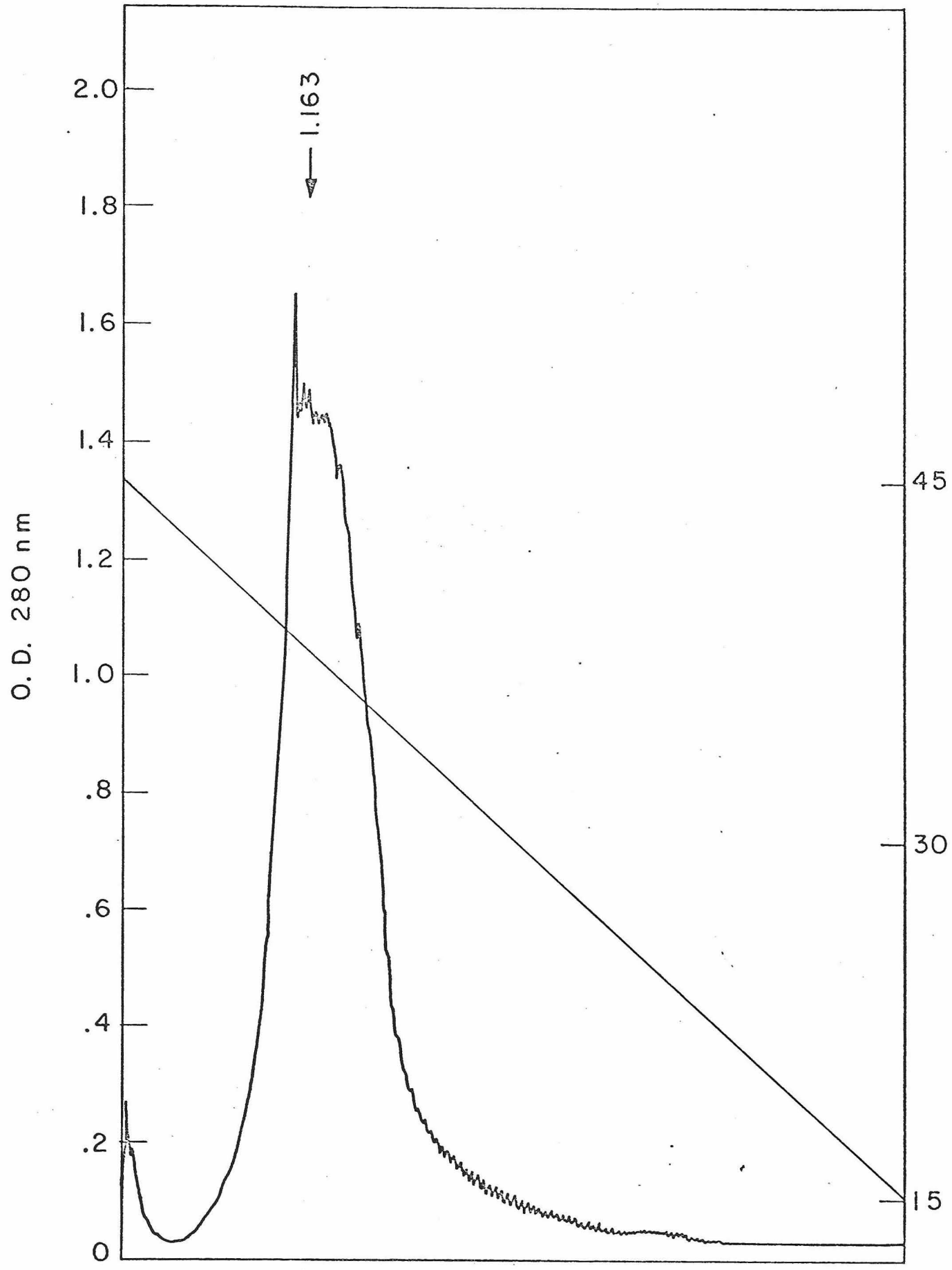
By far the most common technique for the preparation of eucaryotic plasma membranes employs cell lysis by osmotic shock, followed by some type of sucrose gradient purification of the membrane (Oseroff et al., 1973). However, Dictyostelium cells proved to be resistant to osmotic lysis even under conditions of extreme hypotonicity.

A number of lysis procedures were tested before liquid N_2 freeze-thaw was adopted as the method of choice. Extensive mechanical disruption by homogenization in Tris-acetate or Tris-EDTA pH 7.4 resulted in diffuse sucrose gradient profiles with a small membrane peak, denser than expected, and a great deal of material sedimenting in several different shoulders at densities less than 1.15 g/ml. The addition of low concentrations of nonionic detergent (Non iodet P-40, 0.5%) in buffers containing Tris or Hepes or stabilized with glycerol, resulted in varying degrees of improvement in the sucrose gradient profiles, but membranes prepared in this way proved to be inactive in the biological assays. Figure 1 shows a typical OD_{280} profile of membranes prepared by the freeze-thaw technique. Profiles of membranes prepared from vegetative and aggregation phase cells

Figure 1. Isolation of membranes on a sucrose gradient.

Cells broken by freezing in liquid N_2 were thawed and layered onto a 0.2M-1.5M sucrose step gradient. After centrifugation to equilibrium, the turbid interface was isolated, dialyzed again and 0.02M Tris pH 8, and layered on a 15-45% linear sucrose gradient. The gradient was spun to equilibrium at 25,000 rpm in a Spinco SW 27 rotor. The gradient was pumped from the bottom through a recording spectrophotometer and the optical density was measured at 280 nm.

SUCROSE GRADIENT MEMBRANE PROFILE

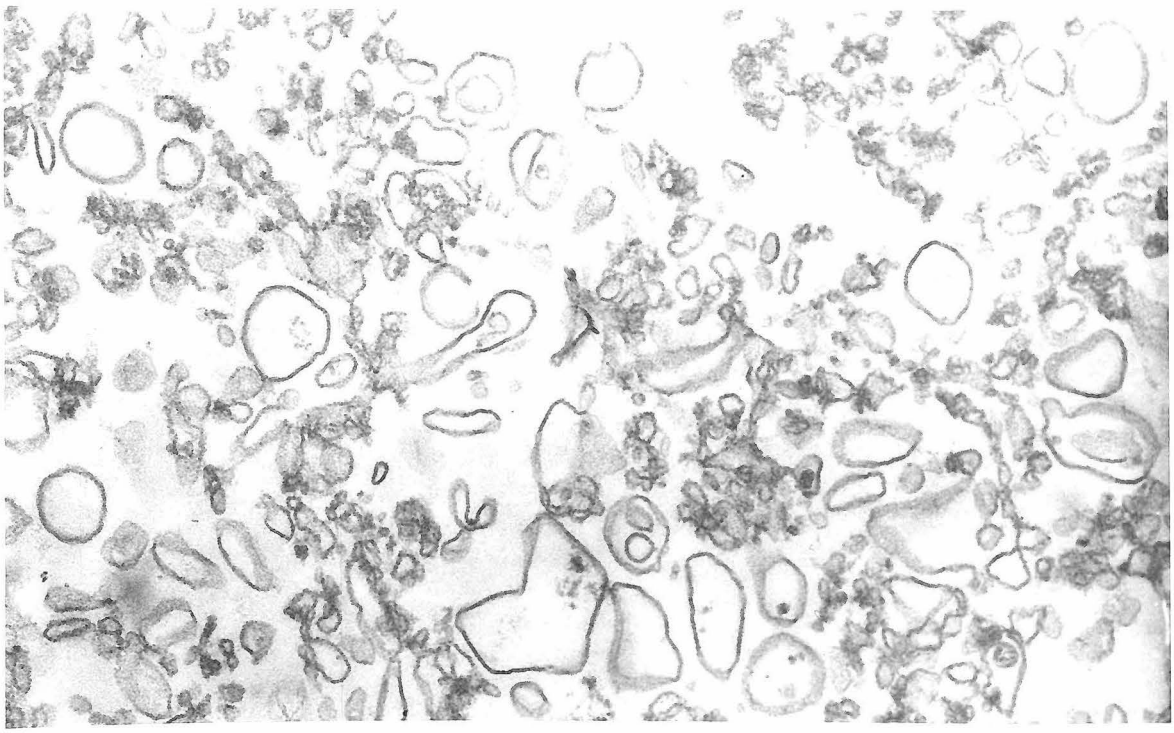


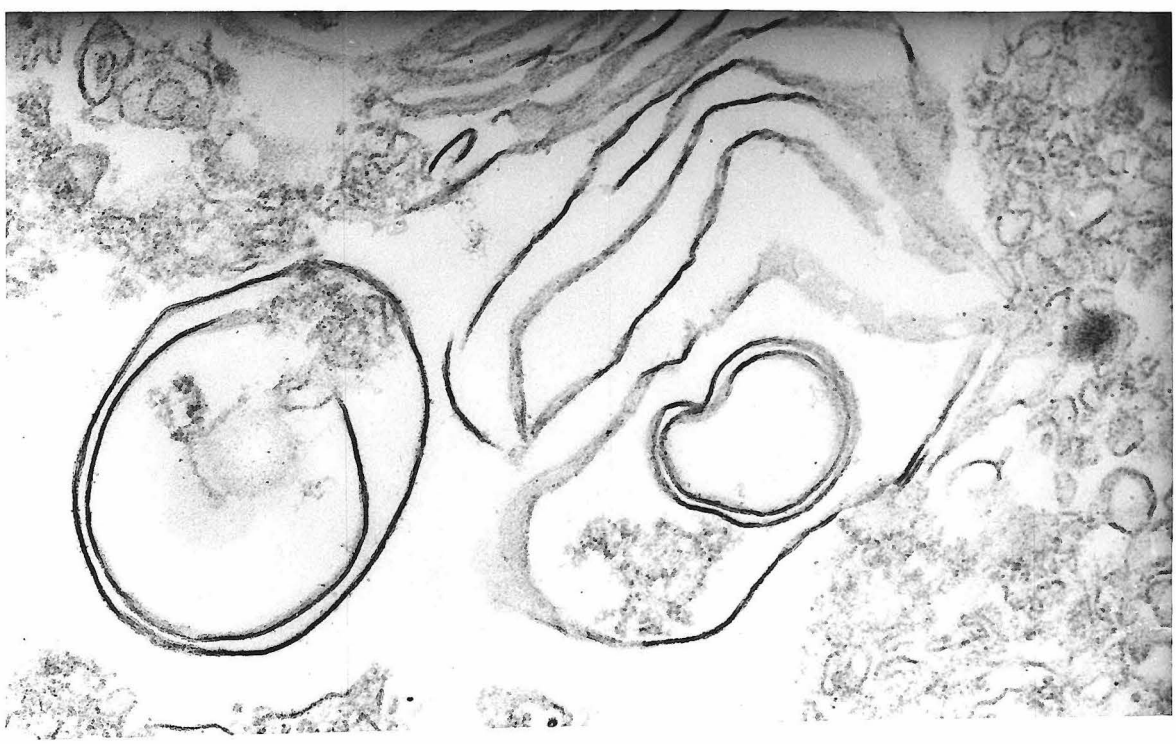
were the same in this respect. The membrane peak regularly banded at a density of 1.161-1.66.

In most cases, membranes were prepared on discontinuous sucrose gradients, by collecting from the 0.2-1.5M sucrose interface. After centrifugation to equilibrium in such a gradient, unbroken cells, nuclei and mitochondria were found in the pellet. Electron microscopy revealed that the turbid membrane band which was washed and pelleted before being fixed, contained no recognizable structures other than membrane (Fig. 2a). Figure 2a shows a field viewed at a relatively low magnification, which is typical of sections all through the pellet. Photographs at higher magnifications (Fig. 2b) confirm the presence of vesicular structures with the trilaminar appearance characteristic of plasma membrane (Olsamer et al., 1971). Extensive treatment of the membranes with RNase and EDTA prior to fixing did not alter the appearance of the membranes, indicating the absence of significant amounts of rough endoplasmic reticulum in these preparations. However the photographs do reveal that there are two distinct size classes of vesicles in these preparations. The larger are thought to be true plasma membrane fragments, while the smaller may be derived from lysozomes (Wiener and Ashworth, 1970).

Figure 2. Electron micrographs of thin sectioned slime mold membranes.

A washed membrane pellet was fixed with glutaraldehyde and stained with osmium tetroxide and uranyl acetate. Thin sections were viewed and photographed at a total magnification of a) x 40,000 and b) x 60,000.





Membranes were further characterized by SDS polyacrylamide gel electrophoresis. Major differences were detected between aggregation and vegetative phase membranes in both protein and glycoprotein composition. After purification through two rounds of centrifugation, membrane proteins were dissolved in a sample buffer containing 2.5% SDS. At the more conventional lower SDS concentrations, membrane preparations did not fully dissolve and would not enter the gel. A variety of gel procedures were tried, but only the Fairbanks method gave satisfactory results with these difficult samples. Figure 3 shows the 550 nm profile of 5.5% gels stained with Coomassie Blue. Several major differences between the vegetative and aggregation phase membranes are readily apparent. The major band/σ (RF .54-.55, MW 47,000) which is present in much greater amounts in the aggregation membranes has been identified as slime mold actin (Tuchman and Lodish, 1973). A very high molecular weight band/α (ca. 200,000) found in vegetative membranes is absent from the aggregation phase preparation, while a new band/λ at approximately 150,000 appears in the latter. There are also a large number of new bands found in aggregation membranes in the range from 70-90,000 molecular weight.

Gels were also stained with periodic acid-Schiff (PAS) for carbohydrate (Fig. 4). Both preparations contained a band at

Figure 3. Profiles of membrane preparations on 5.5% SDS polyacrylamide gels stained with Coomassie Blue (550 nm).

a) Vegetative membranes

b) Aggregation membranes

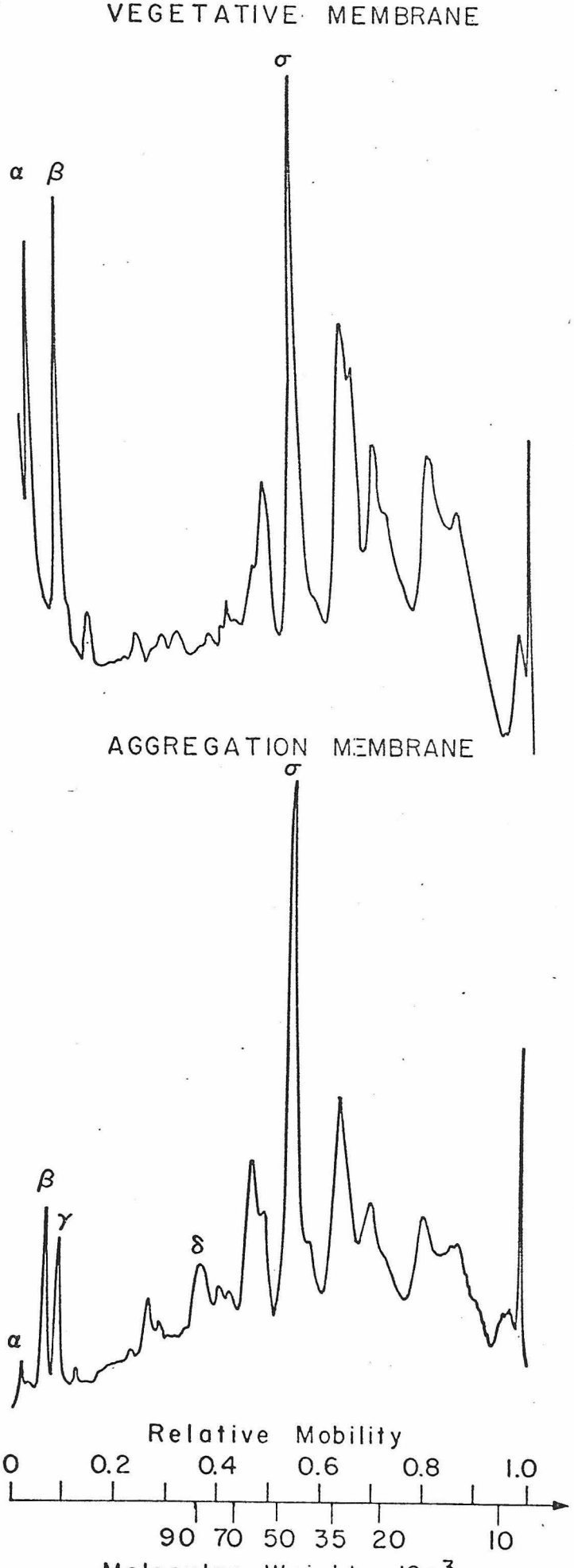
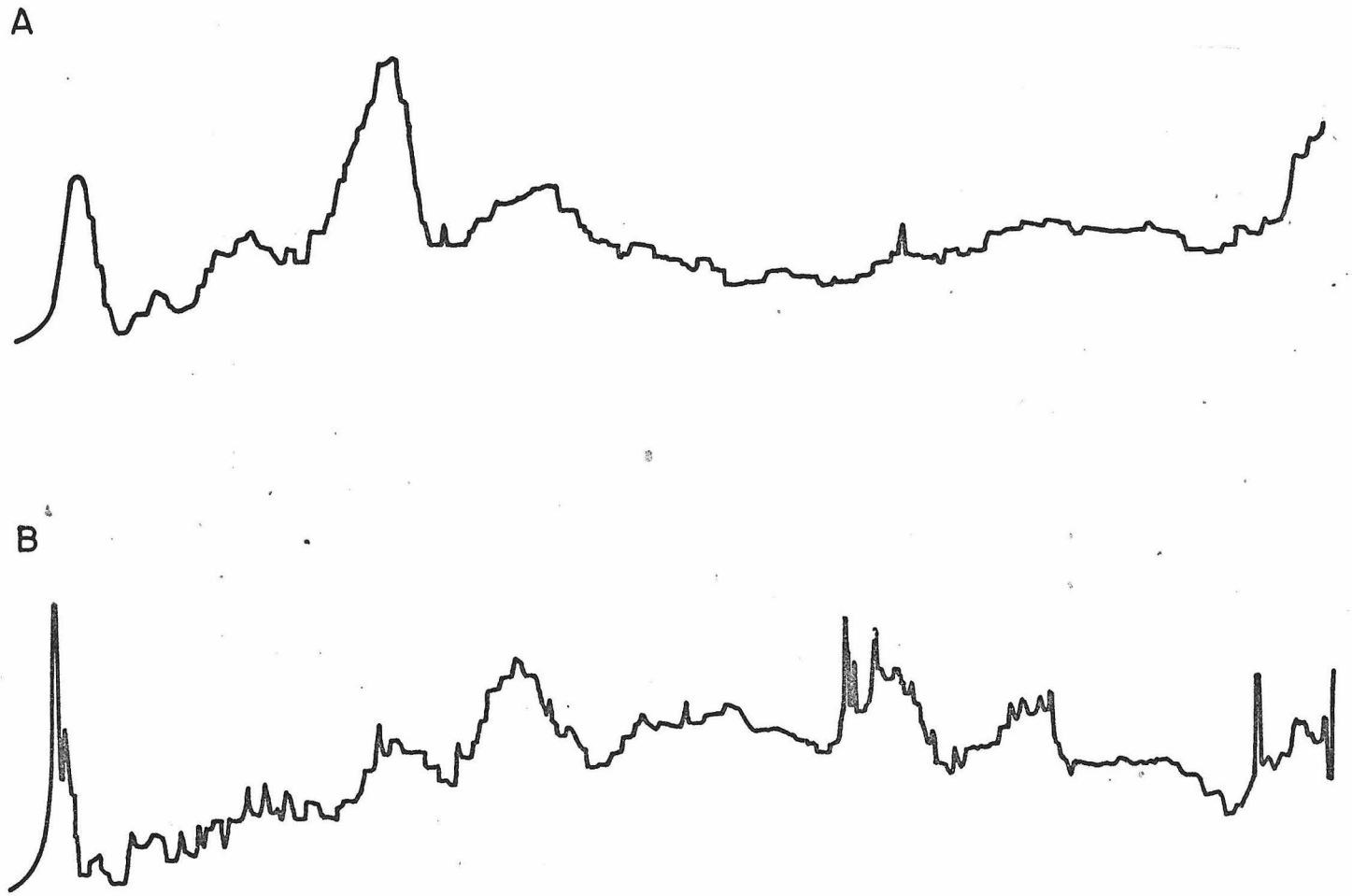

VEGETATIVE MEMBRANE
σ
α
β
AGGREGATION MEMBRANE
σ
β
γ
δ
α
Relative Mobility
0
0.2
0.4
0.6
0.8
1.0
90
70
50
35
20
10
Molecular Weight x 10⁻³

Figure 4. Profiles of membrane preparations on 5.5% SDS polyacrylamide gels stained with PAS (560 nm).

a) Vegetative membranes

b) Aggregation membranes


A
B

RF 0.085 which appears to correspond to the band which vegetative and aggregation membranes show in common when stained with Coomassie Blue. In addition, there are two bands unique to the aggregation membranes, one which increases significantly in the aggregation membranes and one which is unique to the vegetative preparation.

