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AN INHIBITOR OF CELL COHESION FROM DICTYOSTELIUM DISCOIDEUM

BY

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A Thesis Submitted for the Degree of

Doctor of Philosophy

TO MY PARENTS AND KATHRYN

CONTENTS

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ABSTRACT

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AN INHIBITOR OF CELL COHESION FROM *DICTYOSTELIUM DISCOIDEUM*

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The cohesive properties of slime mould cells during axenic growth and during development on solid supports have been studied. Axenically-grown log phase cells are cohesive, but lose this property as they approach the onset of stationary phase. A low molecular weight substance which inhibits cell cohesion has been found to accumulate in the medium of a stationary phase culture. This inhibitor, which has a molecular weight of approximately 500, has been partially characterised by biochemical methods. Its effects are not mimicked by simple sugars or by cyclic nucleotides. A certain concentration has been found to completely inhibit the cohesion of vegetative (feeding and dividing) cells of axenically-grown and bacterially-cultured *Dictyostelium discoideum* cells. It also inhibits the cohesion of vegetative cells of *D.mucoroides*, *D.purpureum* and *Polysphondylium violaceum*.

The cohesion of cells at aggregation-competence and later stages of development is only partially blocked. Evidence is presented which suggests that the cohesion inhibitor binds to the cell surface. It is not homologous with the transcription and division inhibitors also known to accumulate in the medium in stationary phase.

The importance of the cohesion inhibitor as a means of evaluating the necessity for cell contact in development is proposed as an alternative to existing methods.

The effects of this inhibitor are discussed in relation to the known aspects of cell cohesion in the slime moulds. A mode of action is suggested based on the results of this investigation.

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SECTION ONE:

INTRODUCTION.

1.1. CELL ADHESION AND COHESION IN DEVELOPMENT.

1.1.1. The general problem.

Successful development necessitates the correct number of cells of many highly-differentiated types becoming ultimately located in the right position in the body. Regulated division within the embryo is accompanied by a series of morphogenetic movements, both of single cells and of cell groups, resulting in an ordered array of specialised organs and systems. The precision of these morphogenetic movements was first suggested by Townes and Holtfreter (1955) to be determined by the intercellular cohesive properties of the cell types involved. They postulated that at least in the case of the amphibian embryo these cohesions become more specific as development progresses. Once embryonic development has been successfully completed, existing cell cohesions must be maintained and renewed throughout adult life. Aberrations in the cohesive properties of various cell types are one of the chief characteristics of malignancy and other afflictions.

The importance of cell cohesion in many fundamental biological issues, together with the complexity of its mechanisms, has resulted in a field of research which is expansive and far-reaching. Numerous theories on the mechanism of cell adhesion abound and greater consideration is given to these in section 1.1.4., following an introduction to the systems used in cell adhesion studies.

1.1.2. Cell adhesion models.

The complexity encountered in vertebrate systems has prompted attempts to discover simpler systems which can be studied as models. This has resulted in an intensification of research using mainly two invertebrate groups, the sponges (Porifera, Animalia) and the cellular slime moulds (Acrasiales, Protista). The advantages of these two systems are numerous and are best exemplified in a consideration of the assay methods currently employed in cell adhesion studies.

1.1.3. The Assays.

Assay methods for cell adhesion have fallen into four general groups:

(i) Separation of cells from the substratum (inert or cellular)

Studies of this type were initiated by Coman (1944) when he attempted to measure the strength of cohesion between two cells by pulling them apart with a weighted needle. The major criticism of this method is that the force required to generate the separation of a cell from its neighbour is not necessarily the same or even closely related to the strength of the bonds between the two cohering cells. Furthermore, Weiss (1961) pointed out that the line of weakness along which two cells (or cell and substratum) separate may be different from the line of intercellular apposition. He went on to show that if the adhesion of HeLa or rat 16C fibroblasts to a glass surface is disrupted by a shear force, deposits are retained on the glass. These deposits may then be demonstrated immunologically to be species-specific antigenic material derived from the cell types used.

(ii) The aggregation assays.

In principle, these types of assay attempt to measure the cohesion between cells brought together in a single cell suspension. This has the advantage of avoiding the possibility of chemotactic attraction between the cells which may modify cohesive behaviour. It also ensures that cell encounters are completely independent of cell locomotion.

In practice, however, this method suffers two intrinsic shortcomings. First, in many instances, the generation of a single cell suspension requires the addition of proteolytic enzymes or the chelation of divalent cations to separate the cells. Since these procedures are intended to disrupt the cohesive associations of the cells they are probably causing severe alterations in the very system to be investigated. Indeed, Steinberg, et al.(1973) showed that cells dispersed by trypsin treatment reaggregate only after a considerable lag period. This suggests that proteolytic procedures damage adhesive components from the cell surface which must then be resynthesised before normal adhesive and cohesive behaviour can be resumed.

Second, unless the assay is performed over an extended time period, the parameter measured is that of formation of cohesions which may relate to a greater or lesser degree to the strength of cohesions once initial contact has been formed. Passive flocculation can occur even between inert particles when shaken in suspension (supporting Weiss' point (1962) that specificity of adhesion in initial contact is unlikely). There is considerable evidence to indicate that whilst cells of many types will cohere initially under these

shaking conditions, the final pattern of their associative behaviour may be very different. For example, both Moscona (1957) and Steinberg (1964) showed that mixtures of vertebrate embryonic cells will 'sort out' once aggregated to give a precisely-patterned arrangement to the cell clump.

Despite these inherent faults, though, there is some evidence that aggregation methods in general may result in interactions which do have some biological significance. De Long and Sidman (1970) correlated abnormal reaggregative behaviour in mouse brain cells with the mutation 'reeler' which affects the positioning of cerebellar cells during embryonic development. More recently, Lloyd et al. (1976) have presented results which indicate that when cultured rat 16C fibroblasts are aggregated in shaking suspension there is a rapid and extensive reorganisation of their cell surface, resulting in recognisable gap and adhaerens junctions within minutes of contact. In addition, Overton (1977) has shown that mouse and chick embryonic cells will form stable desmosome junctions after some hours in shaking culture.