Inhibition of aggregation by isolated membranes:

Under laboratory conditions, untreated cells harvested from vegetative growth are fully aggregated 12-13 hours after plating and have completed fruiting body construction 26 hours after plating. Treated cells were plated under the same conditions, except that immediately before plating they were mixed with membranes prepared from cells which had been allowed to develop for 14 hours (aggregation phase membranes). When mixed with aggregation phase membranes at concentrations greater than 0.7 mg of membrane protein per plate, cells show no sign of aggregation after 36 hours of incubation (Fig. 5 and 6). At slightly lower concentrations, development is delayed but normal fruiting bodies are eventually formed. In all experiments 5×10^7 cells (approximately 3 mg of cell protein) were deposited on each plate.

Cells mixed and plated with membranes prepared from logarithmically growing cells (vegetative membranes) showed similar

Figure 5. Aggregation inhibiting activity of different membrane preparations.

5×10^7 cells per plate were mixed with membranes at the concentrations shown, and plated as described in Methods, on washed black Millipore filters. The plates were photographed after 13 hours with a Polaroid Land camera mounted on a light microscope.

AGGREGATION INHIBITING ACTIVITY OF DIFFERENT MEMBRANE PREPARATIONS

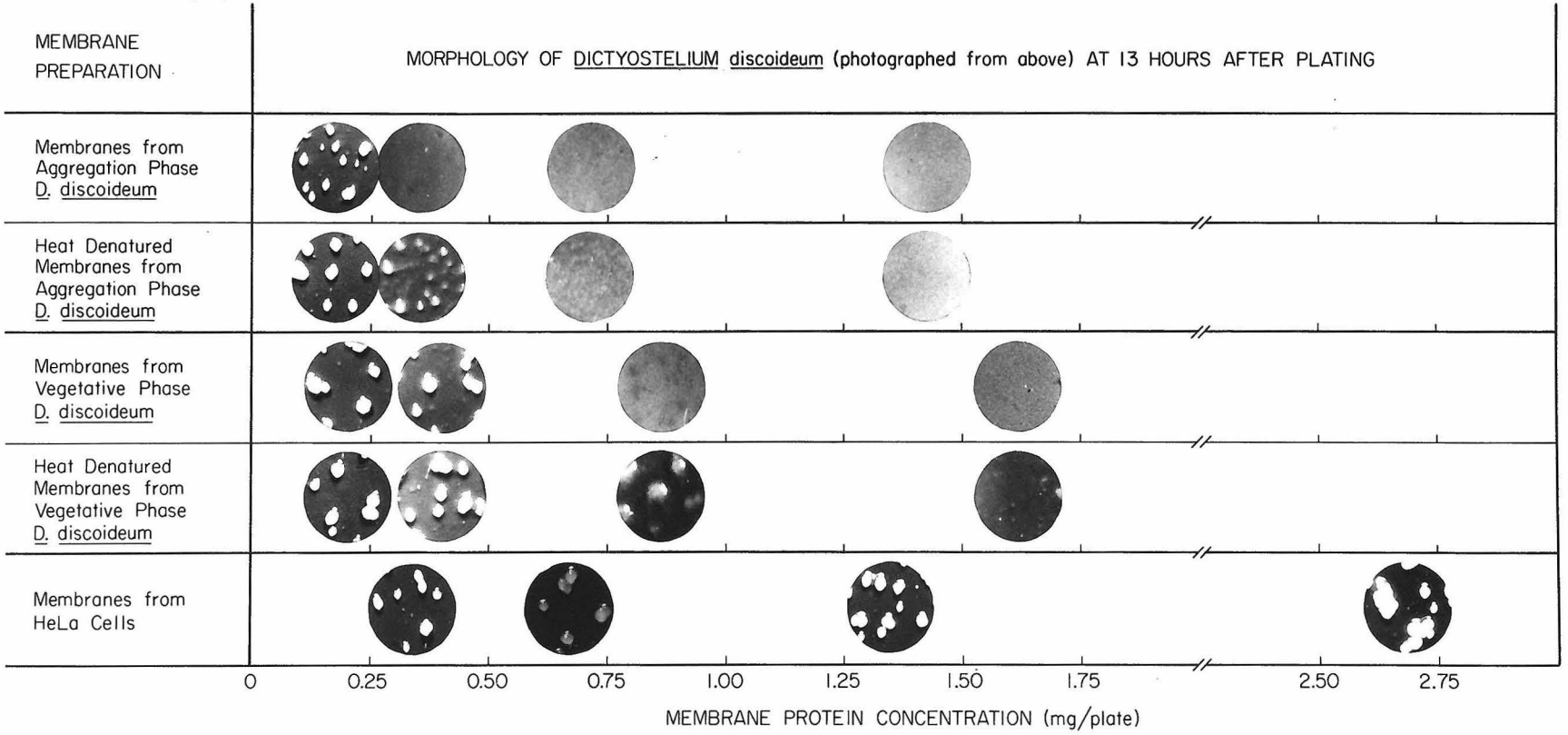
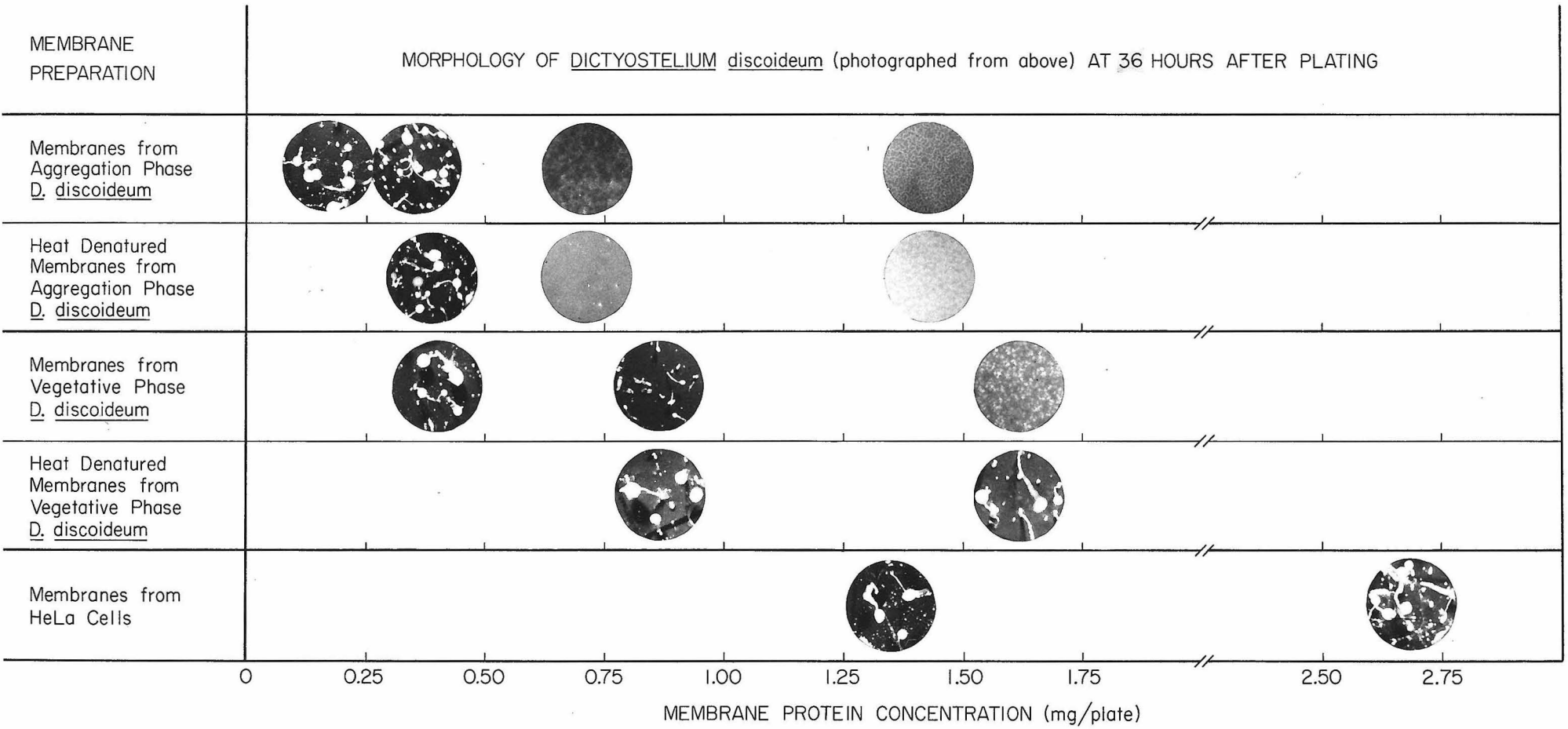


Figure 6. Aggregation inhibiting activity of different membrane preparations.

5×10^7 cells per plate were mixed with membranes at the concentrations shown, and plated as described in Methods, on washed black Millipore filters. The plates were photographed after 36 hours with a Polaroid Land camera mounted on a light microscope.

AGGREGATION INHIBITING ACTIVITY OF DIFFERENT MEMBRANE PREPARATIONS



behavior except that a two-fold greater concentration of membranes was required to produce complete inhibition of aggregation (Fig. 5 & 6). At 0.3 mg per plate, vegetative membranes caused a significant delay in aggregation and the fruiting bodies eventually formed were approximately 25 percent of the normal size. At a membrane concentration of 1.6 mg per plate, which approaches the limit that can be pipetted, the cells do not ever form normal aggregates, although very small irregularly shaped lumps do eventually appear (Fig. 6).

A more striking difference between the activity of aggregation and vegetative phase membranes was revealed by the effect of mild heat treatment (50°C for 15 minutes) on the two preparations. At all concentrations tested, mild heating caused a significant decrease in the ability of vegetative membranes to delay aggregation. Even at the highest concentration tested, such heat treated membranes were unable to prevent the construction of normal fruiting bodies. The activity of aggregation phase membranes on the other hand, showed a considerable resistence to heat treatment. Only at low concentrations of membrane (0.3 mg per plate) was any effect of heat observable, and this was limited to a difference of a few hours in the length of the delay of aggregate formation. At no concentration was the ability of aggregation phase membranes to wholly block aggregate formation

and subsequent development affected by heat denaturation of the membranes. As a result of these experiments, and to avoid the problem of bacterial contamination of the assay plates, aggregation phase membranes were routinely autoclaved before being mixed with cells for plating. Unless otherwise noted all subsequent experiments involving aggregation phase membranes utilized autoclaved membranes.

Both aggregation and vegetative phase membranes thus appear to possess an aggregation inhibiting activity. However the difference in concentration required for activity, and more importantly, the difference in heat sensitivity, indicate that the mode of action of the two preparations is different. In order to determine whether the effects were specific, and not a general effect of added membrane, vegetative cells were mixed and plated with membranes prepared from HeLa cells. At a concentration of 2.75 mg per plate, a solution which is almost too thick to pour, HeLa membranes are unable to block or even to delay cell aggregation. Nearly normal fruiting bodies are formed, protruding through the viscous membrane layer which remains on the supporting filter paper.

Chemical basis of aggregation phase membrane activity

Aggregation phase membranes were subjected to a variety of other treatments in order to shed some light on the chemical

nature of the component(s) active in the assay (Table I). The formation and stability of intercellular contacts during slime mold development are known to be sensitive to EDTA (Takeuchi and Yabuno, 1970; Loomis and Sussman, 1966). The aggregation inhibition assay was therefore performed in the presence of high concentrations of added Ca^{++} and Mg^{++}, to determine whether the membranes might be chelating necessary ions. Membranes were also treated extensively with both DNase and RNase None of these treatments had any effect on the membrane activity. Chloroform extraction of the membranes removed approximately 50 percent of their activity. Digestion with trypsin or pronase also destroyed 50 or more percent of the original activity. α-amylase had no effect on the membrane activity, however, neuradminidase, also known as sialidase, which releases terminal sialic acid residues, did appear to remove some of the aggregation inhibition activity of the aggregation phase membranes.

Cell viability during and after membrane treatment

Simple visual observation established that slow morphogenetic changes were occurring in cells plated with borderline concentrations of membrane even after 40 hours of incubation. To demonstrate that membranes did not affect cell viability, treated cells which had been harvested from filters after incubation with

TABLE I

Effect of Chemical Treatments on the Biological Activity of Aggregation Phase Membranes

Treatment	Highest Membrane Dilution Allowing Complete Inhibition of Aggregation in Vegetative Cells
No treatment	1:5
Added Ca^{++}	1:5
Added Mg^{++}	1:5
DNase	1:5
RNase	1:5
Chlorcform extraction	1:2
Trypsin	full strength
Pronase	full strength
α-Amylase	1:5
Neurominidase (sialidase)	1:3

A membrane preparation at 1.6 mg/ml was divided into aliquots and treated as indicated. After autoclaving each sample was diluted and plates were made at full strength and at dilutions of 1:2, 1:3, 1:5, 1:8. Plates were scored for the presence or absence of aggregates after 26 hours. Details of the enzyme treatments are provided in the Methods section.

membranes for up to 30 hours were washed several times in a high salt solution (0.1M EDTA, 2M NaCl) and replated. It was not possible to achieve a clean separation of cells from membranes, however a large fraction of the added membranes was removed after three washes under these conditions. After washing, subsequent development of the replated cells was observed, although it occurred extremely slowly and very small aggregates and fruiting bodies were formed. The fact that any development occurred in these experiments indicates that the cells had remained viable during membrane treatment in the absence of visible morphogenesis for periods of at least 30 hours. To provide further evidence of cell viability under these conditions, normal and membrane treated cells were pulse-labeled with S^{35} methionine for two hours at varying intervals after plating. The results in Table II show that incorporation of labeled amino acid into TCA precipitable material, occurs at a constant rate for thirty-six hours after plating. The total amount of incorporation is only about ten percent of that found in untreated cells early in development. This difference appeared to be due to decreased uptake of label by the cells in the presence of membranes rather than to a decreased rate of protein synthesis. However difficulties in cleanly separating cells from added membranes after plating, and the known "stickiness" of

TABLE II

Incorporation of S^{35}-Methionine into TCA Precipitable Material During Slime Mold Development

Time of Labeling After Beginning of Development	cpm Incorporated per 2 x 10^7 Cells	cpm Incorporated per 2 x 10^7 Membrane Treated Cells
0-2	1.05×10^6	1.3×10^5
6-8	1.2×10^6	1.9×10^5
14-16	1.0×10^6	1.5×10^5
22-24	7×10^5	1.5×10^5
34-36	-	1.4×10^5

Cells (with or without membranes) were labeled by transferring half filters supporting cells at the required stage of development to barely saturated pads on which 75 μCi of S^{35}-Methionine (New England Nuclear) had just been deposited. After two hours the cells were harvested by washing them from the filter, pelleted and then lysed in 1% SDS. A small aliquot was precipitated with 5% TCA, filtered and counted.

membranes prevent a firm conclusion as to interference with amino acid uptake.

Assay of membrane activity with developing cells

All the experiments described above involved mixing vegetative cells (0 hours of development) with membranes. To further investigate the membrane effect, membranes were also tested with cells at various stages of development. Cells were allowed to develop normally for varying lengths of time. They were then harvested, washed, resuspended and immediately mixed with aggregation phase membranes and replated. Control cells harvested and then replated in the absence of membranes quickly recapitulate their previous morphological development after a short lag (Newell, 1971; Newell et al., 1971). Figure 7 shows the results of an experiment using cells harvested 0, 3, 6, 9 and 12 hours after plating and then replated with autoclaved aggregation phase membranes. When 3 and 6 hour cells were involved (i.e., cells harvested after the indicated number of hours of normal development), the membranes proved just as effective as in the case of 0 hour (vegetative) cells. No differences in the concentration of membranes that was required to block development or in the degree of delay of aggregation was noted. However when 9 or 12 hour cells were used, exogenously added membranes, even at the highest concentrations obtainable,

Figure 7. Morphology of Dictyostelium discoideum during development with and without membranes.

Developmental stages:

0-6 hours - individual cells, seen as a flat lawn, preparing for aggregation.

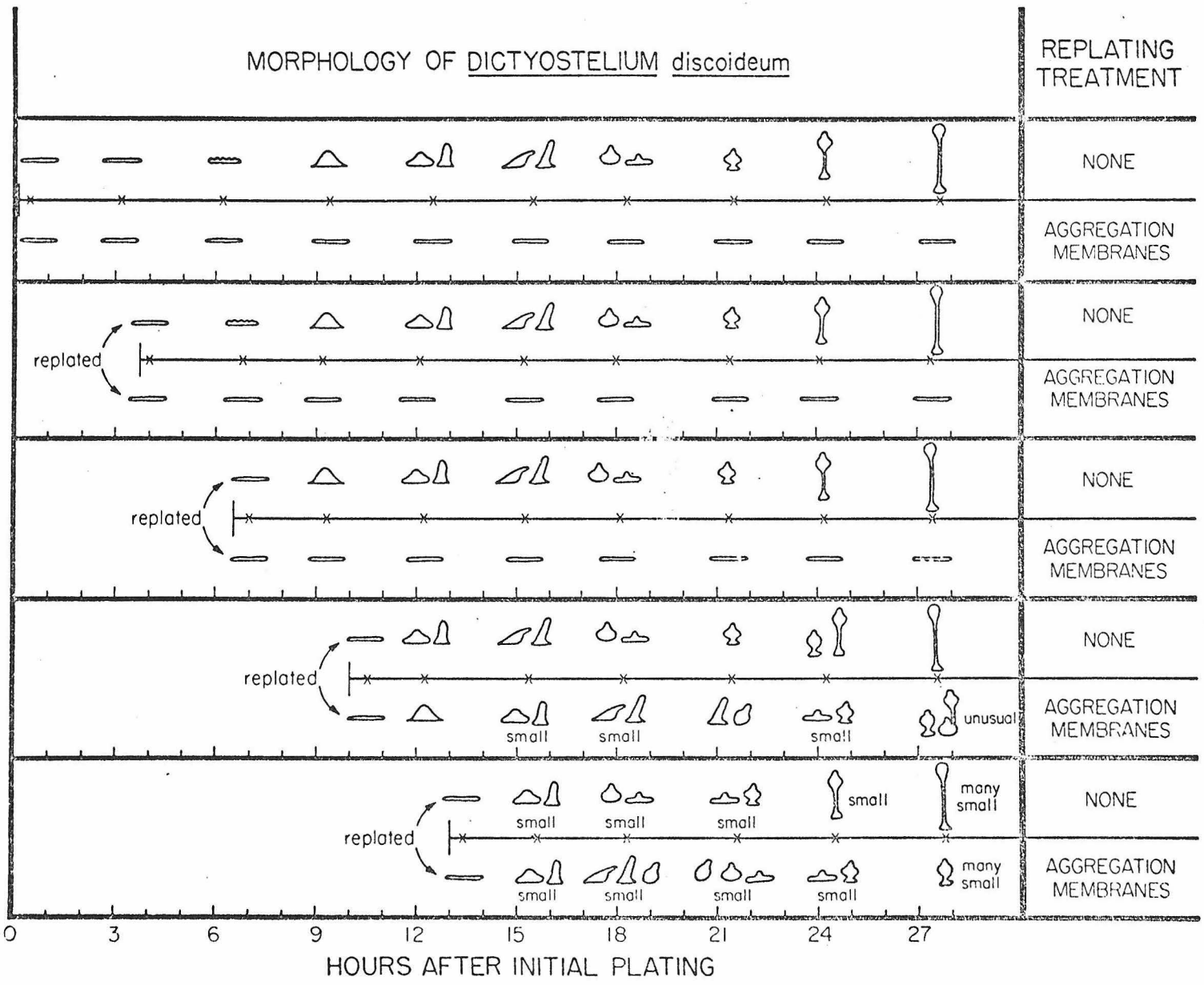
6-10 - rippled lawn followed by formation of discrete aggregates. Increasing number of cell-cell contacts being formed.

10-13 - complete aggregate formation. Cell sorting.

16-20 - Mexican hat stage. Cell reassortment.

20-26 - fruiting body construction and maturation.

Cells were allowed to proceed through normal development to the indicated stage. They were then harvested and pipetted up and down vigorously several times to yield a population of single cells. Cells were then mixed with membranes and replated. Their subsequent development, as well as development of control cells, is depicted above.


MORPHOLOGY OF DICTYOSTELIUM discoideum
REPLATING TREATMENT
NONE
AGGREGATION MEMBRANES
NONE
replated
AGGREGATION MEMBRANES
NONE
replated
AGGREGATION MEMBRANES
NONE
replated
small
small
small
unusual
AGGREGATION MEMBRANES
NONE
replated
small
small
small
small
many small
AGGREGATION MEMBRANES
small
small
small
small
many small
0
3
6
9
12
15
18
21
24
27
HOURS AFTER INITIAL PLATING

proved incapable of blocking aggregation of the cells.

These results imply that once cells have proceeded normally through the first 8-9 hours of development, they become impervious to the action of the membranes. However the experiment is marred by the fact that 8-9 hours of development coincides with time at which cell-cell contacts are normally formed during development. The results described above might therefore have been due to some kind of disruption or alteration of the membrane receptor sites during the harvesting procedure when the naturally formed cell-cell contacts are broken. The following experiment was designed to avoid this drawback. Gerisch (1968) has previously shown that cells grown on bacteria in suspension cultures undergo changes in their ability to aggregate while maintained by agitation in a unicellular state. Specifically, if such cells are harvested and plated eight hours after the exhaustion of the bacterial food source, they can begin to aggregate almost immediately. Gerisch has called this state aggreggation competence. Accordingly, the axenic cells used in these experiments, were harvested from growth medium, washed twice and resuspended in growth flasks containing sterile PDF. After 9 hours on the shaker, the cells were harvested and plated. Under these conditions, the cells showed signs of aggregation in 2 hours and formed mature rounded aggregates in 3 hours. Thus

with respect to their ability to form aggregates, such cells are equivalent to cells harvested after 9 hours of development on filters, except that no cell contacts are formed when the cells are suspended in PDF and continuously agitated on the shaker. Consequently, no cell contacts need be disrupted when the cells are harvested and mixed with membranes.

When aggregation competent cells are harvested and mixed with aggregation phase membranes, the results confirm the earlier experiment. Membranes at concentrations up to 1.5 mg/plate are unable to prevent subsequent aggregation and development of the cells, although aggregation is delayed by 1-12 hours depending on the membrane concentration.

DISCUSSION

Cells surface factors responsible for the formation of intercellular contacts have been the object of study in many biological systems (Humphreys, 1965; Metz et al., 1964; Garber and Moscona, 1972) although in most cases little more than a basic description of the physical and chemical properties of the factors has been presented. Most of the theories which have been proposed to explain the data, have visualized the cell-cell contact as involving a chemical reaction between a cell surface antigen on one cell and a specific receptor site on another cells (Tyler, 1942; Weiss, 1947). Variations of this idea depict the contact as being formed by a ligand molecule which attaches to cellular receptor sites at each end (Moscona, 1968). Virtually all are in agreement that the formation of intercellular contacts requires chemical structures which are to be found on the cell membrane.

The development of the cellular slime mold involves a well-defined transition from a unicellular to a multicellular mode of existence. It is therefore reasonable to expect that at the outset of development the cell membrane must either already possess (in an unexposed configuration) or must develop the ability to form specific cell-cell contacts. Immunological studies

(Sonneborn et al., 1964; Beug et al., 1970) have demonstrated the existence of a new antigenic determinant which increases rapidly in the first twelve hours of development. Immunologically blocking this antigen inhibited cell contact formation without affecting chemotaxis. However these indirect studies do not elucidate the role such cell surface factors play in the normal exchange of information between cells.

The goal of the present studies was to investigate the cell surface factors directly, and to analyze their biological activity by examining a process, namely aggregation, which is a well defined crucial step in normal development. To do so, plasma membranes had to be isolated in a relatively pure and active condition. The specific gravity of the membranes isolated on sucrose gradients in these experiments is characteristic of membrane material (Steck and Wallach, 1970). Further, electron microscopy reveals the absence of any recognizable subcellular organelle other than possibly lysozomes in the final preparation. The presence of 15-20 protein bands and 2-3 glycoprotein bands on SDS gels is typical of animal membranes (Guidotti, 1972), although considerably more complex than amoebae membranes (Korn and Wright, 1973). Finally, chemical and enzymatic treatments reveal that the biological activity of the membranes used in these experiments does not involve nucleic acids or divalent

cations. On the other hand, certain enzymatic treatments affecting protein, carbohydrate and lipid moieities do inhibit biological activity, as might be expected of a membrane component.

The ability of this membrane preparation to inhibit aggregation in developing cells is not the result of a simple physical blockage of the cell surface factors. When plated in a very viscous mixture of cells and HeLa membranes, Dictyostelium cells demonstrate a startling capacity to crawl through the membranous mass and form nearly normal aggregates, excluding the extraneous material. Similarly, autoclaved vegetative membranes at extremely high concentrations do not inhibit normal aggregate formation.

Neither are the chemotactic events of normal aggregation inhibited. Slime mold cells are known to aggregate in response to a gradient of cyclic AMP (Bonner, 1969). If normal chemotaxis is disrupted by added membranes, one would expect cells which are dissociated and replated with membranes after nine hours of normal development to be unable to reaggregate. Instead, the results show normal aggregation on the part of such populations at high membrane concentrations.

Visual observations of morphogenetic events and measurements of the rate of protein synthesis in cells plated with mem-

branes indicate that addition of exogenous membrane does not impair cell viability. Difficulties in separating cells from membranes after plating, prevent a direct evaluation of the developmental capacity of the treated cells. However the small fraction of cells which can be recovered after repeated high salt washes of a cell-membrane mixture, is capable of eventually forming normal fruiting bodies. The available evidence thus strongly implies that cells remain fully viable and capable of differentiating when transferred to favorable conditions, for periods up to 30 hours, in the absence of both a food source and normal development.

Removal of the food source appears to be the only necessary environmental condition for the acquisition of aggregation competence. This state is here defined as the ability to aggregate within 2 hours after plating. The necessity for any physical contact between cells or for gradients of cyclic AMP or any other substance is ruled out by the fact that cells acquire aggregation competence while suspended in PDF and continuously agitated in a manner which prevents intercellular contacts. Once cells have achieved aggregation competence they are impervious to very high concentrations of aggregation membranes. Concentrations of active membranes which totally inhibit aggregation of vegetative (0 hour) cells for up to 36 hours, are only

capable of delaying the aggregation and subsequent development of aggregation competent cells. It is immaterial whether competence is achieved through the processes of normal development on a solid substratum or though 8-9 hours of agitation in a liquid non-nutritive medium. Therefore, the added membranes appear to block the series of biochemical events which normally produce a population of aggregation competent cells eight hours after plating.

It is obviously of interest to learn the stage at which these events are blocked. One way to do this would be to plate vegetative cells with membranes and allow development for increasing lengths of time. If the cells could then be removed from the membranes and replated, the length of time after replating until aggregation would indicate how far the cells had progressed in the normal series of developmentally programmed events while in the presence of membranes. This important experiment must await a procedure for cleanly separating cells from exogenous membranes.

SDS gel analysis reveals that aggregation phase membranes differ from vegetative membranes in both protein and carbohydrate composition, as would be expected of an organelle required to express developmentally controlled biochemical events. Further, aggregation phase membranes, prepared as described, have

the ability to prevent the events which normally result in aggregate formation. The simplest inference from these data is that the isolated aggregation phase membranes display a new, or newly exposed chemical component which is capable of interacting with vegetative cells in such a way as to halt development.

Vegetative membranes are also capable of inhibiting aggregate formation, although differences in required concentration and in heat sensitivity indicate that they do so by a different mechanism from aggregation phase membranes. An attractive explanation for this activity is that it is due to the presence of receptor sites for the aggregation factor(s) on the surface of vegetative cells (and therefore on the vegetative membranes). Vegetative membranes might then interfere with normal cell aggregation by simply interacting with the exposed antigens of aggregation competent cells. This would decrease the availability of cellular antigens for forming the necessary cell-cell contacts. Suggestive experimental support for postulating that vegetative membranes carry such receptor sites is provided by the very fact that aggregation membranes interact with vegetative cells.

Interaction of aggregation phase membranes with the surface of cells which are in the first hour of development blocks the further development of these cells. The block occurs at a stage

which normally occurs sometime within the first eight hours of normal development. The molecular basis of the interaction and the means by which its inhibitory effect is exerted, remain unknown. However experiments described in the following paper show that the membrane-cell interaction causes changes in the expression of some aspects of the cell's developmental program. Thus this system may provide a tool for eventually understanding the molecular nature of these events.

REFERENCES

Beug, H., Gerisch, G., Kempff, S., Riedel, V. & Cremer, G. (1970). Exptl. Cell Res. 63, 147.

Bonner, J. T. (1969). Scient. Am. 220, 78.

Bonner, J.T. (1967). The Cellular Slime Molds, 2nd edition. Princeton University Press, Princeton, N. J.

Bonner, J. T. (1959). Proc. Nat. Acad. Sci. U.S. 45, 379.

Bonner, J.T. & Adams, M.S. (1958). J. Embryol. Exp. Morph. 6, 346.

Bonner, J.T. & Shaw, M.J. (1957). J. Cell Comp. Physiol. 50, 145.

Bonner, J. T. (1932). Am. Nat. 86, 79.

Fairbanks, G., Steck, T.L. & Wallach, D.F.H. (1971). Biochem. 10, 2606.

Francis, D.W. (1964). J. Cell Physiol. 64, 131.

Garber, B. & Moscona, A.A. (1972). Devel. Biol. 27, 235.

Gerisch, G. (1968). Curr. Topics Devel. Biol. 3, 157.

Gregg, J. H. (1965). Devel. Biol. 12, 377.

Guidotti, G. (1972). Ann. Rev. Biochem., p. 731.

Humphreys, T. (1965). Exptl. Cell Res. 40, 539.

Korn, E.D. & Wright, P.L. (1973). J. Biol. Chem. 248, 439.

Loomis, W.F., Jr. & Sussman, M. (1966). J. Mol. Biol. 22, 401.

Metz, C.B., Schuel, H. & Bischoff, E.R. (1964). J. Exptl. Zool. 155, 261.

Moscona, A. A. (1968). Devel. Biol. 18, 250.

Newell, P. C. (1971). Essays in Biochemistry 7, 87.

Newell, P.C., Longlands,M. & Sussman, M. (1971). J. Mol. Biol. 58, 541.

Olsamer, A.G., Wright, P.L., Wetzel, M.G. & Korn, E.D. (1971). J. Cell Biol. 51, 193.

Oseroff, A., Robbins, P. & Bruger, M. (1973). Ann. Rev. Biochem., p. 647.

Raper, K.B. (1935). J. Agr. Res. 50, 135.

Sonneborn, D.R., Sussman, M. & Levine, L. (1964). J. Bact. 87, 1321.

Steck, T.L. & Wallach, D.F. (1970). J. Methods Cancer Res. 5,93.

Takeuchi, I. & Yabuno, K. (1970). Exp. Cell Res. 61, 183.

Tuchman, J. & Lodish, H.F., in preparation.

Tyler, A. (1942). Western J. Surg. Obst. Gynecol. 50, 126.

Weiss, P. (1947). Yale J. Biol. Med. 19, 235.

Wiener, E. & Ashworth, M. (1970). Biochem. J. 118, 505.

II. EFFECTS OF DIFFERENTIATED MEMBRANES ON THE DEVELOPMENTAL PROGRAM OF THE CELLULAR SLIME MOLD

INTRODUCTION

In the preceding paper (Tuchman et al., 1973) we have described the preparation and properties of a partially purified plasma membrane fraction isolated from developing cells of the cellular slime mold Dictyostelium discoideum. Aggregation phase membranes were found to be capable of preventing the aggregation and subsequent development of vegetative cells when mixed with these and plated under normal conditions for slime mold development. However such membranes were found to be incapable of inhibiting the reaggregation of mechanically disrupted cells which had previously been allowed to proceed normally through the first 12 hours of the developmental cycle. Membranes prepared from vegetative cells also proved capable of inhibiting cell development. Unlike aggregation membranes, the activity of vegetative membranes was found to be sensitive to heat denaturation and required significantly higher protein concentrations to yield complete inhibition. The results of several experiments indicate that the action of of aggregation phase membrane lies in blocking the biochemical events which result in the attainment of aggregation competence (Gerisch, 1968) and not in blocking the physical events involved in the formation of the actual cell-cell contacts. Vege-

tative membranes on the other hand apparently exert their effect through a direct competition for the available cellular binding sites.

It has been shown in many different biological systems that biochemical interactions on the outside of the cell surface may affect, and in some cases direct events inside the cells (Anderson and Huebner, 1968; Lilien, 1969; Sutherland et al., 1965; Kohn and Fuchs, 1971). Often these interactions occur between molecules on the surface of neighboring cells and play a role in intercellular communication. As yet however the molecular mechanisms of such interactions and the ways in which a signal can be transmitted from the cell exterior to the nucleus and/or cytoplasm remain unknown.

In this paper we describe experiments designed to shed light on the effect of membrane treatment on some of the biochemical events which make up what is known of the slime mold developmental program. The most thoroughly investigated aspect of this program is the pattern of induced enzyme synthesis. The induction, synthesis, and subsequent disappearance of nearly twenty so-called developmentally controlled enzymes have been studied (Sussman and Sussman, 1969; Newell, 1971, for review). Six enzymes from among this group are investigated in this paper. They cover the temporal range from

the enzyme induced earliest in development (N-acetyl glucosaminidase) to the latest (alkaline phosphatase, isozyme II) of the newly synthesized enzymes. One of them, UDPG, pyrophosphorylase is involved in polysaccharide synthesis, while the others are thought to have a primarily catabolic function. The precise role of most of them *in vivo* is not known. Partially purified membranes isolated from aggregated cells of this species are shown to affect the induction of some of these enzymes in ways which may provide some clues to the mechanism of the cell-membrane interaction and the nature of the slime mold developmental program.

MATERIALS AND METHODS

Materials:

p-nitro-phenylphosphate, p-nitro-phenyl β-D glucoside, p-nitro-phenyl-N acetyl-β D glucosamine, α-ketoglutarate and pyridoxal phosphate were obtained from Sigma Chemical Co. Uridine diphosphoglucose, NADP and dinitrophenylhydrazine were from Calbiochem. Glucose G-P dehydrogenase and phosphoglucomutase were from Boeringer Mannheim. S^{35}-methionine (NEG-009) 100-150 Curies/mmole, 1 μCi/μl was obtained from New England Nuclear.

Methods:

Organism: Strain AX-3, an axenic haploid strain derived from the wild type NC-4 (Raper, 1935) was used throughout these experiments. Under our conditions, mature fruiting bodies were formed in 26-27 hours.

Growth and plating conditions and membrane preparation were described previously (Tuchman et al., 1973).

Enzyme assays:

Cells were plated with and without membranes and allowed to develop for varying times, after which they were harvested from the filter by washing with 3 ml of PDF (per liter: 0.111 g $CaCl_2$, 4.6g $NaH_2PO_4 \cdot H_2O$, 1.51g Na_2HPO_4, 1.5g KCl and 0.61g

$MgSO_4$ final pH 6.4) and immediately stored at -20°C. Cells were lysed by sonication for 30 seconds with a Branson sonifier equipped with a microtip. Samples harvested after 22 hours, were sonicated for two 30 second periods to allow for the increased strength of the spore coat. Cells to be assayed for threonine deaminase were harvested and lysed in PDF containing 20 µg/ml pyridoxal phosphate.

N-acetylglucosaminidase was assayed according to the procedures of Loomis (1969b). Threonine Deaminase was assayed following Pong and Loomis (1973). Tyrosine transaminase was assayed according to Pong and Loomis (1971). UDPG pyrophosphorylase was assayed according to Ashworth and Sussman (1967). β-glucosidase and alkaline phosphatase were assayed according to the procedures of Coston and Loomis (1969) and Loomis (1969a). In all cases membranes plated alone were also harvested and assayed for activity. If any activity was found, it remained constant throughout the plating period and was subtracted from the final activity of the cells plus membranes. Likewise membrane protein remained constant and was subtracted from the cell plus membrane total. Protein was measured by the method of Lowry et al. (1951). A unit of enzyme activity is defined as that amount which will liberate 1 nmole of product per minute. Specific activity is defined as units per mg protein.

Enzyme localization:

Cells were harvested after 16 or 24 hours of development. They were pelleted and resuspended in 1 ml of 0.02M Tris pH 8.0 (or Tris containing 20 µg/ml pyridoxal phosphate in the case of threonine deaminase) and frozen and stored in liquid nitrogen. After quick thawing, they were layered onto 12 ml discontinuous sucrose gradients (6 ml 1.5M sucrose, 0.02M Tris pH 8.0 under 6 ml 0.2M sucrose, 0.02M Tris) and spun to equilibrium for at least eight hours in an SW 41 Spinco rotor at 28,000 rpm and 4°C. The gradients were fractionated by pumping from the bottom and enzyme activity was immediately assayed in aliquots of the fractions.

RESULTS

Changes in enzyme activities:

The normal developmental time course of N-acetylglucosaminidase activity is shown in Figure 1. There is a 2-3 fold increase in activity in the first 15 hours of development. The level of initial (0 hours) activity and the amount of increase depends on the concentration at which cells are harvested from the growth medium, and on the composition of the medium (Quance and Ashworth, 1972). When cells are plated with aggregation phase membranes, there is a very rapid decrease in the level of enzyme activity in the first three hours after plating. Thereafter the enzyme activity remains constant. There is no decrease in the specific activity of N-acetylglucosaminidase at 24 hours after plating as is found in untreated cells.

Figure 2 shows the very different effect of membrane treatment on threonine deaminase. In normal cells the enzyme displays a 3-5 fold increase in specific activity followed by a decrease to approximately 60 percent of peak activity. In membrane treated cells on the other hand, the enzyme activity rises steadily throughout the period tested. The initial increase in activity parallels that found in untreated cells.

Figures 3 and 4 depict the normal and membrane-treated

Figure 1. N-acetylglucosaminidase activity in normal and membrane treated cells.

Cells were plated with and without added membranes and were harvested and assayed at intervals as described in Methods. Membranes plated alone were also assayed and the activity subtracted from the total of cells and membranes.

O normal cells

● membrane treated cells.