The use of this basic assay method has been extended to a study of the interactions between cells from two tissue or species types (Moscona, 1969; Steinberg, 1964). The cells attain an equilibrium configuration from which Steinberg, in his "Differential Adhesion Hypothesis" has constructed a hierarchy of relative adhesive strengths for the different cell types. Aggregates of single cell types, when placed in juxtaposition, will ultimately assume the same equilibrium configuration (Steinberg, 1970).

The findings here have been supported by computer simulations produced from differential adhesiveness data (Steinberg, 1975).

(iii) The collecting aggregate technique.

First employed by Roth and Weston (1967), and Roth (1968), this method uses previously-formed aggregates of one or more tissues to collect single cells from a suspension of freshly-dissociated, labelled cells of one tissue type. In essence, the method introduces a measurement of 'choice' or specific adhesion between unlike cell types and attempts to establish a hierarchy of heterotypic adhesive interactions. Again, the problem of dissociation procedures is encountered although at least in this case one of the cell types is allowed to recover before use. The main weakness with this technique lies in the low percentage of single cells collected, coupled with the fact that the single cells co-aggregate during the course of the assay, thus severely reducing the chance of single cell-heterotypic aggregate collisions.

Variations on the collecting aggregate technique have been employed. Edelman, et al. (1971) coated nylon fibres with antibodies or plant lectins and bound cells to these fibres. These cells then acted as a collecting surface for other cells. The advantage here is a surface of uniform size and shape, which enables the use of cell types which may be incapable of forming stable aggregates themselves (e.g., many adult tissue cells).

Walther, et al. (1973) used a cell monolayer as their collecting surface. This also provides a uniform collecting surface for comparative work and circumvents the problem of cell types which form poor aggregates. Also this method involves a lag period of 48 hours while the dissociated cells form the monolayer and thereby recover from the trypsin dispersal. This lag period is a comparatively long one, however, during which,in the case of embryonic cells, the adhesive properties may change drastically. Gottlieb, et al. (1975) modified this technique by using centrifugation to establish the monolayer in one hour on glass surfaces pre-treated with cross-linked amino groups. More recently, Vosbeck and Roth (1977) used Sephadex beads, covered with a layer of single cells, as the collecting surface. This again has the advantage of being a uniform surface and it enables the collection of a larger proportion of the suspended single cells.

The avoidance of disruptive dissociative procedures is obviously a major asset in any cell adhesion study. This is one of the main advantages conferred by the use of the cellular slime moulds. These organisms incorporate both a unicellular and multicellular phase in their life cycle. The unicellular stage offers a ready-dissociated population of single cells, the cohesive properties of which can be altered simply by depriving them of nutrients. These single cells will develop cohesive associations and enter a social, multicellular phase. They then pass synchronously

through the later stages of development to the final differentiated state which consists of only two cell types, spore and stalk. The patterning of these two types may be a reflection of their different cohesive properties (cf. section 1.2.6.).

The ease of manipulation of the non-cohesive and cohesive phases makes the slime moulds an ideal model system for adhesion studies. The vegetative (unicellular) phase cells can also be grown axenically in large numbers in shaking liquid culture providing a ready source of material for biochemical analysis. Furthermore, these organisms show in their adhesive properties and behaviour a closer resemblance to vertebrate cells than do the other invertebrates commonly favoured for studies of cell adhesion and cell recognition, namely the sponges.

The slime moulds exhibit three types of adhesive behaviour also seen in vertebrate embryonic cells. First, their adhesive properties alter with time during development. These "chrono-specific adhesion" properties (Garrod 1974) occur in vertebrate embryonic development and are thought to control morphogenetic movements (Gustafson and Wolpert,1967; Trinkaus, 1963).

Second, the slime moulds show species-specific recognition which has also been demonstrated in vertebrate embryonic cells (Burdick and Steinberg, 1969; Burdick, 1974a,b,). When cells of two species of slime mould are agitated together in suspension, they are readily mutually cohesive, forming aggregates consisting of both cell types. Such aggregates

later 'sort out' (Nicol and Garrod, 1977) to assume a spatial relationship analogous to that obtained under similar conditions using cells from two vertebrate tissues (Steinberg, 1964; Moscona, 1967; Roth, 1968).

Third, the slime moulds also exhibit a degree of tissue-specific cohesion when prespore and prestalk cells 'sort out' (Forman and Garrod, 1977a, b; Garrod and Forman, 1977) in a manner similar to that of embryonic vertebrate cells (Steinberg, 1964).

Sponges have been used extensively since Wilson (1907) showed that disaggregated sponge cells will reaggregate into species-specific cell clumps. The work on sponges has been reviewed by Burger (1974). In contrast to the slime moulds, sponge cells exhibit little or no interspecific, tissue-specific or chronospecific adhesions. Consequently, the cellular slime moulds are now more extensively used for studies of cell cohesion and adhesion. These terms are not synonymous; cohesion refers to cell-cell interactions and adhesion to cell-substrate interactions.

1.1.4. Adhesive mechanisms.

Before considering the detailed mechanisms by which cells cohere and adhere, it is advantageous to review the functional requirements of an adhesive system. First, it must possess sufficient strength to maintain a cell in juxtaposition with others under normal conditions of stress. Second, it must in many cases allow movement of the cell relative to its substratum (inert or cellular) whilst still

maintaining adhesive contact; and third, it must allow for specificity of adhesion i.e. a selective or recognition aspect must be involved. There is no reason to suppose that all three requirements are fulfilled by the same adhesive component. There is considerable evidence to indicate that different adhesive functions are performed by separate components of an integrated system, according to the specialised requirements of the cell types concerned.