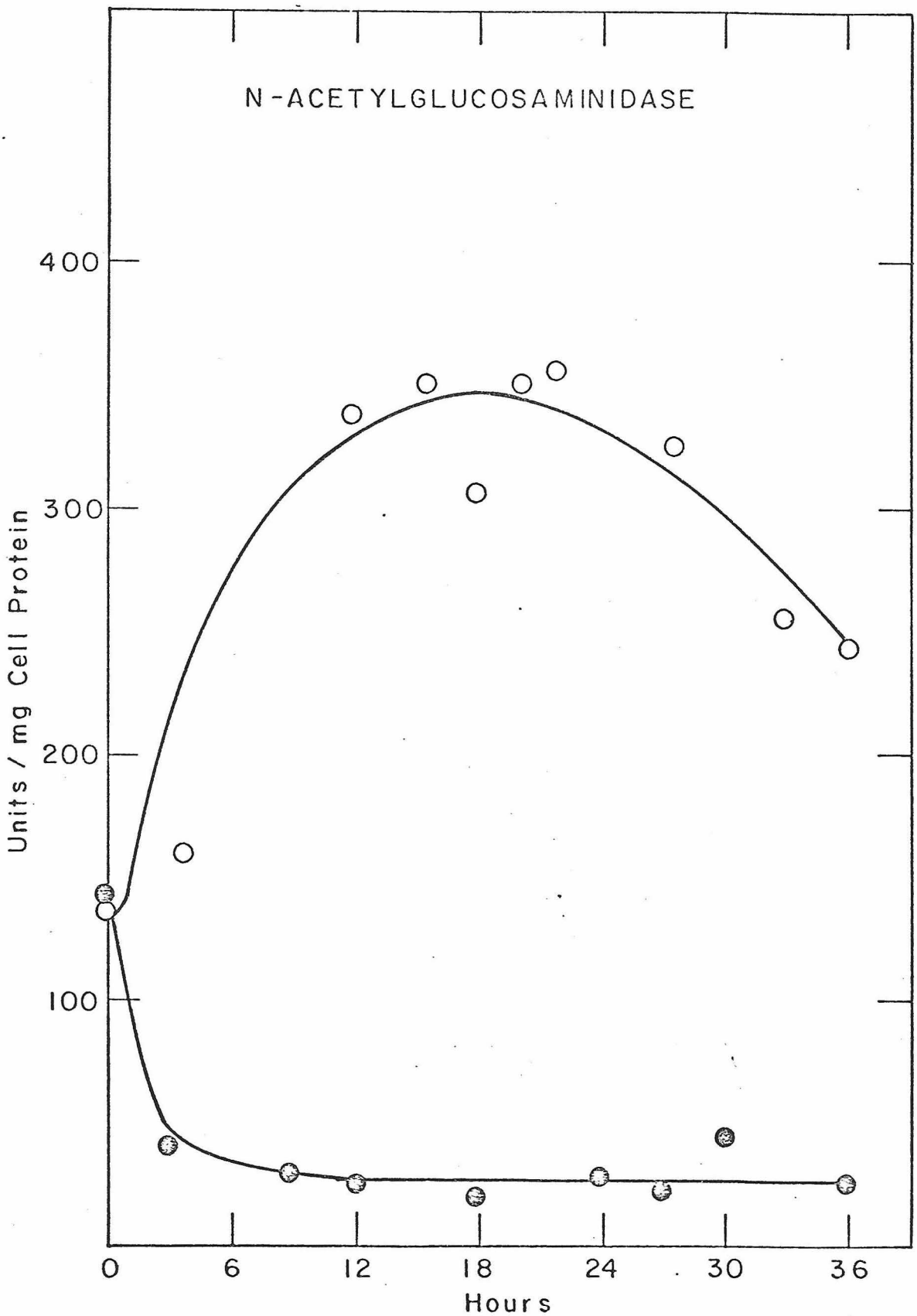
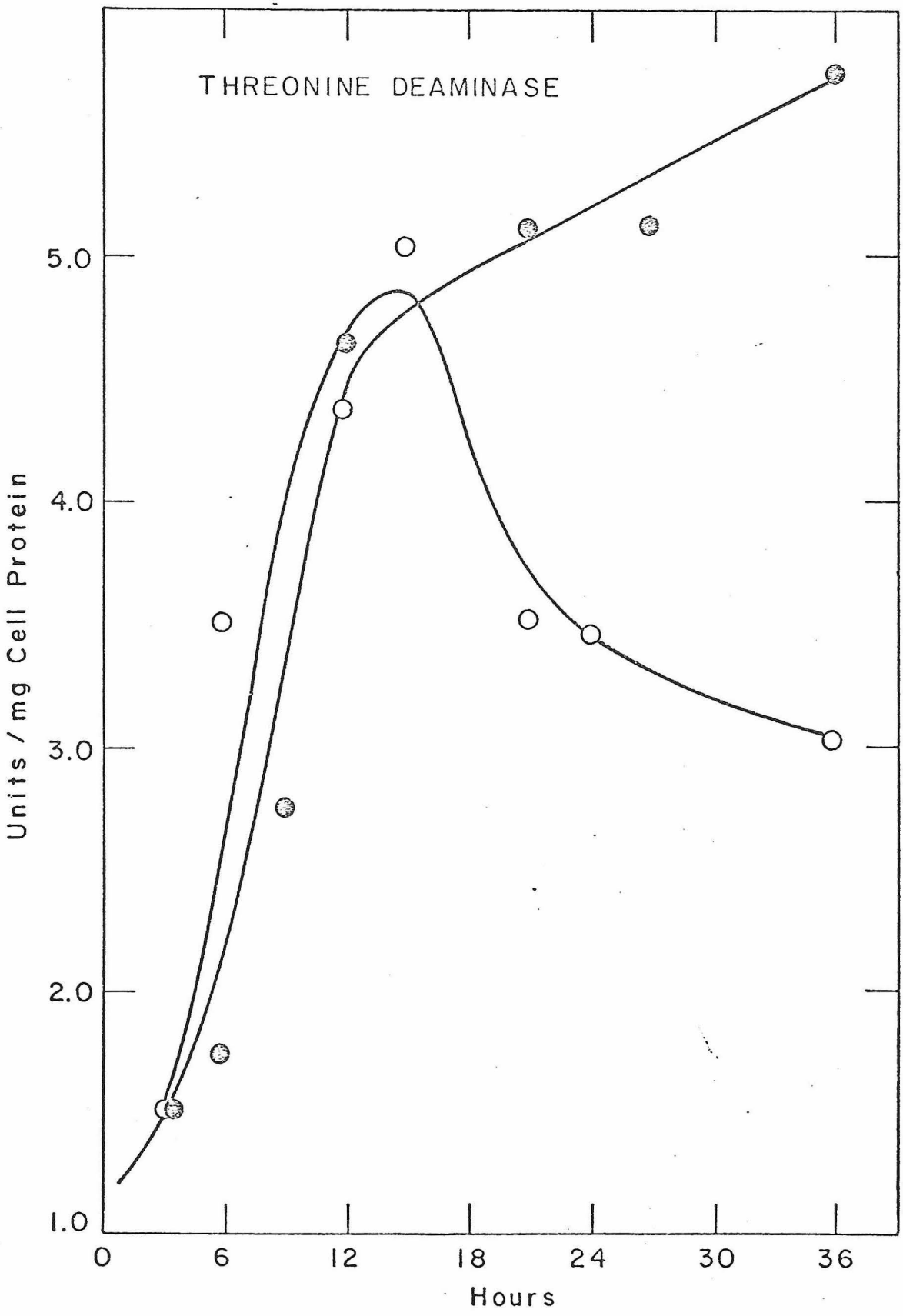

N-ACETYLGLUCOSAMINIDASE
400
300
200
100
Units / mg Cell Protein
0
6
12
18
24
30
36
Hours

Figure 2. Threonine Deaminase activity in normal and membrane treated cells.

Cells were plated with and without added membranes and were harvested and assayed at intervals as described in Methods. Membranes plated alone were also assayed and the activity subtracted from the total of cells and membranes.

O normal cells

● membrane treated cells


THREONINE DEAMINASE
Units / mg Cell Protein
5.0
4.0
3.0
2.0
1.0
0
6
12
18
24
30
36
Hours

activities of two other developmentally controlled enzymes. Tyrosine transaminase normally undergoes a two fold increase in activity, reaching a maximum after eighteen hours of development. The activity then drops to less than its 0 hour level during culmination. In membrane treated cells there are no such changes. There is an initial decrease in activity which thereafter remains constant. Similarly, there are no changes in the activity of UDPG pyrophosphorylase in membrane treated cells. The very large increase (about 15 fold) and subsequent decrease seen in untreated cells are absent, and the activity remains constant at its 0 hour level.

The situation in membrane treated cells in the case of β-glucosidase, depicted in Figure 5, is similar to tyrosine transaminase and N-acetyl glucosaminidase. The normal activity profile represents the composite activities of two isozymes, of which only the second is considered to be an integral feature of the developmental program (Coston and Loomis, 1969). There is no indication of the induction of this second isozyme in membrane treated cells. Rather the first isozyme decreases gradually over the first 15 hours of development, after which the measurable activity remains constant.

Another developmentally controlled enzyme whose activity is composed of two isozymes is shown in Figure 6. Alkaline phos-

Figure 3. Tyrosine transaminase activity in normal and membrane treated cells.

Cells were plated with and without added membranes and were harvested and assayed at intervals as described in Methods. Membranes plated alone were also assayed and the activity subtracted from the total of cells and membranes.

O normal cells

● membrane treated cells

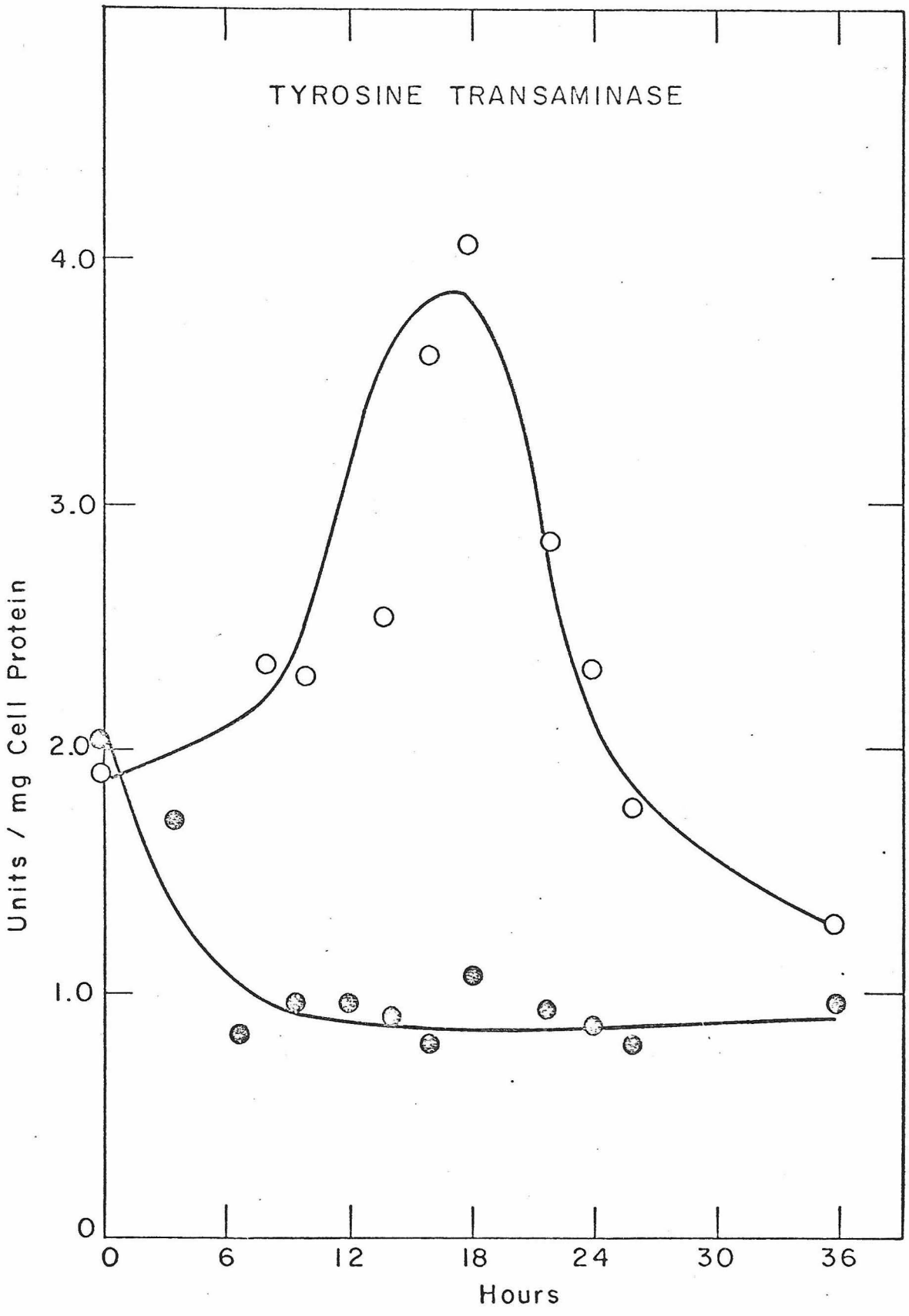

TYROSINE TRANSAMINASE
Units / mg Cell Protein
4.0
3.0
2.0
1.0
0
0
6
12
18
24
30
36
Hours

Figure 4. UDPG pyrophosphorylase activity in normal and membrane treated cells.

Cells were plated with and without added membranes and were harvested and assayed at intervals as described in Methods. Membranes plated alone were also assayed and the activity subtracted from the total of cells and membranes.

O normal cells

● membrane treated cells

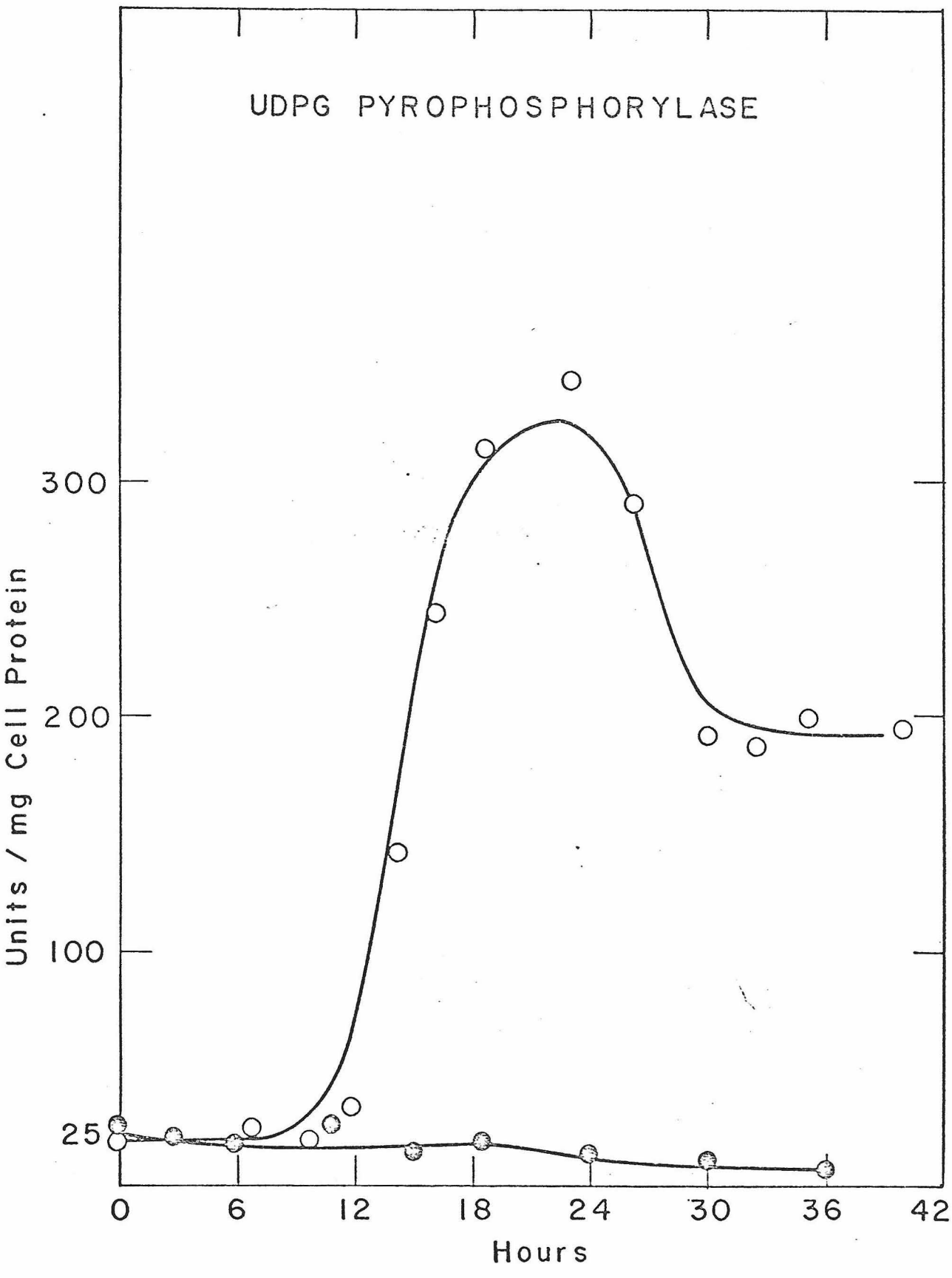
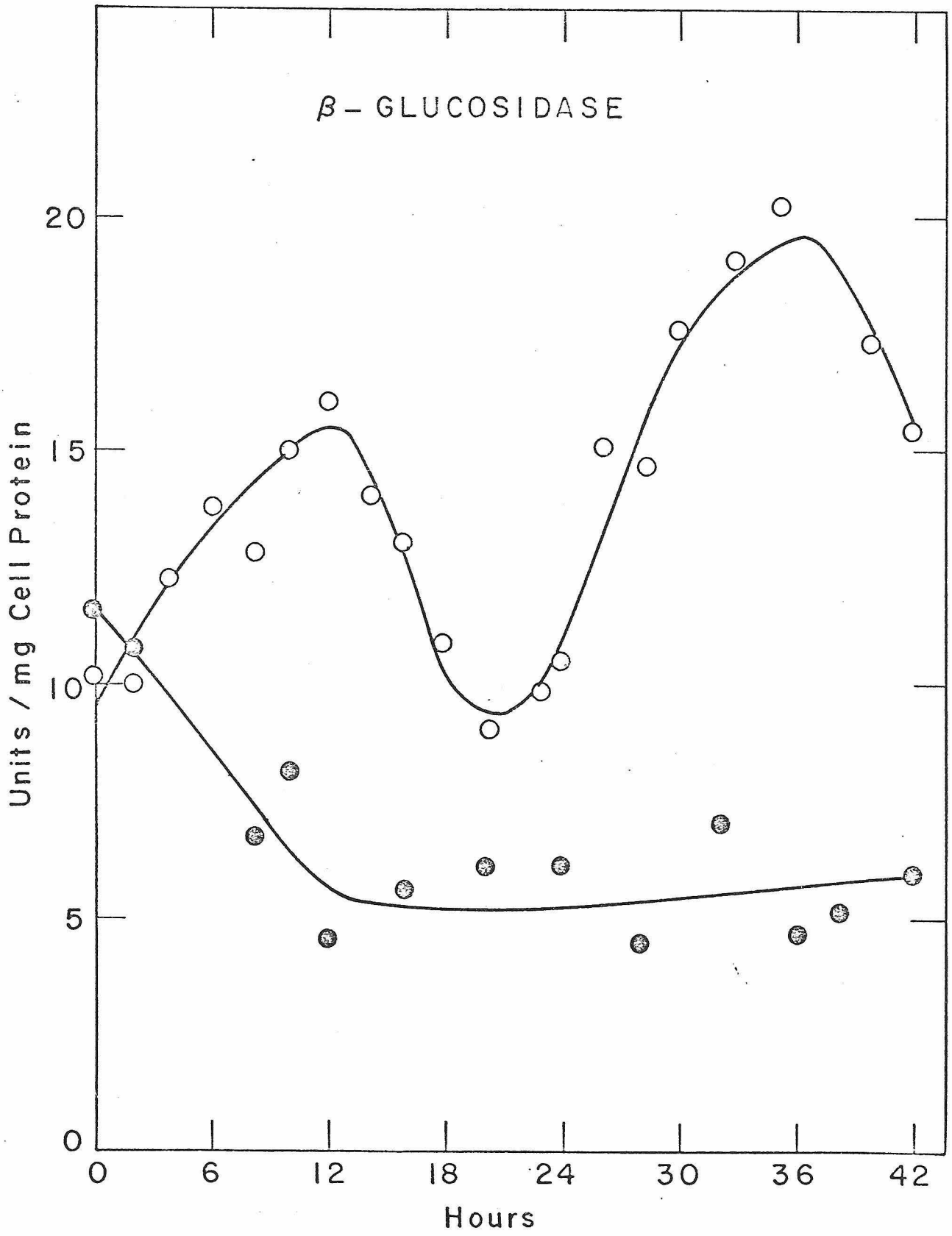

UDPG PYROPHOSPHORYLASE
Units / mg Cell Protein
300
200
100
25
0
6
12
18
24
30
36
42
Hours

Figure 5. β-glucosidase activity in normal and membrane treated cells.

Cells were plated with and without added membranes and were harvested and assayed at intervals as described in Methods. Membranes plated alone were also assayed and the activity subtracted from the total of cells and membranes.

O normal cells

● membrane treated cells


β – GLUCOSIDASE
Units / mg Cell Protein
0
5
10
15
20
0
6
12
18
24
30
36
42
Hours

phatase isozyme II normally peaks at 26-28 hours after initiation of development. The peak activity attained is approximately 35 units/mg protein. However in membrane treated cells, the enzyme reaches a peak activity of nearly 70 units/mg. This maximum is reached at about 18 hours after plating or 10-12 hours before normal cells.

Intracellular localization of developmentally controlled enzymes:

The intracellular localization of several enzymes was investigated by gently breaking the cells in the absence of detergent and spinning the resulting extract on a discontinuous sucrose gradient. It has been previously demonstrated that such gradients produce a turbid band between the heavy and light sucrose solutions which contains a high proportion of membrane vesicles of two size classes, which are thought to be plasma membrane possibly contaminated by lysozymes (Tuchman et al., 1973). No other recognizable cellular organelles are found at the interface or in the supernatant. Nuclei, ribosomes and mitochondria are found in the pellet. Figure 7 shows that nearly 70 percent of the total alkaline phosphatase activity found in 24 hour cells is localized in the membrane fraction, whereas in vegetative cell extracts only half that amount is found in this fraction. If the last five fractions are taken to represent the soluble, supernatant component, 20 percent of the activity

Figure 6. Alkaline phosphatase activity in normal and membrane treated cells.

Cells were plated with and without added membranes and were harvested and assayed at intervals as described in Methods. Membranes plated alone were also assayed and the activity subtracted from the total of cells and membranes.

O normal cells

● membrane treated cells

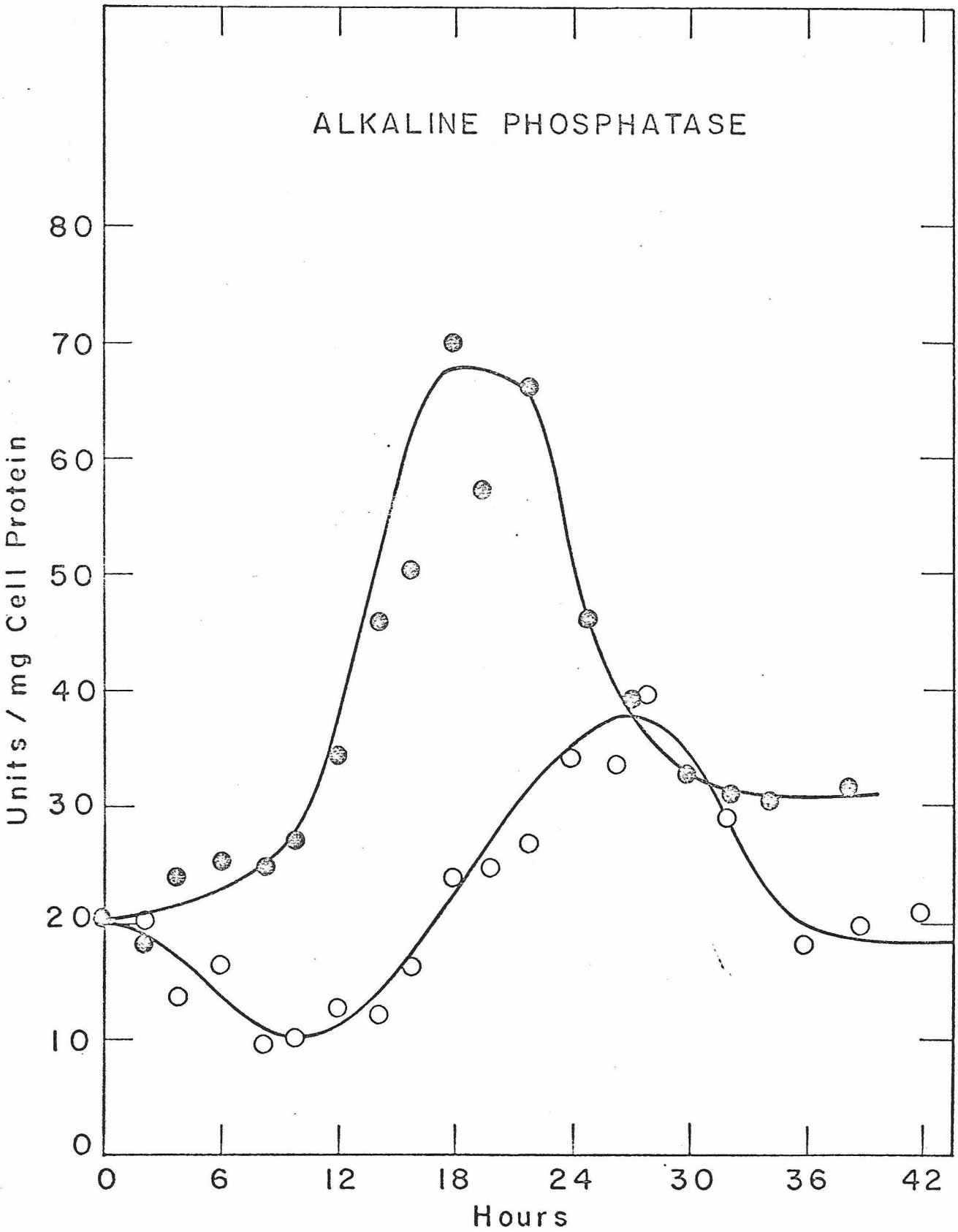
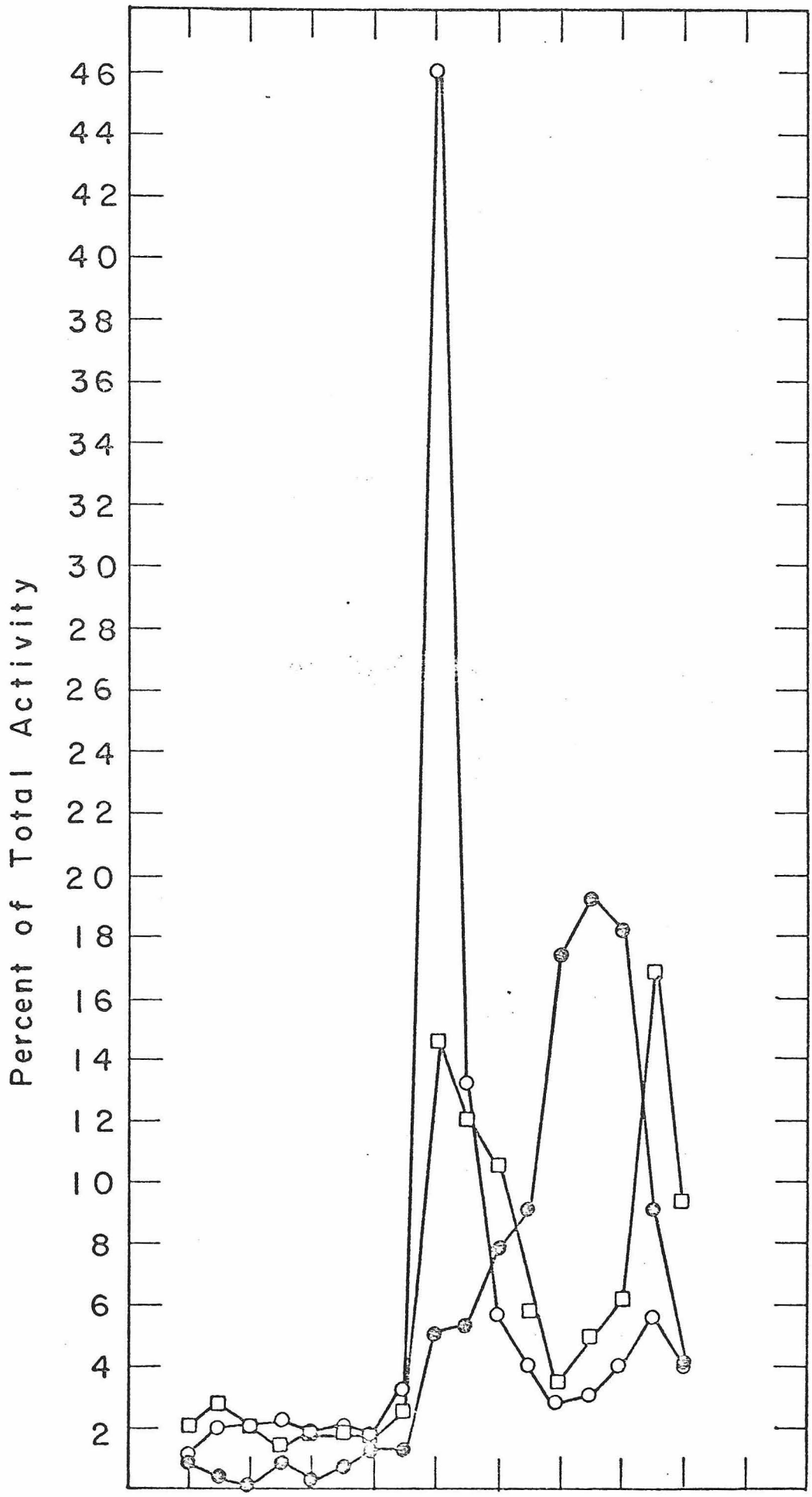

ALKALINE PHOSPHATASE
Units / mg Cell Protein
0
10
20
30
40
50
60
70
80
0
6
12
18
24
30
36
42
Hours

Figure 7. Intracellular distribution of some developmentally controlled enzymes.

Cells lysed by freeze-thaw in liquid nitrogen were layered onto discontinuous sucrose gradients and spun to equilibrium. The gradients were fractionated and enzyme activities assayed in aliquots of the fractions.

- O alkaline phosphatase activity in 24 hour cells
- □ alkaline phosphatase activity in vegetative cells
- ● β-glucosidase activity in 24 hour cells


Percent of Total Activity
46
44
42
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
1
3
5
7
9
11
13
15
17
19

found during development is soluble, while 40-45 percent is soluble in vegetative cells.

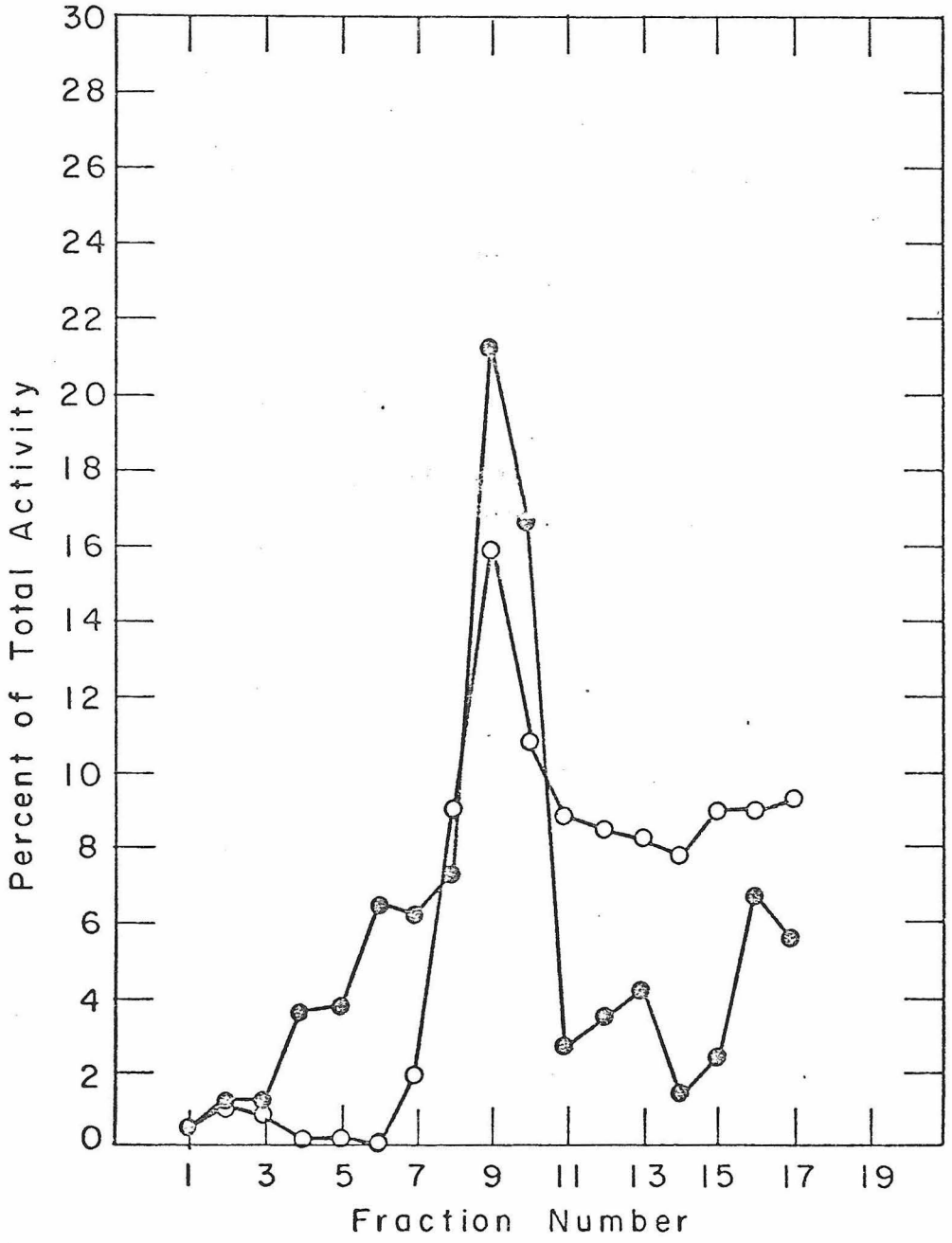
Beta-glucosidase, isozyme II, after 24 hours of development is seen to be 70 percent soluble. Approximately 20 percent is found in the membrane fraction (Fig. 7). N-acetylglucosaminidase shows an intermediate distribution (Fig. 8): approximately 45% is soluble while 35% can be clearly defined as membrane-associated. The distribution of threonine deaminase (Fig. 8), is more complicated. Approximately 20 percent of the activity is found in fractions denser than membrane, 45 percent is found in the membrane band, and 20 percent is soluble.

Figure 8. Intracellular distribution of some developmentally controlled enzymes.

Cells lysed by freeze-thaw in liquid nitrogen were layered onto discontinuous sucrose gradients and spun to equilibrium. The gradients were fractionated and enzyme activities assayed in aliquots of the fractions.

O N-acetylglucosaminidase activity in 16 hour cells

● Threonine deaminase activity in 16 hour cells


Percent of Total Activity
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
1
3
5
7
9
11
13
15
17
19
Fraction Number

DISCUSSION

Those enzymes which are currently considered to be developmentally controlled have been shown to satisfy some or all of the following criteria.

(1) The increase, and in some cases the subsequent decrease in enzyme activity depends on concomitant protein synthesis (Sussman and Sussman, 1969; Franke and Sussman, 1971). Early experiments using the inhibitor actinomycin D indicated that the necessary RNA synthesis preceded protein synthesis, and that translational control was therefore required. However more recent work (Firtel et al., 1973) indicates that these results may have been misleading and that regulation is exercised at the level of gene transcription.

(2) Enzyme induction is generally not found in morphological mutants whose development is blocked at a stage before the enzyme normally appears, but usually is found in mutants blocked at a later morphological stage than that associated with normal enzyme synthesis (Loomis, 1969b; Coston and Loomis, 1969). There are however exceptions to this rule, particularly in the case of aggregation minus mutants (Pong and Loomis, 1971, 1973).

(3) The sequence of induction of the known developmentally controlled enzymes is altered in both morphological and temporally

deranged mutants (Sonneborn et al., 1963; Loomis, 1971).

All these data have contributed to the notion that such enzymes are developmentally controlled, and that together with other biochemical events, they constitute a developmental program which is read off in a linear, sequential manner and primarily controlled at the level of transcription.

The experiments described in this paper, together with those of others, point to two general conclusions which indicate that this conception of developmental control may be misleading. First, they indicate that there are important differences in control among the enzymes which satisfy the criteria for developmentally controlled synthesis. Secondly, they imply that the formation and maintenance of specific cell-cell contacts play a crucial role in the control of slime mold development. Specifically, the membranes have been shown to be capable of three kinds of effects on enzyme activity. They can induce, repress, or leave unchanged the activity of a given developmentally controlled enzyme, but in no case can the effect be simply analyzed in terms of the enzyme's normal time of induction in the developmental sequence.

N-acetyl glucosaminidase was originally thought to be a true developmentally controlled enzyme, but recent experiments of Ashworth and co-workers (Quance and Ashworth, 1973; Ashworth and

Quance, 1972) have indicated that this may not be the case. Ashworth has postulated (Ashworth and Quance, 1972) that the developmentally controlled event is not the synthesis but rather the excretion of this enzyme. Normal axenic cells show a decrease in N-acetyl glucosaminidase activity beginning at about the time of aggregation, while membrane treated cells show a rapid drop in activity immediately after plating. It is possible that the excretion of this enzyme is coordinated with aggregation and that the interaction of cells with aggregation phase membranes mimics this event. However conclusions drawn from the study of this enzyme are weak, since the available evidence is equivocal as to whether it is or is not a bona fide developmentally controlled enzyme.

The normal developmentally controlled increase in the activity of threonine deaminase precedes aggregation, and does not appear to be linked to it in any way, as the enzyme accumulates normally in two mutants (206, VA-4) which fail to aggregate (Pong and Loomis, 1973). However another aggregationless mutant, DA-2, which is blocked at an earlier stage (Pong and Loomis, 1973) does not accumulate any enzyme. This may indicate that the event(s) which control the synthesis of threonine deaminase follow the stage at which DA-2 is blocked, but precede that at which VA-4 and 206 are blocked. Membrane treated cells show an increase in

threonine deaminase activity which closely parallels normal cell synthesis for the first 12 hours of development. The block in the events leading to aggregation competence in membrane treated cells would therefore appear to occur after the block in DA-2 and before the block in VA-4. Membrane treated cells continue to accumulate threonine deaminase activity for up to 36 hours. Normal cells show a decrease in activity beginning at about 15 hours, and morphological mutants, blocked later than aggregation, accumulate the enzyme and show neither an increase nor a decrease but rather a constant level of activity after 15-16 hours. Thus the normal pattern of activity is dependent on complex regulation and is difficult to analyze. It does seem to require the correct execution of events which normally occur at approximately 15-16 hours of development. These events are not carried out in membrane treated cells.

The enzyme activity profiles of β-glucosidase in morphological mutants indicate that some further development after aggregation is required for induction of the second isozyme. No increase in the activity of this isozyme is found in membrane treated cells. On the other hand, the increase in the first isozyme in normal cells precedes aggregation and is independent of it. The decrease of isozyme I coincides with normal aggregation in axenic cells, but is probably also independent of it,

since a decrease is found in the aggregation minus mutant VA-4. Thus there is no simple explanation for the initial decrease in activity both of β-glucosidase (isozyme I) and of tyrosine transaminase in membrane treated cells. It is of interest in this respect that Coston and Loomis (1969) found a similar dip in β-glucosidase activity after replating normal cells, harvested at 18 hours of development. The decrease occurred in untreated cells, and was even more pronounced in cells which were prevented from reaggregating by the presence of a high concentration of EDTA. This is not a general effect of such treatment, since alkaline phosphatase showed no transient decrease when assayed in the same conditions.