Hypotheses on the mechanism of cell adhesion fall into two main groups — those which attribute adhesion to a purely physicochemical basis and those which favour the binding of complementary molecules on the cell surface.

(i) Physicochemical theories.

(a) Calcium bridging between cells.

The proposal that divalent cations link two anionic groups on apposing cell surfaces and maintain them in juxtaposition has been made by many workers. Chelation of cations by ethylene diamine tetraacetic acid (EDTA) or use of cation-free media is extensively employed for dissociation of cell clumps. Armstrong (1966), studied the effect of several divalent cations on the reaggregation of dissociated chick cells. He concluded that their presence was essential for cell adhesion and his evidence suggests that their role was not solely to reduce the

negative electrostatic cell surface charge. Armstrong and Jones (1968), studying the effect of divalent cations on the dissociation of Rana pipiens embryos by EDTA, reported that these cations, notably Ca^{2+}, were essential for maintaining cell cohesion in the presence of this chelating agent.

McGuire (1976) also showed that the removal of Ca^{2+} ions inhibited cohesion in a liver cell/liver aggregate system. In contrast, however, Edwards, et al.(1975) found that the aggregation of baby hamster kidney (BHK) cells was independent of divalent cations. Whether divalent cations are directly involved in cohesion, or are necessary for the functioning of sub-surface organelles which in turn affect cohesive properties, is as yet undetermined.

(b) Surface charge.

Curtis (1973) views cell adhesion as occurring at the point of balance between the negative charge of cell surfaces (causing electrostatic repulsion) and the van der Waals-London forces (which are forces of attraction). Thus any factors upsetting one or other of these components will diminish or increase adhesiveness accordingly. Kemp and Jones (1970) reported a diminution of adhesiveness in embryonic chick cells when treated with tannic acid and p-benzoquinone, two substances which are known to alter cell surface charge.

Also, McGuire (1976) presented evidence that disruption of the electrical potential across liver cell membranes with ionophores also inhibits adhesion.

(c) Hydrogen bonding.

Roseman (1970) suggested that the basis of adhesion between cells lies in the hydrogen bonding between oligosaccharide chains on the cell surface. Hydrogen bonding is known to be of importance in the maintenance of the secondary and tertiary structure of proteins, and could conceivably be involved in intercellular adhesion. The limitation of these physicochemical theories is their inability to account for the degree of specificity apparent in cellular adhesion. It is unlikely, therefore, that any one of these causes is solely responsible for the adhesive properties of cells. It seems probable that these mechanisms act in conjunction with one or more of the 'lock and key' processes outlined in section 1.1.4.(ii) which could provide the specificity component of the interaction between cells.

1.1.4.(ii) Specific cell surface components in intercellular cohesion.

Tyler (1946) and Weiss (1947) independently proposed the theory of lock-and-key type interactions in cell cohesion. The 'key' molecule on one cell would bind to the 'lock' molecule on another cell, effecting cohesion between the two. The major area of support for these theories to date is in the glycosyl transferase system (Roseman 1970; Roth 1973; Shur and Roth, 1975).

(a) Glycosyl transferases.

The glycosyl transferases are a family of enzymes located on the cell surface and in the Golgi apparatus. They elongate oligosaccharide chains on the cell surface

by the addition of monosaccharide units. The receptors are either glycoproteins or glycolipids. Adhesion would occur when the enzyme (on one cell) and the substrate acceptor (on another cell) bind together. So far, glycosyl transferases have been detected in many systems including mouse fibroblasts (Bosmann 1972a, Roth and White, 1972), chick embryonic neural retina cells (Roth et al, 1971) and human blood platelets (Barber & Jamieson, 1971a,b; Jamieson et al.1971; Bosmann 1971; 1972b). In these cases altered levels of enzyme activity have been correlated with alterations in cell adhesiveness.

(b) Other surface macromolecules.

Other cell surface macromolecules have also been assigned roles in cell cohesion (Lilien, 1969; Curtis, 1973; Moscona, 1974). Some of these enhance cell adhesion, others inhibit it. Garber and Moscona (1972) isolated a factor from cultures of embryonic mouse cerebral cells which was able to enhance only the aggregation of this cell type. Cells from other mouse brain tissues were unaffected. Chick embryonic cerebrum cells, however, responded to the aggregating ability of this factor. A glycoprotein which stimulates adhesion of chick embryonic neural retina cells has also been isolated by Hausmann and Moscona (1975).

Specific aggregation factors have been found in the marine sponges. Henkart, et al (1973), working with Microciona parthena, isolated such a factor, a proteoglycan comprised of at least half carbohydrate by weight. A similar factor has been found from Microciona prolifera

by Burger, et al.(1975). This factor is inhibited by glucuronic acid and cellobiuronic acid.

Aggregation inhibitors have also been isolated from vertebrate systems. In 1965, Curtis and Greaves reported the inhibition of aggregation of embryonic chick cells by a specific protein present in horse serum. A similar protein which specifically inhibits aggregation has been purified from chick embryonic neural retina cells by Merrell, et al.(1975), while Rutishauser, et al.(1976) were able to inhibit the cohesion of these cells by antibodies specific to a protein recovered from the tissue culture supernatant.

Curtis and Van de Vyver (1971) reported that two strains of the sponge Ephydatia fluviatilis produce a factor which increases the adhesiveness of homologous cells but decreases that of the heterologous type. A similar system appears to operate in the positioning of mouse T and B lymphocytes (Curtis and de Sousa, 1975).

(c) Surface carbohydrates.