UDPG pyrophosphanylase is perhaps the most thoroughly studied enzyme in this organism. Its credentials as a developmentally controlled enzyme have been established in a variety of ways (Ashworth and Sussman, 1967; Newell and Sussman, 1970, 1971; Newell et al., 1971). However there is doubt as to the necessary role of this enzyme in development. Evidence exists which indicates that the intracellular concentration of UDP glucose is controlled by other factors (Wright, 1968; Edmundson and Ashworth, 1972) and cells treated with penicillin form only slightly abnormal fruiting bodies although they show no increase in UDPG pyrophorylase activity (Tuchman, unpublished data).

Membrane treated cells also show no increase in this enzyme, nor do they show the rapid initial decrease characteristic of several other of the enzymes tested.

Certainly the most intriguing effect of aggregation membranes on developing cells is that on alkaline phosphatase. The increase in activity, which normally reaches a maximum after 2-hours of development, does occur in membrane treated cells, but the increase occurs approximately twelve hours earlier in the treated cells, and the enzyme reaches approximately twice the normal activity. Interaction of aggregation phase membranes with developing cells might be expected to set in motion events closely linked to normal aggregation. However in a temporally controlled, linear developmental program alkaline phosphatase would seem to be the least likely candidate to be induced by membrane treatment since its normal induction is the most distantly removed from aggregation. Based on activities in morphological mutants blocked at different stages of development, Loomis (1969) concluded that the increase in alkaline phosphatase activity is dependent on the ability to culminate. However the increase occurs in membrane treated cells in the absence of any morphogenesis. In this case aggregation membranes can apparently act as a specific inducer of at least one unit of the developmental program.

The effect of membrane treatment on enzyme activity appears to be correlated with the intracellular localization of the affected enzyme. Figure 7 demonstrates that vegetative cells contain approximately equal amounts of a membrane-associated and a soluble alkaline phosphatase activity, while the activity in developing cells is almost entirely membrane bound. These activities most likely correspond to the two isozymes of alkaline phosphatase described by Solomon et al. (1964). Threonine deaminase activity, also strongly affected by membrane treatment, is likewise associated with membrane to a large degree. The nature of the activity which bands at a greater density than membrane is unknown. On the other hand two enzymes that are almost unchanged by membrane treatment, β-glucosidase and UDPG pyrophosphorylase (data not shown) are found in the soluble region of the gradient. Finally, N-acetyl glucosaminidase shows an intermediate distribution. The activity which bands in the membrane region may be due to contamination of this band with lysozomes (Ashworth and Quance, 1972). The reason for this apparent correlation of membrane localization and sensitivity to the effects of aggregation phase membranes is not yet known, however the coincidence appears to be beyond the realm of chance.

In summary, aggregation phase membranes appear to be capable of influencing biochemical events inside Dictyostelium cells

through their interaction with the cell surface. The membranes lead to the induction of some developmentally controlled enzymes and the repression or excretion of others. Further, the distribution of affected and unaffected enzymes bears no resemblance to the normal sequence of enzyme induction. The only characteristic with which the membrane effect can be linked appears to be the intracellular localization of the affected enzyme. Although many questions remain to be answered, it can be said that isolated plasma membranes, bearing an aggregation factor(s) are capable of affecting the normal development of slime mold cells in highly specific ways. Together with the previously reported effects of cell disaggregation experiments (Newell et al., 1971; Gregg, 1971; Raper, 1940) these results point out the crucial role played by the formation and maintenance of cell-cell contacts during normal development. The results indicate that there are some difficulties in the generally accepted view of the slime mold developmental program, and that there may be important differences in the control of various developmentally controlled enzymes. Finally, it is shown that changes in the specific activity of some developmentally controlled enzymes may in certain conditions be wholly divorced from both morphogenesis and the normal sequence of induction.

REFERENCES

Anderson, F. & Huebner, E. (1968). J. Morphol. 126, 163.

Ashworth, J.M. & Quance, J. (1972). Biochem. J. 126, 601.

Ashworth, J.M. & Sussman, M. (1967). J. Biol. Chem. 242, 1696.

Coston, M.B. & Loomis, W.F. (1969). J. Bact. 100, 1208.

Edmundson, T.D. & Ashworth, J.M. (1972). Biochem. J. 126, 593.

Firtel, R.A., Baxter, L. & Lodish, H.F. (1973). J. Mol. Biol., in press.

Franke, J. & Sussman, M. (1971). J. Biol. Chem. 246, 6381.

Gerisch, G. (1968). Curr. Topics in Devel. Biol. 3, 157.

Gregg, J.H. (1971). Devel. Biol. 26, 478.

Kohn, A. & Fuchs, P. (1970). Curr. Topics in Microbiol. & Immun. 52, 95.

Lilien, J.E. (1969). Curr. Topics in Devel. Biol.

Loomis, W.F. (1971). Exptl. Cell Res. 60, 285.

Loomis, W.F. (1969a). J. Bact. 100, 417.

Loomis, W.F. (1969b). J. Bact. 97, 1149.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). J. Biol. Chem. 193, 265.

Newell, P.C., Longlands, M. & Sussman, M. (1971). J. Mol. Biol. 58, 241.

Newell, P.C. (1971). Essays in Biochemistry 7, 87.

Newell, P.C. & Sussman, M. (1971). J. Biol. Chem. 246, 2252.

Newell, P.C. & Sussman, M. (1970). J. Mol. Biol. 49, 267.

Pong, S.S. & Loomis, W.F. (1973), in preparation.

Pong, S.S. & Loomis, W.F. (1971). J. Biol. Chem. 246, 4412.

Quance, J. & Ashworth, J.M. (1972). Biochem. J. 126, 609.

Raper, K. (1940). J. Elisha Mitchell Sci. Soc. 56, 241.

Raper, K.B. (1935). J. Aggr. Res. 50, 135.

Solomon, E.D., Johnson, M. & Gregg, J. (1964). Devel. Biol. 9, 314.

Sonneborn, D.R., White, G.J. & Sussman, M. (1963). Devel. Biol. 7, 79.

Sussman, M. & Sussman, R. (1969). Symp. Soc. Gen. Microbiol. xix, 403.

Sutherland, E.W., Oye, I. & Butcher, R.W. (1965). Recent Prog. in Hormone Res. 21, 623.

Tuchman, J., Smart, J.E. & Lodish, H.F. (1973), in preparation.

Wright, B.E. (1968). J. Cell Physiol. 72 Suppl. 1, 145.

III. PREFERENTIAL SYNTHESIS OF ACTIN DURING EARLY DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

INTRODUCTION

Upon removal of nutrients, the cellular slime mold Dictyostelium discoideum ceases to grow, and enters the developmental phase of its life cycle. The unicellular vegetative amoebae grow and divide by fission, are motile and chemotactic. During the non-dividing developmental phase, individual cells, and the organisms which they form by aggregation, display a variety of new properties. They become sensitive to cyclic AMP and capable of forming gradients of this substance along which they are thought to aggregate (Bonner, 1971; Gerisch et al., 1972). They display new cell surface antigens and become capable of forming specific cell-cell contacts (Sonneborn et al., 1964; Beug et al., 1970; Tuchman et al., 1973). After aggregating, they display elaborate cell sorting phenomena within each organism, and can regulate the proportions of the two new differentiating cell types, pre-spore and pre-stalk, regardless of the total number of cells in the organism (Bonner and Adams, 1958; Bonner, 1967; Gregg, 1965). The developing pseudoplasmodium is highly mobile; it is attracted towards light, humidity and higher temperature (Bonner et al., 1950; Bonner and Shaw, 1957). The migrating slug is thought to move through a tube

of slime sheath, composed mainly of glycoprotein, which it continuously synthesizes and leaves behind as it moves (Loomis, 1972). The actual mechanism by which the organism moves relative to the sheath is unknown. Finally, the two distinct cell types mature, producing hard coated spores and large vacuolated stalk cells, and development ends with the formation of an erect fruiting body.

Because of its relative simplicity, biochemical sophistication and ease of handling in the laboratory, Dictyostelium makes an attractive candidate for an intensive study of the molecular mechanisms controlling development in eucaryotes. With this in mind, we undertook a study of the changes in protein synthesis during vegetative growth and throughout development by pulse-labeling the cells and autoradiography of total cell protein on SDS-polyacrylamide gels. An unexpected dividend of this work was the discovery that a single protein is synthesized in extremely large amounts during the early stages of development. In this paper we describe the patterns of protein synthesis during development and the purification and identification of the major component as slime mold actin.

MATERIALS AND METHODS

Cells:

The axenic haploid strain AX-3 derived from Raper's (1935) wild-type NC-4 was used.

Growth and plating conditions:

Cells were grown and harvested as described in Tuchman et al. (1973). The harvested cell pellet was resuspended at a concentration of 1.5×10^8/ml and 0.33 ml of this was deposited onto a 47.5mm #50 Whatman filter saturated in PDF. The filter was supported on a Millipore pad also staurated in PDF. Cells were incubated in covered glass dishes, in direct light at $22^{\circ}C$ during the developmental phase.

Labeling with S^{35}-Methionine

S^{35}-Methionine (New England Nuclear NEG-009, 100 Ci/mmole) was diluted in PDF to a concentration of 75 mCi/ml. 0.075 ml of the label was deposited on one-half of a Millipore pad barely saturated in PDF. Immediately after adding the label, one-half of a filter supporting cells at the desired stage of development was lifted from its pad, briefly touched to a dry pad, and transferred to the radioactive pad. After the desired interval, the labeled cells were washed off the filters in 3 ml of PDF and harvested by centrifugation.

SDS gel electrophoresis:

SDS polyacrylamide gels were prepared, electrophoreised and stained according to the method of Fairbanks et al. (1971). Samples were prepared for electrophoresis by boiling for 5 minutes in an equal volume of buffer containing 2.5% SDS, 10% sucrose, 10mM Tris-HCl (pH 8), 1mM EDTA (pH 8), 40mM Dithiothreitol and 10 µg/ml Pyronin Y (tracking dye, obtained from Allied Chemical). Gels were run for 3-4 hours at 8 mamps/gel. The leading edge of the tracking dye was marked by pricking the gels with a needle dipped in India ink, to allow for accurate calculation of RF values.

Gels were vertically sliced and dried under vacuum for autoradiography. Developed films were scanned with a Joyce Loebl microdensitometer.

Actin purification:

Actin was prepared by a modification of the procedure of Spudich (1973, personal communication). 2-3 x 10^{10} vegetative cells were harvested in 0.2% NaCl and washed 3 times. The cell pellet was taken up in 900 ml cold acetone and stirred for 5 hours at 4^{o}C. The acetone extract was then filtered once through a conventional funnel by gravity and twice under pressure through a Buchner funnel with a small pore filter. The retained material was washed several times with cold acetone.

The successive washings were accomplished by scraping the filters and transferring the cell mass to a new filter with a spatula. After the final wash, the filter was left to dry overnight at room temperature. The dried extracts were ground in a mortar and pestle and left for one hour at room temperature. The powder was then taken up in 50 ml of Buffer A (1mM Dithiothreitol, 5mM Tris-HCl, 0.5mM ATP, 0.2mM $CaCl_2$ final pH 8.0) and stirred in the cold for 3-4 hours. This extract was then centrifuged in a Sorvall SS-34 rotor at 10,000 rpm for 10 minutes. The resulting supernatant was then made 0.05M in $MgCl_2$ and 0.1M in KCl and stirred at room temperature for 2 hours. This solution was centrifuged at 20,000 rpm for 30 minutes in the Sorvall SS-34 rotor at 20°C. This procedure yielded a large loosely packed brown pellet over a small very hard, clear white pellet. The brown material was resuspended in 10 ml Buffer A and stirred overnight in the cold. The solution was centrifuged as before and the supernatant made 0.1M KCl and 0.05M $MgCl_2$. After stirring at room temperature for 2 hours, the solution was centrifuged in a Spinco SW 50.1 rotor at 30,000 rpm for 30 minutes at 20°C. This yielded several milligrams of homogeneous, translucent whitish pellet.

Demonstration of actin polymerization in high salt:

A small amount of the purified actin pellet was resuspended in Buffer A. After 2 hours of stirring in the cold, this was layered onto a 15-30% sucrose gradient containing 0.2mM $CaCl_2$ and 0.01M Tris pH 8.0. A second 15-30% gradient containing 0.1M KCl was overlayed with actin which had been resuspended in 0.1M KCl and stirred at room temperature. The gradients were centrifuged at 48,000 rpm for 3 hours at 4°C in an SW 50.1 Spinco rotor. The tubes were punctured from the bottom and collected in fractions. The optical density of each fraction at 280 nm was measured.

Isolation of labeled actin from membranes:

3.5×10^8 cells were plated and labeled for the first eight hours of development with a total of 700 µCi of S^{35}-Methionine. The labeled cells were harvested in 0.01M Tris HCl pH 6.5, centrifuged, resuspended in 1 ml of the same buffer, and frozen in liquid nitrogen. After thawing, the cell extracts were layered onto discontinuous sucrose gradients (8 ml of 0.2M sucrose, 0.02M Tris pH 8 over 8 ml 1.5M sucrose, 0.02M Tris pH 8) and centrifuged to equilibrium in a Spinco SW 27 rotor at 25,000 rpm for 8 hours at 4°C. This procedure produces a turbid band composed of plasma membranes at the 0.2-1.5M sucrose interface (Tuchman et al., 1973). This band was re-

moved by pipetting and found to contain a total of 1×10^8 cpm. The isolated membrane material was diluted ten-fold with 0.02M Tris pH 8.0 and pelleted at 18,000 rpm for 40 minutes in the SW 27 rotor. 90-95% of the counts are recovered in the pellet. 0.6 ml of a solution containing 1mM Dithiothreitol, 5mM Tris-HCl, 0.5mM ATP, 0.2M $CaCl_2$ and 0.5% Cemusol (NP-40) pH 8.0, was added and the pellets were stirred in the cold. After 30 minutes this solution was layered on top of 5 ml of 7% sucrose and spun for 1 hour at 35,000 rpm in an SW 50.1 rotor at 4^oC. After centrifugation the top 1 ml of the tube was removed and tested for the presence of depolymerized actin.

RESULTS

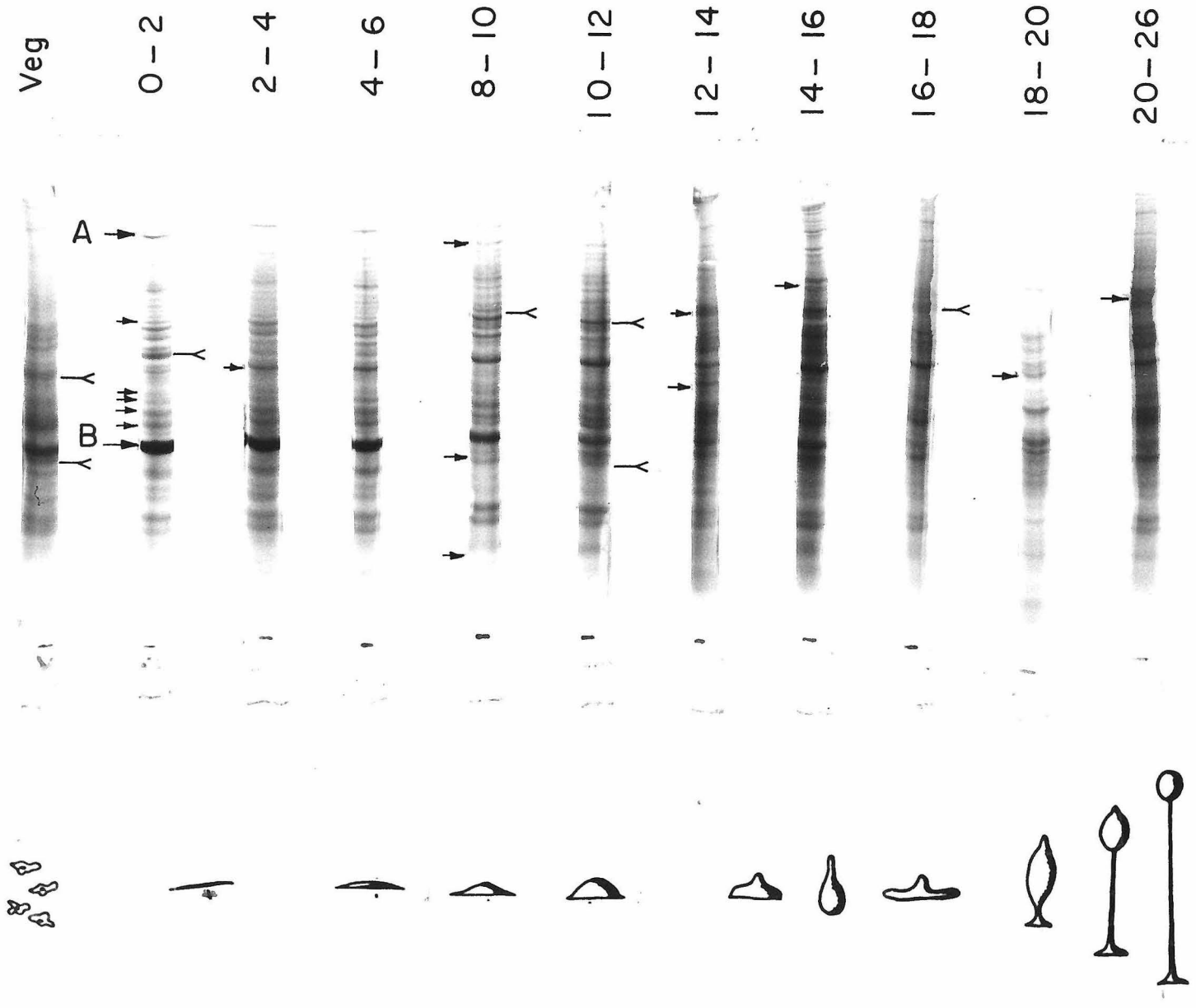
Patterns of protein synthesis:

Figure 1 shows the pattern of protein synthesis during slime mold development resolved on 5.5% SDS polyacrylamide gels. Each gel represents a two hour labeling period except the vegetative sample which was labeled for six hours or one-half of one division time and the final sample which was labeled from 20-26 hours of development. The most dramatic differences occur between the vegetative stage and the first two hours of development. Several new bands appear on the 0-2 hour gel which are not resolved on the vegetative gel. It should be noted however that samples labeled during vegetative growth regularly have a very high background on SDS gels. This implies that a very large number of proteins is being made, greater than the power of the gel to resolve. Nevertheless there are changes in the relative intensities of bands which have been marked in the Figure. Arrowheads on the left of a gel denote bands which are new at that position or increase markedly in intensity of the indicated time. Arrowtails on the right of a gel indicate the disappearance or sudden decrease in intensity of a given band. The first eight hours of development appears to be a time of fairly constant protein synthesis. However there are changes apparent in the period from

Figure 1. Changes in the patterns of protein synthesis during Dictyostelium growth and development.

Cells were pulse-labeled as described in Materials and Methods, harvested, lysed and electrophoresed on 5.5% polyacrylamide gels. The morphological stages corresponding to the period of labeling are shown schematically below each gel.

DEVELOPMENTAL CHANGES IN PROTEIN SYNTHESIS

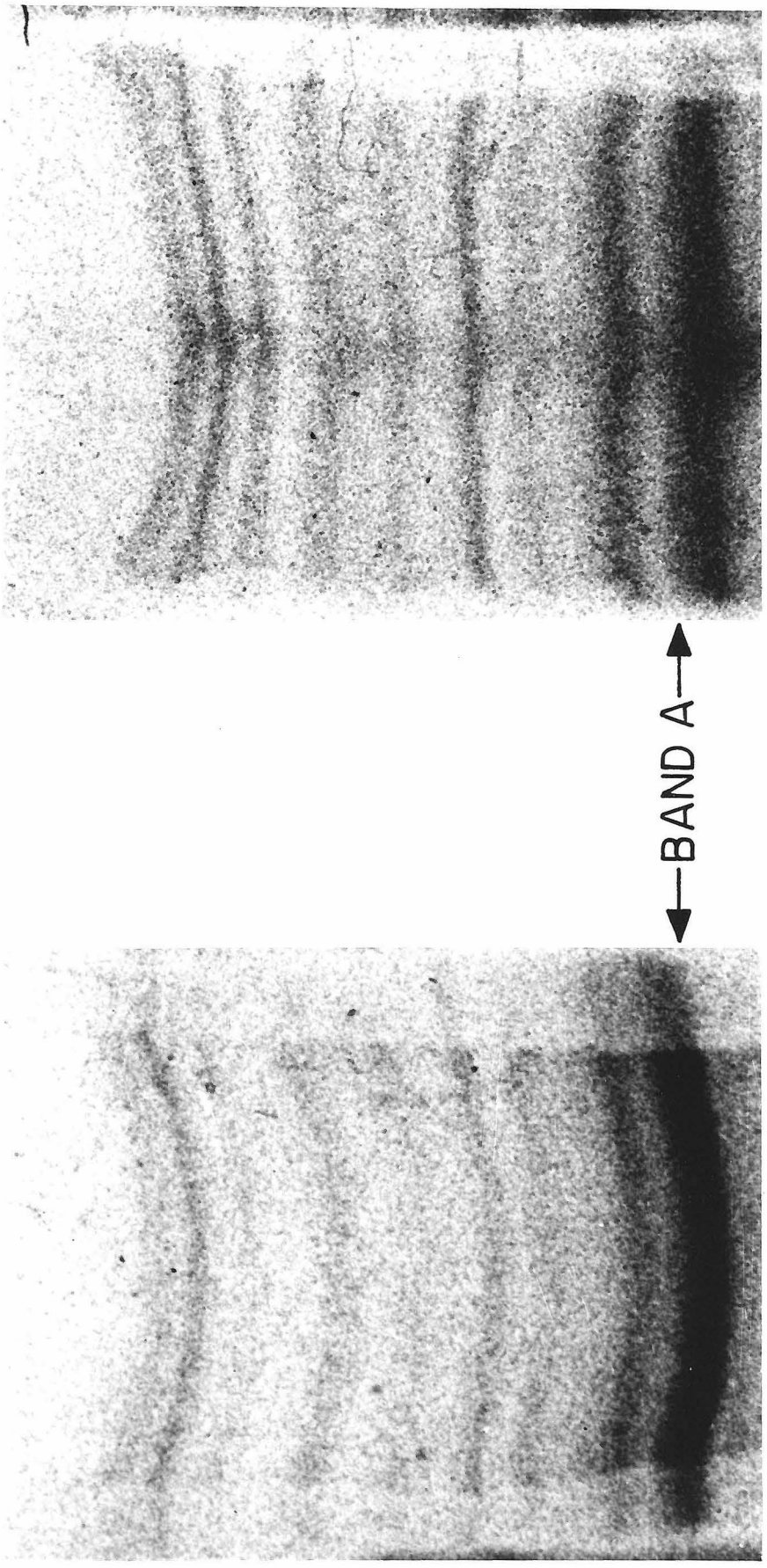


eight to twelve hours. As indicated in the drawings below the gels, this corresponds to the period when a large number of cell contacts are first being made and cell aggregates are forming and maturing. The gels representing the period after 16 hours of development give a less accurate picture of actual events, because by this stage of development there are two distinct cell types in the organism and the gel sample contains a mixture of both. In addition, as the cell mass rises off the supporting surface the two cell types are unequally labeled.

No one exposure time allows for optimal resolution of all bands. The gels shown were chosen as the best compromise, and consequently certain interesting facets of these gels are necessarily lost in this reproduction. For example, the more heavily exposed autoradiographs shown in Figure 2 reveal the presence of eight distinct and clearly resolved bands above the band marked A. These bands are present throughout development, and each represents a protein of subunit molecular weight greater than 150,000. Different exposures reveal several bands of molecular weight between 10,000 and 20,000. There are changes in the pattern of these light proteins. However detailed study of this range of molecular weights requires resolution on gels of higher percent acrylamide.

Figure 2. High molecular weight region of 5.5% gel.

A photographic magnification of the high molecular weight bands seen on an autoradiograph of a 5.5% polyacrylamide gel. The gel samples were labeled from 0-2, and, 4-6 hours.


BAND A

Quantitation of band intensity:

Lightly exposed autoradiographs were scanned in a microdensitometer. Two representative profiles are shown in Figure 3. A curved background line was drawn as shown in the figure and the extent of the major band (Band B; RF 0.55) was marked. The profiles were then xeroxed and the resulting profiles were carefully cut out and weighed.

Band B was found to constitute 22% of the total intensity on the 0-2 hour gel. Between four and six hours of development this dropped to 18%. Between eight and ten hours the figure was 9 percent and this fell to 4% in the 14-16 hours period.

Purification of slime mold actin:

The molecular weight of the heavily labeled band, 47,000, led us to speculate as to whether it might be slime mold actin. Consequently we attempted to purify actin according to a known procedure and compare the properties of the isolated protein to that of the protein made in large amounts during early development.

Spudich (1973) and Woolley (1972) have shown that vegetative slime mold cells contain actin. Following a modification of Spudich's procedure, actin was purified from an acetone extract of vegetative cells. After extraction the dried and powdered extract was stirred with ATP in the presence of low salt (Buffer

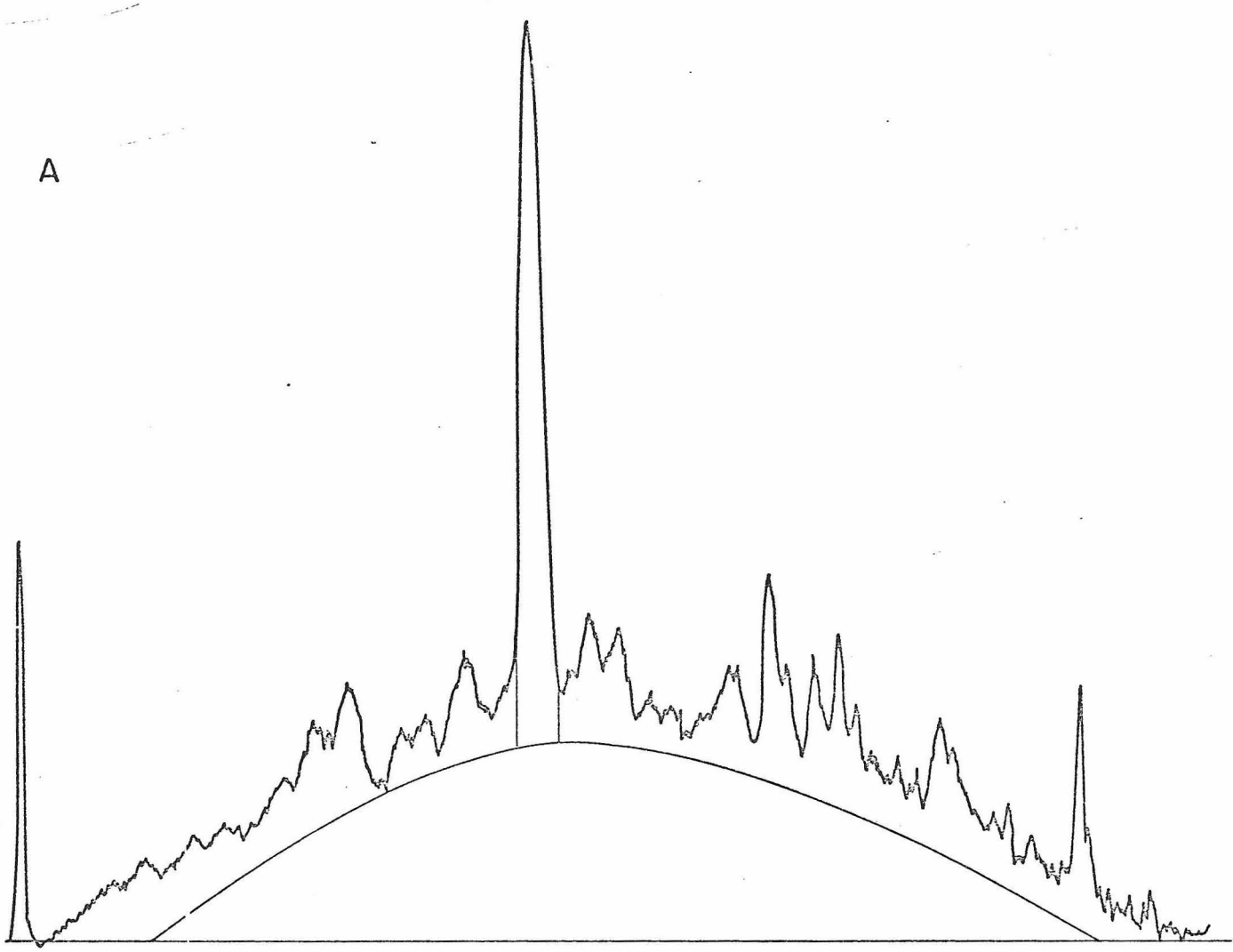
Figure 3. Profiles of Autoradiographed SDS Gel from Different Stages of Development.

Exposed autoradiographs of polyacrylamide gels were scanned on a microdensitometer. The curved line represents the approximate background that was assumed for purposes of quantitation.

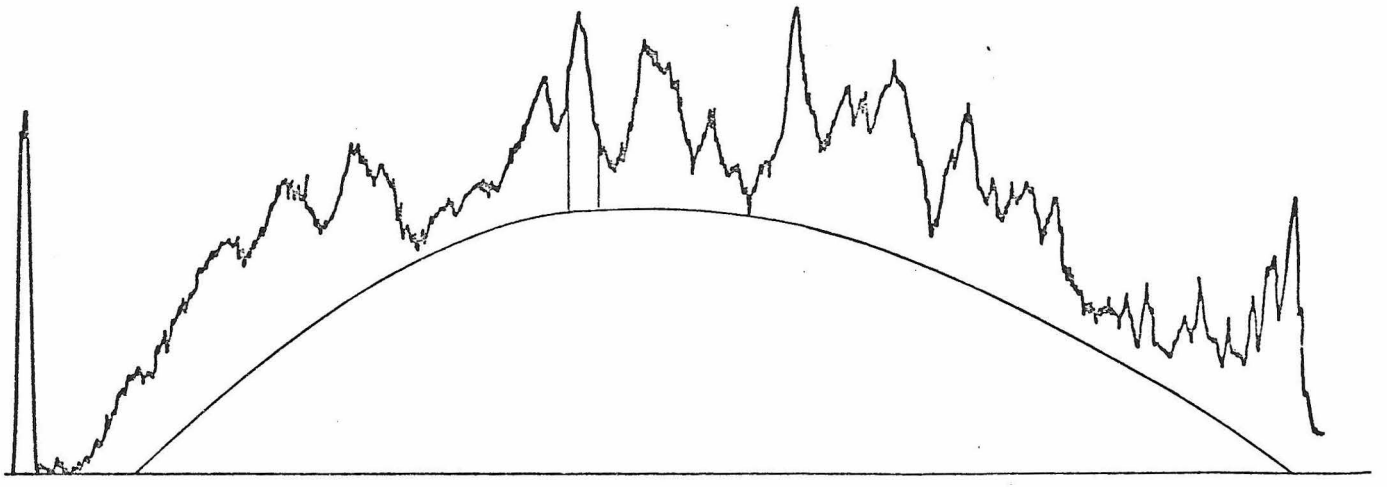
A.) Sample labeled from 0-2 hours of development.

B.) Sample labeled from 14-16 hours of development.

A



B



A) in the cold. These conditions lead to the depolymerization of actin filaments into monomeric subunits. The solution is then centrifuged to remove insoluble material. The actin in the supernatant is then repolymerized by the addition of $MgCl_2$ and KCl and is transferred to room temperature. A second centrifugation then should yield pure actin filaments in the polymerized state. In our hands, it was found necessary to repeat the low salt extraction and the succeeding steps in order to obtain a relatively pure product.

Figure 4 shows the final product of an SDS polyacrylamide gel stained with Coomassie blue. Approximately 90% of the material on the gel is found in a band which runs at an RF of 0.55. The molecular weight of this band was calculated from a logarithmic plot of the standards as shown in Figure 5 and estimated to be 47,000. A minor band of molecular weight 35,000-36,000 is also present. It is not known whether this represents a contaminant or a degradation product of the heavier band. The gels in Figure 4 demonstrate that the purified actin co-electrophoreses with Band B both in autoradiographs and on stained gels of vegetative and developing cells. In addition it should be noted that this protein is found exclusively in the membrane fraction of labeled cells.

Figure 4. SDS polyacrylamide gels of various Dictyostelium samples.

A.) Autoradiograph of 0-2 hour labeled sample.

B.) Coomassie Blue stain of Dictyostelium membrane fraction from developing cells.

C.) Coomassie Blue stain of purified Dictyostelium.

D.) Autoradiograph of 0-6 hour labeled membrane fraction.

E.) Autoradiograph of 0-6 hour labeled supernatant fraction.

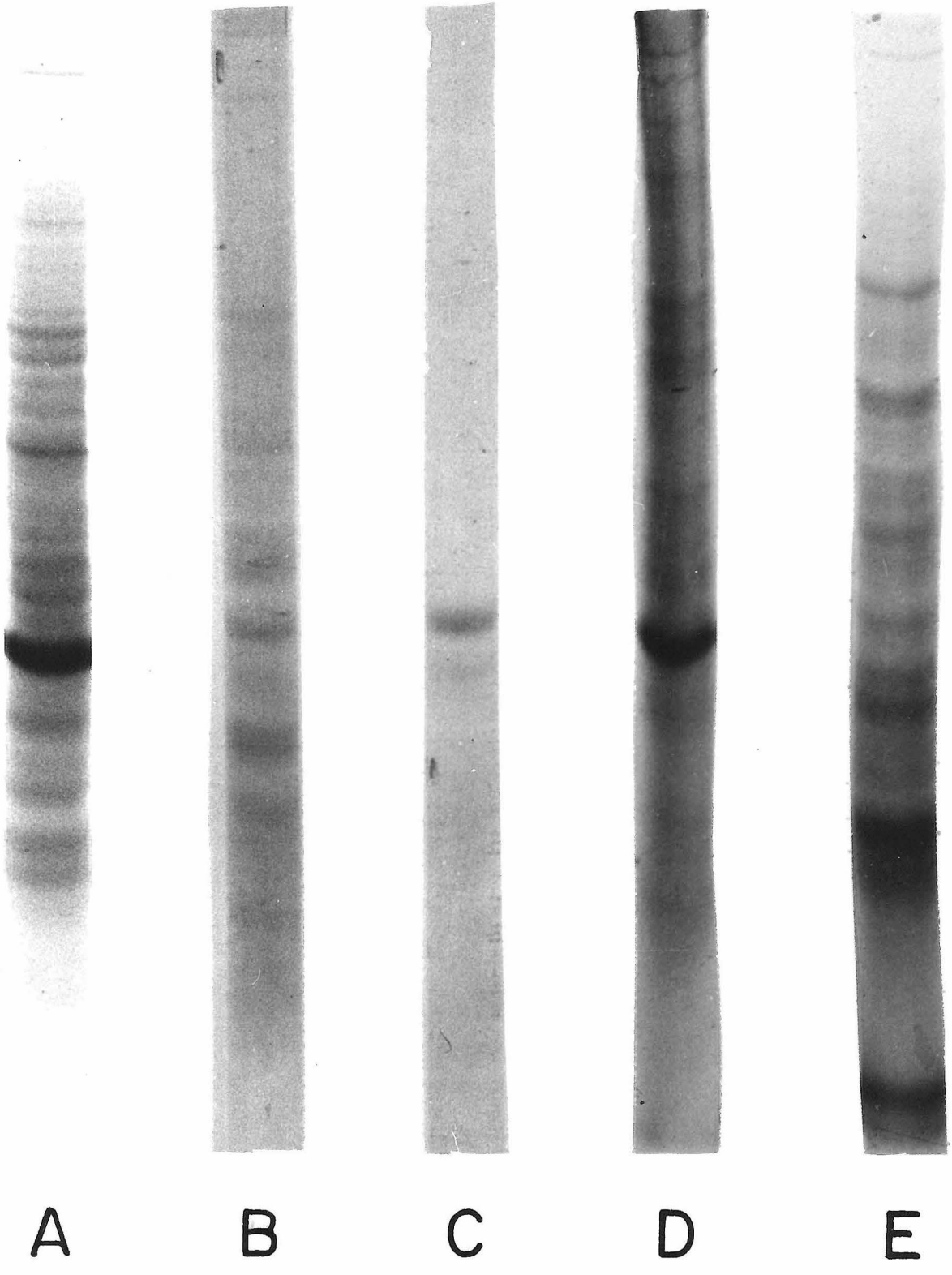
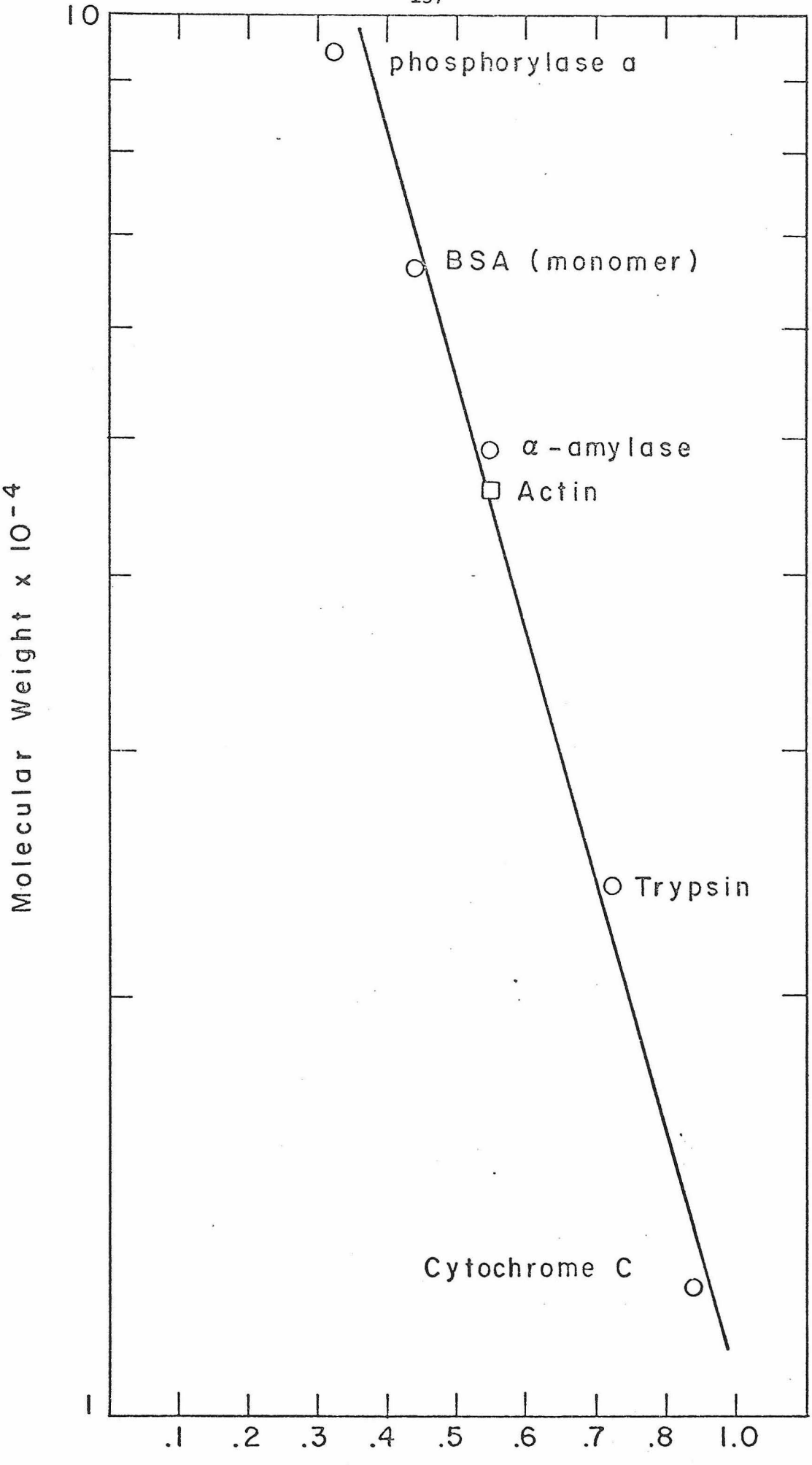

A
B
C
D
E

Figure 5. Molecular weight calculation of SDS gels.