Surface carbohydrates have been implicated to play a role in cell cohesion by many workers in recent years (Roseman, 1970; Cook and Stoddart, 1973; Harrison and Lunt, 1975). Moscona (1957) found that periodate treatment inhibited the reaggregation of dissociated chick embryonic cells. Similar treatment also severely reduces the ability of liver cells to cohere to homotypic aggregates (McGuire, 1976). Lloyd and Kemp (1971) and Garber and Moscona (1972) reported

that a high concentration of glucosamine in the medium results in smaller chick cell aggregates. Oppenheimer et al.(1969) showed that a lack of hexosamine synthesis (effected by using a medium free of the precursor, glutamine) rendered mouse teratoma cells non-cohesive. Addition of hexosamines reversed this effect. Conversely, addition of acetylated hexosamines decreased the binding of chick neural retina cells to homotypic aggregates (Roth,et al. 1971a). Furthermore, Hausmann and Moscona (1973) reduced hexosamine, and consequently glycoprotein, synthesis in chick neural retina cells. This resulted in an inhibition of reaggregation. Edwards, et al.(1975), have correlated areas rich in glycoproteins on the cell surface with adhesiveness. Terminal B-galactoside residues on surface oligosaccharide chains are considered to be important in cohesion of chick neural retina cells (Roth, et al. (1971b). They found that B-galactosidase treatment increased cell cohesion.

Neuraminidase has been used extensively to investigate the role of sialic acids in cohesion. These sialic acid residues, when present, are always terminally-sited on the oligosaccharide chains of the cell surface, and are cleaved off by neuraminidase. This treatment, because it removes the negatively-charged sialic acid groups, also alters the surface charge. Although neuraminidase treatment increases aggregation in cultured cell lines (Wickers, et al. 1972; Deman, et al.1974; Lloyd and Cook, 1974) it does not have the same effect on primary chick

muscle cells (Kemp, 1970) or neural retina cells (McQuiddy and Lilien, 1971).

(d) Gross cell surface properties.

Coupled with these very specific intermolecular bonding reactions are the larger-scale alterations in the cell surface. The number and distribution of various junction types, as seen using electron microscope techniques e.g. desmosomes (Fawcett, 1966) can be correlated with cohesive strength although the molecular bases of these structures are unknown. For example, Overton (1977) in an aggregation study using mouse and chick embryonic cells was able to equate the degree of desmosome formation to the strength of cohesions between the cells.

Recently, various sub-surface organelles, notably microtubules (Tilney 1968) and microfilaments (Wessels, et al, 1971), have been shown to be important in regulating cell shape and motility and are, therefore, indirectly involved in cell adhesion. The extent of the role played by the flow of membrane components within the plane of the membrane is still unsolved (Singer and Nicolson, 1972).

The mechanisms by which two cells cohere are far from being fully understood. The next section reviews the current understanding of these mechanisms in the cellular slime mould.

1.2. THE CELLULAR SLIME MOULDS.

1.2.1. Introduction.

The cellular slime moulds are widely used to study certain problems of cell interactions during development, e.g. cell adhesion, cell differentiation, pattern formation and morphogenesis. This organism is considered generally to be a model system — a simple eukaryote which exhibits the same developmental phenomena as higher organisms, but without the complexity found in the higher plants and animals. The cellular slime moulds are, therefore, the tools by which many biologists are attempting to understand the central processes relevant to successful development in higher organisms. In the following section the organism and its life cycle are described and the advantages of using this organism to investigate these problems become more apparent. Of particular interest in this instance is the usefulness of the organism in studies of cell adhesion. Considerable attention, therefore, has been paid to this area, and a review of the relevant literature is incorporated in this chapter.

1.2.2. The organism and its life cycle.

One of the reasons why the slime moulds are most suitable is because their life cycle incorporates both a unicellular and a multicellular stage. The developmental cycles of the two main genera, Dictyostelium and Polysphondylium differ only in detail (Bonner, 1957) and since the bulk of the research reported in this thesis was performed using Dictyostelium species, the developmental pattern of D.discoideum is described. A diagrammatic

scheme is shown in Figure 1.1.

During the growth or vegetative stage the cells exist as solitary amoebae, feeding phagocytically on bacteria and dividing regularly while a food source persists. The individual cells exhibit no 'social' behaviour towards any neighbouring cells, e.g. no directional movement, no apparent cell-cell communication and form no stable cell-cell cohesions. Upon depletion of the nutrient source, the cells cease to divide and begin to enter the social phase of the life cycle. After a short lag period (interphase) which lasts a few hours (see time-scale of development in Fig.6.1.) the amoebae begin to migrate towards agglomerates of (now) cohesive cells which are termed 'aggregation centres'. Amoebae migrating the long distances involved (several millimetres) form chains of cells elongated in the direction of movement, and cohering at anterior and posterior ends, See Fig.6.4. Shaffer, 1964). Where these chains begin to converge on the aggregation centre they may form streams several cells wide. At this point the cells also cohere by side-to-side contact and with more than one cell layer. Streaming is a non-random process, the amoebae aggregating in response to a chemical attractant "acrasin". In the case of D.discoideum, D.mucoroides and D.purpureum acrasin has been shown to be adenosine 3' : 5' cyclic monophosphate (cyclic AMP) (Konijn et.al.1967; Bonner et.al. 1969; Robertson et.al.1972; Shaffer, 1972). The Polysphondylium species and D.minutum are apparently insensitive to cAMP

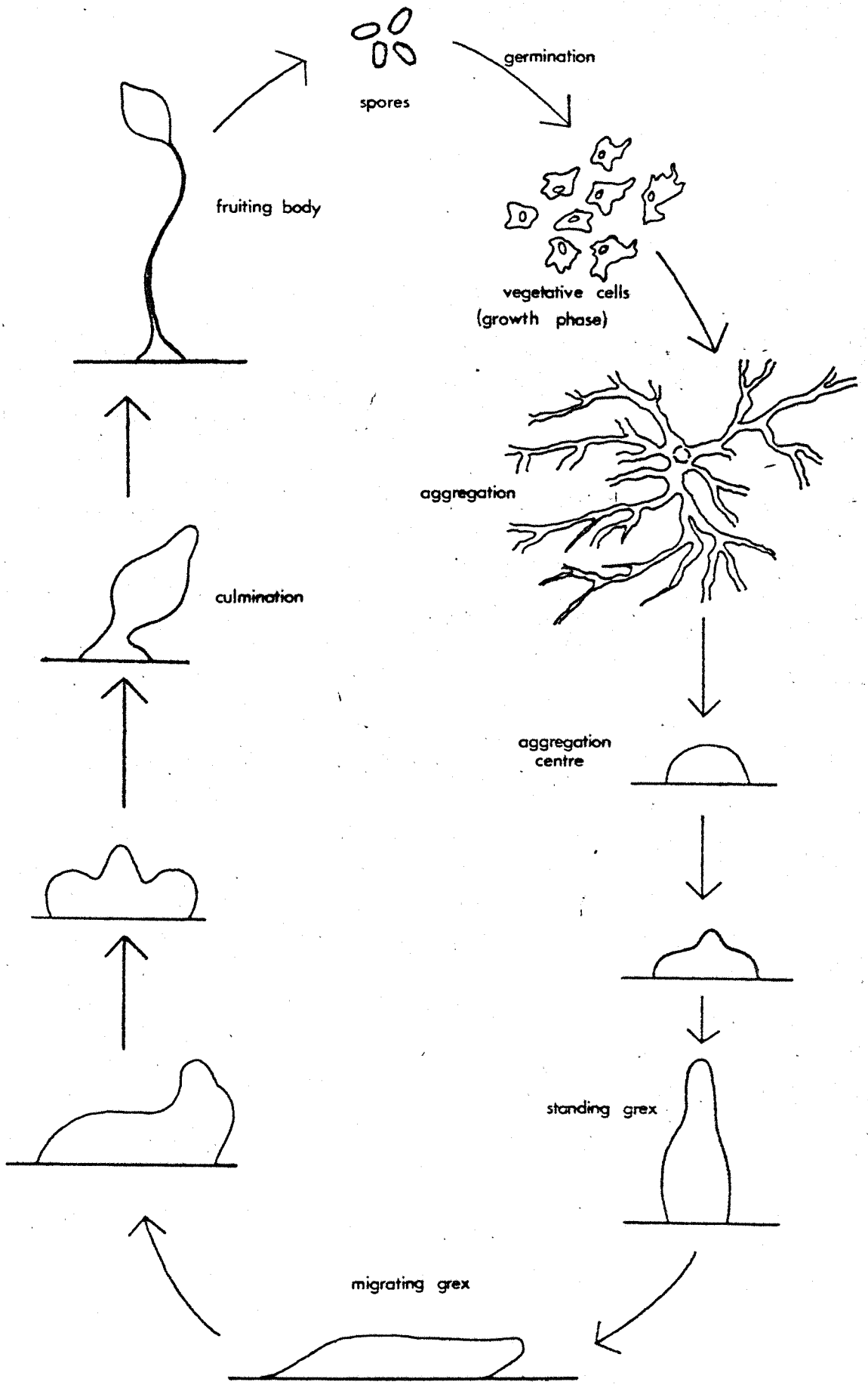

Fig.1.1. The life cycle of Dictyostelium discoideum

gradients (Jones and Robertson, 1976).

Once a discrete multicellular aggregate has formed, the cell mass extends upwards to assume a sausage-like shape which then falls laterally to lie lengthways on the substratum. This is termed the slug, grex or pseudoplasmodium. By this stage the cell mass, containing normally about 10^5 cells, is surrounded by a sheath of slime material (mucopolysaccharides). The grex then migrates for a greater or lesser period of time, dependent upon environmental conditions, leaving behind a trail of collapsed slime sheath. At the end of this migratory period, the grex tip rises vertically while the cell mass rounds up beneath it. The tip then begins to elongate upwards, forming the stalk of the future fruiting body. The stalk cells die and are strengthened by an enclosing cellulose sheath. The spore cells, which were previously situated at the posterior end of the grex, then migrate up the stalk and form the spore mass at the distal end.

1.2.3. Chemotaxis and aggregation-competence.

Although the problem of chemotaxis in this organism is not central to the present thesis a brief review of the salient points is in order. The onset of aggregation, i.e. the point at which the fundamental behaviour and the cohesive properties alter drastically, correlates with the development of chemotactic sensitivity and the numerous components of this guidance system.

The chemotactic agent in D.discoideum, 'acrasin', was found to be responsible for attracting aggregating amoebae

towards the centres by diffusion outwards from the middle of the aggregation centre (Runyon, 1942; Bonner, 1947; Shaffer, 1953). Acrasin has now been identified as 3', 5' cyclic AMP. mainly by work of Konijn et.al(1968). When a population of amoebae are starved, some of the cells (randomly distributed) begin to secrete bursts of cAMP at regular intervals (Cohen and Robertson, 1971). Adjoining amoebae 'sense' these pulses and subsequently emit a similar pulse of cAMP (Robertson et al, 1972; Shaffer, 1972). These cells also make a directional movement toward the original source of the chemotactic signal which then becomes an aggregation centre (Konijn et al,1968) (Cohen and Robertson, 1971). After each pulse there is a refractory period during which the cell cannot relay the signal. This results in a series of pulsatile movements towards the aggregation centre. These movements, when seen under time-lapse photography appear as regular wave-like surges. As the cells become aggregation-competent the number of cAMP receptors, situated on the cell surface, increases (Malchow and Gerisch, 1974) and the cAMP is detected by these receptors without being internalised by the cell (Malchow and Gerisch, 1973). Paralleling the appearance of the cAMP receptors is the appearance on the cell surface of the degradative enzyme, phosphodiesterase (Malchow et.al, 1972). Phosphodiesterase breaks down the cAMP which has been detected by the receptors (Nanjundiah and Malchow, 1976). This surface-located enzyme supercedes the extracellular phosphodiesterase present during growth

(Gerisch et al.1972). The extracellular phosphodiesterase seems to be inactive after the onset of aggregation-competence (Gerisch, 1976).