Determination of molecular weight of Dictyostelium actin on a linear-logarithmic plot of several protein standards, on 5.5% SDS polyacrylamide gels.


10
1
phosphorylase a
BSA (monomer)
α-amylase
Actin
Trypsin
Cytochrome C
Molecular Weight x 10-4
.1
.2
.3
.4
.5
.6
.7
.8
1.0

It has been shown frequently that actin subunits will polymerize upon the addition of 0.1M KCl to large polymers with reported $S_{20,w}$ values of 25-30 (Woolley, 1972; Adelman and Taylor, 1969; Hatano et al., 1967). When layered onto 15-30% sucrose gradients, with and without the addition of 0.1M KCl, the presumptive slime mold actin behaved similarly (Fig. 6). An aliquot stirred in the cold in low salt and the presence of ATP behaved as a low molecular weight, soluble species on the gradient, whereas the same material, allowed to sit at room temperature for 15 minutes in 0.1M KCl, sedimented in the region of $S_{20,w}30$.

The purified, unlabeled actin was then compared to the highly labeled material synthesized by cells in early development. Since earlier experiments (Fig. 4) demonstrated that Band B was found only in the membrane fraction, we attempted to purify it from highly labeled membranes. A highly labeled membrane pellet was therefore stirred in Buffer A with the addition of non-ionic detergent which was found to be necessary for the release of radioactivity into the supernatant. In the absence of detergent, 0.1 percent of the counts in the membrane pellet were released after 5 hours of stirring in the cold. Thirty minutes after addition of the detergent 10 percent of the total counts had been released. After an additional ninety

minutes, a total of 40 percent of the original radioactive material had been released.

Figure 7 demonstrates that the in vivo labeled material exactly parallels the purified actin on sucrose gradients, with and without added KCl. The unlabeled material in Figure 7 was stirred in depolymerizing conditions for only thirty minutes before being layered on the gradient. This is reflected in the small peak of residual heavy material near the bottom of this gradient.

Figure 6. Slime mold actin on 15-30% sucrose gradients.

Purified Dictyostelium actin was stirred in depolymerizing conditions, as described in Methods. One aliquot was layered directly onto a sucrose gradient and a second aliquot was made 0.1M in KCl before layering. Gradients were centrifuged for 3 hours at 48,000 rpm.

● optical density profile of actin in depolymerizing conditions.

○ optical density profile of actin in polymerizing conditions, containing 0.1M KCl.

The arrows indicate the position of co-sedimented P^{32}-labeled HeLa cell RNA.

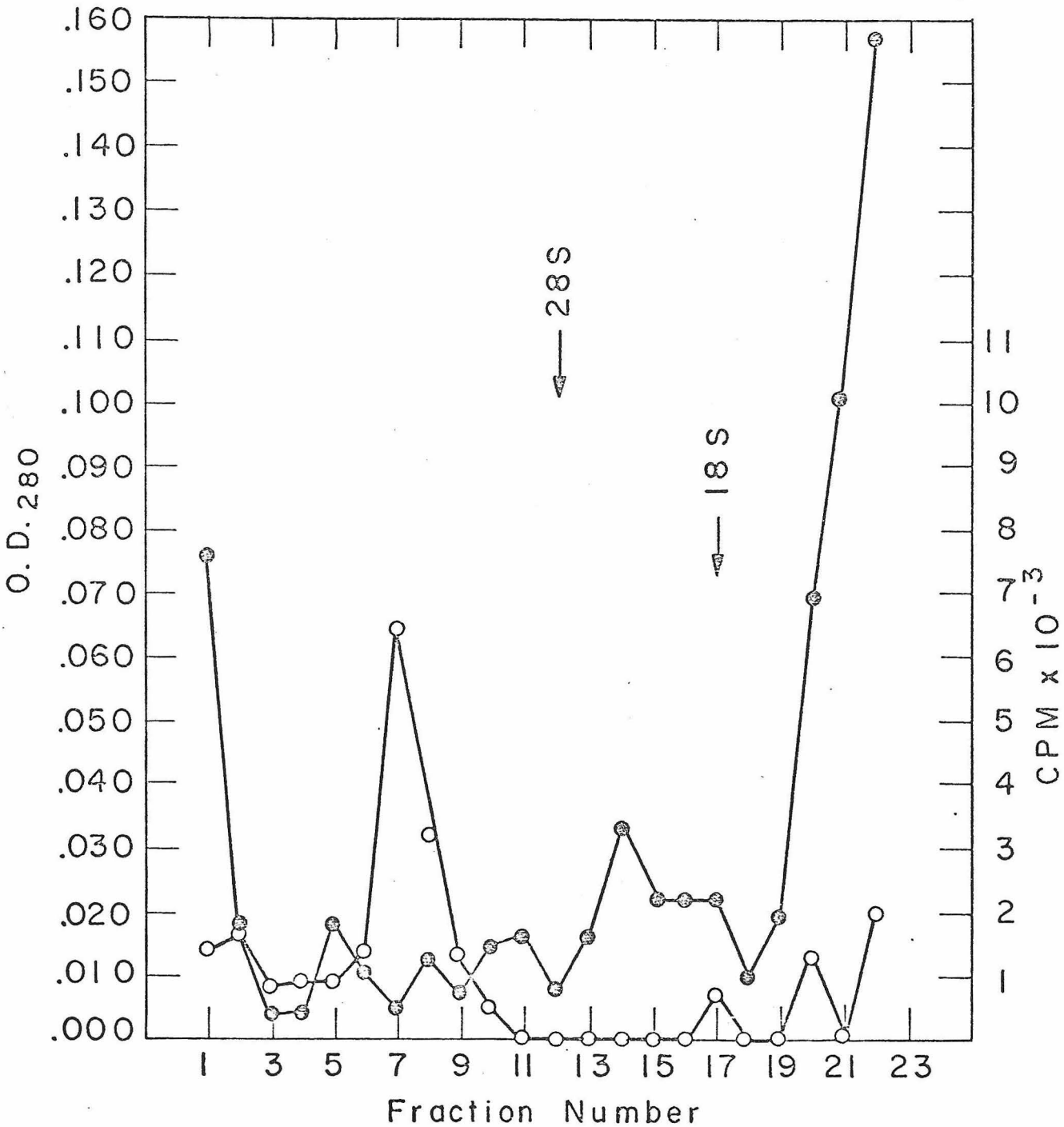
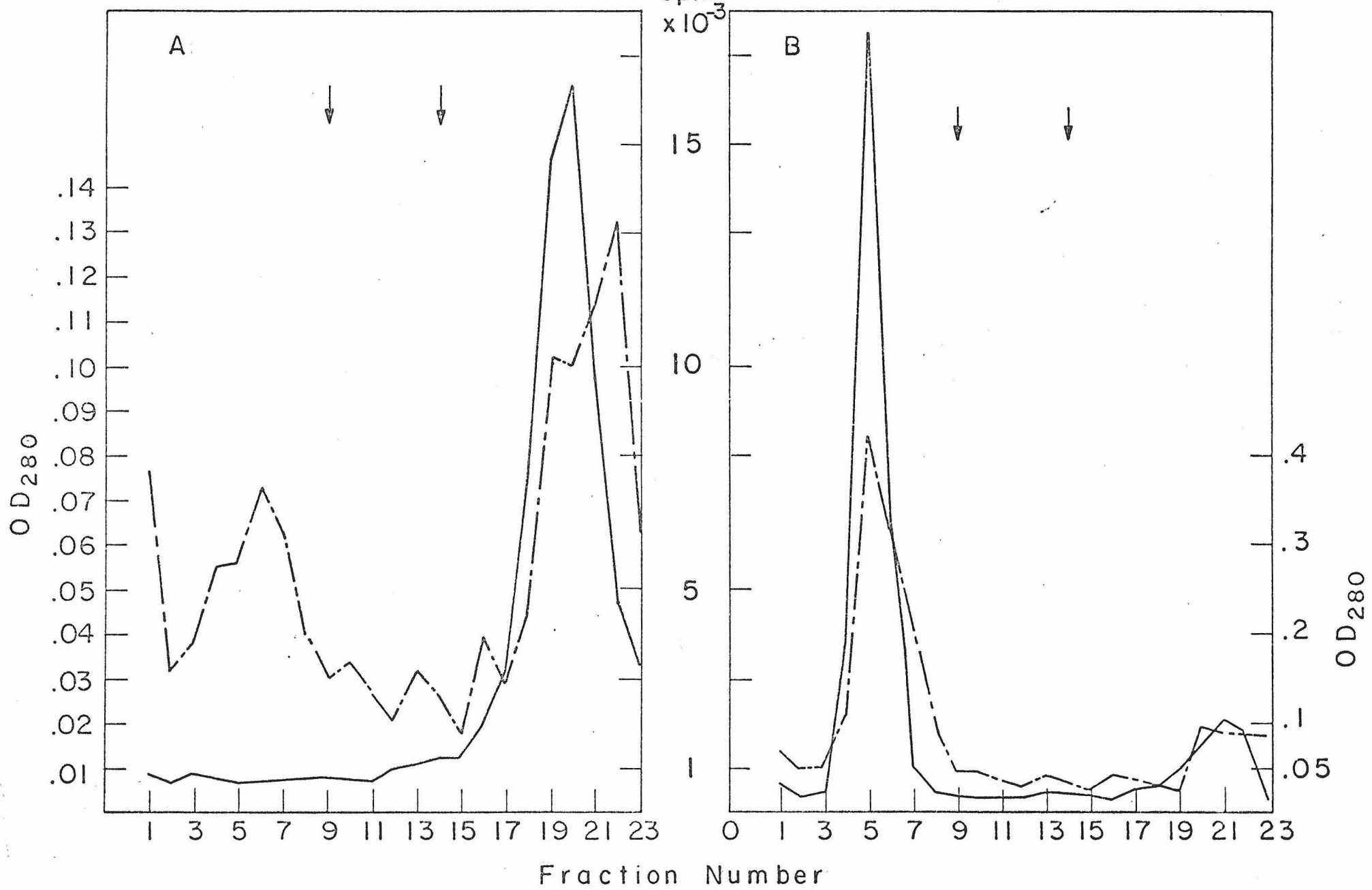

.160
.150
.140
.130
.120
.110
.100
.090
.080
.070
.060
.050
.040
.030
.020
.010
.000
O. D. 280
28S
18S
11
10
9
8
7
6
5
4
3
2
1
CPM x 10⁻³
1 3 5 7 9 11 13 15 17 19 21 23
Fraction Number

Figure 7. Behavior of highly labeled membrane bound material on sucrose gradients.

Purified slime mold membranes were treated so as to release actin into the supernatant as described in Methods. Aliquots of the released material was mixed with purified slime mold actin and treated as described in the legend to Figure 5.

A. Depolymerizing conditions: ----- optical density of purified actin; ——— radioactivity of released membrane associated material.

B. Polymerizing conditions: ---- optical density of purified actin; —— radioactivity of released membrane bound material.


A
B
cpm
x10⁻³
OD280
OD280
Fraction Number
.14
.13
.12
.11
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
15
10
5
1
.4
.3
.2
.1
.05
1 3 5 7 9 11 13 15 17 19 21 23
0 1 3 5 7 9 11 13 15 17 19 21 23

DISCUSSION

Pulse-labeling of slime mold cells during growth and development reveals several interesting aspects of overall protein synthesis, at the different stages. Vegetative cells, whether labeled for a two hour pulse, for half of one division time, or for two full generations, demonstrate a high background on gels of various percent acrylamide (Fig. 1 and unpublished observations). Gels of developing samples show many clearly resolved bands against a much lower background. This indicates that vegetative cells probably synthesize a much larger number of proteins than do developing cells. An alternative explanation is that the total number of proteins made in the two cell types is approximately the same, but that in developing cells 30-40 proteins are made in significantly greater amounts than all the others. Since gel samples are equalized as to counts, this situation would give the appearance of a lower background in the developing cell samples.

Thirty to forty bands can be distinguished on a cyclindrical SDS polyacrylamide gel, although cells are certainly synthesizing a good many more proteins than this at any one time. Therefore the bands resolved on a gel reveal only the major protein species in the sample. With this caveat in mind, it is of

interest to look at the periods of major change in protein synthesis revealed on gels, and to reflect on what this implies of the general structure and timing of the developmental program in Dictyostelium. The gels reveal a large number of band differences between vegetative amoebae and 0-2 hour developing cells. Thereafter the pattern of protein synthesis remains virtually identical for the next six hours of development. Assuming that the pattern of protein synthesis reflects, at least partially, the outcome of a developmental program, one could conclude that removal of nutrients and plating on a solid substratum initiates a large number of biochemical processes in slime mold cells, and that these events occupy the cells for the eight hours thereafter. Between eight and twelve hours, the time during which the cells actually change from a unicellular to a multicellular existence, another set of changes in protein synthesis occurs. After twelve hours there is no one period which can be singled out as a time of major change, although a number of changes can be identified. Gels of samples labeled in this later period may not accurately reflect major events which occur in only one of the two cell types which are present by this stage in development.

The unusual number of extremely large proteins made in developing Dictyostelium cells is also worthy of note. At least

nine clearly resolved bands whose molecular weights exceed 200,000 are synthesized throughout development. Since the background is much lower in this region of the gel, these bands may actually be present in much lower molar amounts than those species in the 40,000-150,000 molecular weight region of the gel.

The most outstanding feature of Dictyostelium protein synthesis as revealed on these gels is the finding that a single species of protein, Band B, rises from 4-6 percent of the total protein synthesis in vegetative cells to 22 percent during the first two hours of development. This protein has been identified as slime mold actin by comparing its properties to those of purified actin.

To do this, slime mold actin was first purified by a modification of a procedure previously developed for isolating this protein. The purified material was found to be approximately ninety percent pure on an SDS gel, and was estimated to be of 47,000 molecular weight (Fig. 4 & 5). This was in good agreement with the molecular weights reported for actin isolated from a variety of organisms and tissues, including rabbit and chick muscle, a primitive amoebae and the acellular slime mold Physarum polycephalum all of which have been

reported to lie in the range of 45,000-49,000 molecular weight (Korn and Wright, 1973; Rees and Young, 1967; Adelman and Taylor, 1969). Woolley (1972) has also purified Dictyostelium actin and reported a molecular weight of 48,000. The purified material was further identified as actin by its characteristic behavior on addition of KCl to a solution of the protein subunits. As has been reported for the other actins mentioned above, the purified material moved from a soluble position of less than 5S, to a sharp peak on a sucrose gradient of 25-30S upon addition of 0.1M KCl (Fig. 6).

The properties of the purified actin were then shown to parallel those of the highly labeled material. As shown in Fig. 4, the stained band runs with the same relative mobility as Band B on 5.5% polyacrylamide gels. Band B was shown to be localized in the membrane fraction and was isolated from this fraction under conditions designed to cause depolymerization of actin filaments. These conditions caused the release of 40 percent of the membrane counts. The released counts were then found to be identical to the purified actin in their behavior on sucrose gradients, before and after the addition of 0.1M KCl (Fig. 7).

Thus it appears that the protein synthesized in very large amounts during the first hours of development is slime mold

actin. The actin is associated with the plasma membrane, and is quite tightly bound to it. Actin-like filaments, identified solely by their appearance in electron micrographs, have been reported to be associated with the plasma membranes in a large number of systems (Zucker-Franklin, 1970; Tilney and Cardell, 1970; Yamada et al., 1971; McNutt et al., 1971). More detailed studies have been carried out by Korn and his colleagues on the primitive Amoeba, Acanthamoeba castellanii. These workers have firmly established the identity of the filaments as actin by a variety of criteria (Pollard and Korn, 1973). Highly purified plasma membrane preparations display associated actin filaments localized on the inner (cytoplasmic) surface of the membranes which form characteristic arrowhead structures after interaction with heavy meromyosin. Korn (1973) has postulated that the terminal few subunits of the actin filament may actually be inserted into the membrane. Thus the bulk of the actin would appear to be loosely bound to the membrane, while a small fraction appears to be tightly bound. However the conditions which lead to the release of actin from isolated membranes of Acanthamoeba are ineffective on Dictyostelium preparations unless a substantial concentration of non-ionic detergent is added. Therefore the attachment of actin to the much more complex slime mold membrane may be very different

from the situation in Acanthamoeba.

It is not clear why the slime mold cells should require such greatly increased synthesis of actin at the outset of development. Vegetative amoebae are motile and do synthesize actin though in much lesser relative amounts. It is possible that developing amoebae utilize a different means of locomotion from the growing cells. Nevertheless, unusual rates of synthesis have not previously been reported for actin in other motile systems, and the need for it in Dictyostelium demands a satisfying explanation. Even though unexplained, the greatly enhanced synthesis of actin in these cells promises to provide a very powerful tool for the isolation of a specific messenger RNA molecule, and for an intensive study of all the factors involved in regulating protein synthesis in a eucaryotic organism.

REFERENCES

Adelman, M.R. & Taylor, E.W. (1969). Biochem. 8, 4976.

Beug, H., Gerisch, G., Kempff, S., Riedel, V. & Cremer, G. (1970). Exptl. Cell Res. 63, 147.

Bonner, J. T. (1971). Ann. Rev. Microbiol., p. 75.

Bonner, J. T. (1967). The Cellular Slime Molds, end edition, Princeton University Press, Princeton, N.J.

Bonner, J. T. & Adams, M.S. (1958). J. Embryol. Exp. Morph. 6. 346.

Bonner, J.T. & Shaw, M.J. (1957). J. Cell Comp. Physiol. 51, 145.

Bonner, J.T., Clarke, W.W., Jr., Neely, C.L., Jr. & Slifkin, M.K. (1950). J. Cell Comp. Physiol. 36, 149.

Fairbanks, G., Steck, T.L. & Wallach, D.F.H. (1971). Biochem. 10, 2606.

Gerisch, G., Malchow, D., Riedel, V., Muller, E. & Fuery, N. (1972). Nature 235, 90.

Gregg, J.H. (1965). Devel. Biol. 12, 377.

Hatano, S., Totsuka, T. & Oosawa, F. (1967). Biochim. Biophys. Acta 140, 109.

Korn, E.D. & Wright, P.L. (1973). J. Biol. Chem. 248, 439.

Loomis, W.F., Jr. (1972). Nature 240, 6.

McNutt, N.S., Culp, L.A. & Black, P.H. (1971). J. Cell Biol. 50, 691.

Pollard, T.D. & Korn, E.D. (1973). J. Biol. Chem. 248, 448.

Raper, K.B. (1935). J. Agr. Res. 50, 135.

Rees, M.K. & Young, M. (1967). J. Biol. Chem. 242, 4449.

Sonneborn, D.R., Sussman, M. & Levine, L. (1964). J. Bact. 87, 1321.

Spudich, J. (1973). in preparation.

Tilney, L.G. & Cardell, R.R. (1970). J. Cell Biol. 47, 408.

Tuchman, J., Smart, J.E. & Lodish, H.F. (1973). in preparation.

Woolley, D.F. (1972). Arch. Biochem. Biohys. 150, 519.

Yamada, K.M., Spooner, B.S. & Wessells, M.K. (1971). J. Cell Biol. 49, 614.

Zucker-Franklin, D. (1970). J. Cell Biol. 47, 293.