Exogenous cAMP, when pulsed onto a field of amoebae, can induce the onset of aggregation – competence in the cells (Gerisch et al, 1975; Darmon et al.1975) and recent information suggests that pulsing may not be necessary so long as a continuous gradient of the attractant is provided. cAMP continues to be secreted at the grex stage although seemingly only by the cells at the anterior tip, the prestalk cells (Rubin and Robertson, 1975). Grex cells, however, do not appear to be chemotactically sensitive since they will not enter an aggregation stream if placed nearby, whilst aggregation-competent cells readily move towards the stream and become incorporated within it. (Garrod 1974).

1.2.4. Cell cohesion during development.

Cells in the vegetative phase on a substratum are virtually non-cohesive. Cell-cell contacts do not result in stable adhesions and the cells move apart again (Shaffer 1957). Approximately 2 hours after the beginning of starvation, however, such cells begin to develop cohesive properties, paralleled by changes in morphology to an elongate, flattened shape. These changes form the basis for the new behaviour patterns observed during aggregation. Such clusters of cells are not merely the result of chemotactic attraction, since these aggregates cannot be mechanically disrupted (Shaffer 1957).

This development of cohesiveness has been demonstrated and to some extent quantified by work of Beug et al (1973a) and of Garrod (1972). They showed that when a single cell suspension was shaken or rotated the cells agglomerated. The degree of agglomeration was assessed by determining either the mean particle size or the reduction in number of single cells. The level of agglomeration increased with developing aggregation competence. A low level of agglomeration is present in suspensions of feeding-stage cells (Gerisch, 1961). This can be prevented by the addition of a calcium-chelator, e.g. EDTA to a final concentration of 0.01 M in the suspension medium.

As the cells begin to develop the cohesive properties associated with aggregation, their ability to cohere in EDTA-phosphate buffer increases (Beug et al, 1973a). This parallels to the cells' ability to enter an aggregation stream on a solid substratum. Between 6 and 8 hours post-feeding the cohesion level of cells in EDTA-phosphate buffer increases to that in buffer alone.

At the grex stage of development the cells lose their elongate shape, maintain their high cohesiveness and are packed tightly within the mass. Maeda and Takeuchi (1969) reported that the anterior (pre-stalk) cells are far more tightly-packed than the cells of the posterior (pre-spore) region of the grex. The anterior cells were correspondingly much more difficult to dissociate than posterior cells (Takeuchi and Yabuno, 1970) by proteolytic and EDTA treatments.

1.2.5. Cohesive mechanisms.

The elucidation of cohesion mechanisms in slime mould cells has been approached from several directions. Three major areas of research have emerged. First, the series of elegant and incisive experiments by Gerisch, Beug and coworkers on the immunochemistry of cells as they develop; second, the discovery of native lectins, their appearance, properties and functions by Rosen, Barondes and their group, and third, the investigations into cell surface properties by a number of workers.

i) Cell surface Properties.

(a) Physical alterations during development.

Rossomando, et al (1974) undertook a scanning electron microscope study which revealed that gross changes in cell surface architecture occur between the log and stationary phases of axenic growth, and between these and aggregation-competent cells. Log cells are irregular in shape and possess fine filopodia over the cell surface, whereas stationary cells are more rounded, with shorter surface projections and more extensive surface ruffling. Aggregation-competent cells exhibit a fine ruffling, the pattern and shape of the surface extensions being more complex than in either log or stationary cells.

Several membrane fluidity studies have been initiated with somewhat disappointing results. Changes in membrane lipid composition during development (Ellinston, 1974; Long and Coe, 1974) were not paralleled by changes in the fluidity of lipids as measured by Van Dreele and Williams(1977).

In addition, Molday, et al.(1976) coupled Concanavalin A (ConA) or wheat germ agglutinin (WGA) to methacrylate microspheres and mapped their distribution on the cell surface using a scanning electron microscope. Fixed and labelled vegetative cells showed a dense uniform array of ConA and WGA receptors over the entire cell surface, including the microvilli. This pattern was also detected in the aggregation stage cells which suggests that the difference in agglutinability by ConA between these two stages (Weeks and Weeks (1975) - discussed later) is not the result of different receptor arrangement. Molday et al.(1976) also demonstrated that WGA receptors in unfixed cells remain as a mosaic over the cell surface after several hours, while ConA sites show patching and subsequent capping around a cluster of microvilli localised at areas of cell-cell or cell-substratum contact. A concurrent change in cell morphology from a flattened irregular form to a spherical shape was also observed. Patching and capping are generally attributed to the flow of receptors within a fluid membrane (Singer and Nicolson, 1972). Since these topographical changes in ConA microsphere distribution were observed in both newly-plated cells and those which had developed for 12 - 18 hours, there appears to be little difference between these two stages regarding this property of membrane fluidity.

The cell surface charge during development has also been investigated, since alterations in this could conceivably alter cell cohesiveness (Yabuno, 1970; Lee, 1972).

The electrophoretic mobility was found to decrease during the transition from the solitary to the aggregative state, although the change was gradual and did not parallel the onset of aggregation-competence (Garrod and Gingell, 1970).

(b) Biochemical changes.

Certain components of the cell surface (the plasma membrane and associated structures) have been shown to alter with the change in cohesiveness of the cells. Plant lectins have been employed to detect alterations in the glycoprotein profile of the surface. Gillette and Filosa (1973) demonstrated a delay in aggregation of NC-4 (wild type) cells by ConA, and suggested that the cAMP chemotactic system was affected since incubation of the cells in the presence of ConA greatly increases the activity of the cAMP-specific phosphodiesterase. However, in a subsequent study, Weeks and Weeks (1975) demonstrated that aggregation of the NC-4-derived axenic strain Ax-2 is delayed to a much lesser extent than the parent strain, suggesting that the ConA-binding system has no fundamental involvement in the differentiation process. This is further substantiated by the fact that succinyl-ConA (effectively univalent and binding to the ConA sites) does not affect Ax-2 cell development. From Weeks' study, however, it transpires that the susceptibility of the cells to agglutination by ConA decreases as the cells pass from log to stationary phase of axenic growth, and from vegetative to aggregated amoebae on a solid substratum. This indicates a change in carbohydrate-binding sites during differentiation and

probably reflects surface differences between growing (dividing) and non-growing states. This phenomenon is markedly apparent in ConA agglutinability studies on malignant and normal cell types. ConA binds to ∝-glucopyranosyl or ∝-mannopyranosyl residues. Wheat germ agglutinin (binding to N-acetylglucosamine residues) also agglutinates growth phase cells more effectively than aggregation cells although Ricinus communis agglutinin (RCA-I) which binds to D-galactopyranosyl residues (Reitherman, et al.1975) had the opposite effect.

Labelled lectins have also been employed to trace specific surface receptors. Weeks and Weeks, (1975) have shown that the decreased agglutinability of the cells by ConA during differentiation cannot be correlated with a change in the number of binding sites on the cell surface. There is either no change, or a small increase at aggregation-competence (Darmon and Klein, 1976) and in fact when calculated from the viewpoint of sites per unit area there is a sharp increase with differentiation (Weeks, 1975; Geltowsky, et al, 1976). Weeks (1975) has suggested that the decrease in agglutinability is due to a decrease in the binding affinities of the receptors for ConA, which implies a modification of the surface glycoproteins in some way.

McMahon and colleagues (West and McMahon, 1977; Hoffman and McMahon, 1977) have demonstrated the presence of membrane glycoproteins by using a modified electrophoresis method using ConA as 'antibody'. By staining corresponding sodium dodecyl sulphate (SDS) gels with periodic acid/

/Schiff's reagent (PAS), they demonstrated that most (>35) of the glycoproteins on the surface of vegetative axenic strain (A3) cells were ConA receptors. Of these, nearly all were still present in cells which had been starved for 12 hours (aggregation-competent). A minority had disappeared, and a few novel glycoproteins were detected. Geltowsky, et al.(1976) on the other hand, having labelled the cell surface components by the sodium ^{125}I - lactoperoxidase method, demonstrated the presence of 15 ConA receptors in vegetative cells by using affinity chromatography on a ConA column followed by gel electrophoresis. They found that as the cells started to differentiate after 6 hours' starvation there was a dramatic increase in intensity of the 150,000 molecular weight band on the gel, and a decrease in the 180,000 molecular weight band.

This is of particular interest in relation to the work of Smart and Hynes (1974) who also labelled cell surface proteins on Ax-2 cells using the sodium ^{125}I-lactoperoxidase method. Four bands were found to increase with differentiation while one decreased over twelve hours of development. One of those that increased, a polypeptide of 130,000 molecular weight, appeared within 12 hours of development. This was confirmed by Siu, et al.(1975) but was found to be absent on aggregation-competent cells which had differentiated in suspension (Smart and Hynes, 1974) and, therefore, this polypeptide seems to be of little direct significance in the cohesion of aggregation-competent cells. This polypeptide may, however, correspond to the band found by Geltowsky, et al (1976).

The study of cell surface proteins was stimulated by a report by Takeuchi and Yabuno (1970) which indicated that proteolytic enzyme treatment coupled with a disulphide reducing agent, 2,3,dimercapto-1-propanol (BAL, British Anti-lewisite) would dissociate grex cells. The cohesiveness of these dissociated cells can be drastically reduced by treatment with trypsin (Alexander et al, 1975) thus suggesting that surface proteins were playing a direct role in the cohesion process. Hoffman and McMahon (1977) studied the polypeptide profile of plasma membranes on SDS gels, stained with Coomassie Blue. Over twelve hours of development, eight bands decreased and five bands increased or appeared. Although it is possible that some of the stained bands were internal cytoplasmic proteins, pronase digestion of intact 12-hour cells removed three of the five new bands, indicating that at least these three were surface proteins. The correlation of these results with those of Smart and Hynes, (1974), however, is disappointing, showing no apparent correspondence between these three bands and the 'external' proteins labelled in the latter work. Some of the proteins present throughout development, however, did become more pronase-sensitive, suggesting that they are being externalised. Whether they have a true function in cell cohesion or not is indeterminate especially since the topographical arrangement of these proteins on the surface is unknown.

(ii) Immunochemistry of the developing cell surface.

As early as 1956, Gregg reported that new antigens

appeared between the vegetative and aggregation stages of D.discoideum, D.purpureum, and P.violaceum. Mutants of D.discoideum which were unable to aggregate had a different surface antigen profile to that of the wild type cells, (Gregg and Trygstad, 1958). Gerisch (1961) showed that while both vegetative and aggregation-competent cells would agglomerate in shaken suspension, only the latter would do so when EDTA was present. Sonneborn, et al.(1964) raised an aggregation-specific antibody to D.discoideum cells which could block aggregation, but it was found to be absent in an aggregation-deficient mutant. It was also ineffective in blocking the cohesion of this chemotactically defective mutant and, therefore, did not appear to be directly involved in cell cohesion.

Following Sonneborn's findings, Beug and colleagues began work on a series of experiments in which they raised antibodies against vegetative and aggregation-competent D.discoideum (wild type) cells (cf.Beug et al 1970). Three different classes of antigens were demonstrated. Two of these classes, "antigens I and II" (AG1/2) and "contact sites B" (CSB) are present on the surface of feeding vegetative cells. The serological activities of antigens I and II reside in their carbohydrate moiety. That of AGI contains fucose, mannose, behenic acid (lipid) and a polypeptide component. Univalent antibodies (Fab) against these carbohydrate-containing antigens do not affect aggregation, although they have been demonstrated to react with the surfaces of living cells and to be diffusely distributed over

the entire surface. The third class of antigen is the so-called "contact sites A". The Fab fragments made against the two kinds of contact sites, A and B, (CSA and CSB) have proved extremely useful in elucidating alterations in surface antigens during development.

Anti-aggregation-cell-homogenate Fab was found to block aggregation of competent amoebae with no apparent alteration in cell morphology, motility, or chemotaxis (Beug, et al 1970). This Fab also prevented agglomeration of aggregation-competent cells in shaken suspension, suggesting that it was specifically blocking cell cohesion (Beug et al 1973a). In addition, this Fab, at high concentrations, was able to dissociate slices of grexes (Beug et al, 1971). To purify the Fab fraction, Beug, et al (1973a) absorbed the antiserum with broken vegetative cells. This removed all Fab fragments to antigens common to both vegetative and aggregation-competent cells, leaving only those specific to differentiated cells. The resultant Fab fraction completely blocked the cohesion of aggregation-competent cells, but did not affect that of vegetative cells. The target antigens of these Fab fragments are the "contact sites A". The anti-CSA Fab was not absorbed by aggregation-competent P.pallidum cells indicating species-specificity.

Fab fractions prepared against vegetative cells and absorbed with aggregation-competent cells have the reverse effect to above. In this case, cohesion of vegetative but not of aggregation-competent cells in suspension is blocked. The target antigens for this Fab are the "contact sites B".

Beug, et al.(1973b) were able to demonstrate that contact sites A are detectable on the cell surface only after 4 hours' starvation, and greatly increased in number until 9 hours, paralleling the development of EDTA-insensitive cohesion.

Anti-CSB Fab specifically inhibits the side-to-side cohesion of aggregation-competent cells on a solid substratum, resulting in chains or star-like rosette shapes formed by the maintenance of the end-to-end contacts. Anti-CSA Fab, in contrast, blocks these end-to-end cohesions, and results in clumps of laterally-cohering cells (Beug et al 1973a; Gerisch et al.1974). Labelled anti-CSA Fab, however, was found to be distributed over the entire cell surface, suggesting that either it is specifically activated only at the terminal sites (Gerisch 1974) or that the Fab is not entirely specific to CSA and is coupling to another antigenic species as well.

The number of contact sites A per cell, as determined by labelled Fab binding is at maximum 3×10^5, and hence covers only about 2% of the surface (Beug et al, 1973b). CSB could be completely blocked by 2×10^6 specific Fab molecules Anti-carbohydrate Fab, bound at 2.5×10^6 molecules per aggregation-competent cell and at 2×10^6 molecules per vegetative cell, had little or no effect on cell agglomeration in suspension. This indicates that the contact sites are a discrete population of surface antigens.

Anti-homogenate Fab will completely block aggregation and also renders the cells non-adhesive to glass. This

antibody is also active against the carbohydrate moieties of antigens I and II.

To directly implicate the contact sites in cell cohesion, Gerisch and Beug made use of a series of "aggregateless" mutants which had lesions in various developmental stages. One of these was unable to agglomerate in EDTA after starvation, and also lacked CSA (Beug et al, 1973a). Another important mutant absorbed only part of the anti-CSA Fab. The residual Fab still blocked normal aggregation and was absorbed by wild type cells, indicating that anti-CSA Fab has two components and, therefore, presumably, contact sites A are bi-partite (Gerisch, et al, 1974). Both the components are necessary for cohesion, since the mutant possessing only one was unable to aggregate, and Gerisch suggests that they may be complementary sites on adjacent cells.

Solubilised CSA have been prepared from crude membrane extracts and purified by column chromatography (Heusgen and Gerisch, 1975). The molecular weight was estimated to be 130,000, and the activity of the semi-purified CSA was sensitive to pronase and periodate. Fab binding to the CSA is also inactivated by periodate, indicating the involvement of carbohydrate in this process (Beug. 1973b). It is possible that the 130,000 dalton band detected by Smart and Hynes (1974) and Geltowsky, et al (1976.) represent CSA.

(iii) Native lectins.

Lectins are proteins which can agglutinate erythrocytes. They are generally isolated from plants but

may be isolated also from other sources. Lectins contain at least two sites which bind carbohydrates. Cell agglutination is caused by lectin molecules binding to a carbohydrate on two adjacent cells (Sharon and Lis, 1972). If simple sugars are present they will compete for the carbohydrate-binding sites and hence inhibit lectin-induced agglutination.

Carbohydrate-binding proteins have now been isolated from six species of cellular slime mould. These proteins have distinct specificities in their carbohydrate-binding properties as shown by saccharide inhibition of erythrocyte agglutination. Due to this specificity of action, these lectins have been suggested as a potential basis for species-specificity in cellular recognition among slime mould cells.

'Discoidin', the native lectin from D.discoideum and 'pallidin', from P.pallidum, have been characterised most fully regarding their location, appearance and activity (Rosen et al 1973; Rosen et al, 1974). The appearance of the agglutinating activity in cell extracts parallels the development of cellular cohesiveness. It has been shown by using fluorescent antibody techniques that discoidin first appears in detectable amounts on the surface of NC4 cells some 6 hours after food deprivation (Chang et al., 1975). (Pallidin, on the other hand, is present in quite significant amounts even in vegetative cells.) The fluorescence is diffuse over the whole cell surface, but after 3 hours at room temperature with living cells, capping is observed indicating fluid flow within the membrane. 8-to 10-hour aggregates are brightly fluores-

cent and in these, and in the elongated radially-oriented streaming cells, the staining is diffuse. Ferritin labelling studies have confirmed the uniformity of spacing of discoidin molecules over the cell surface, although in cohering cells no ferritin can be seen in the 2 nm gap between apposed cell surfaces.

Discoidin has been purified on Sepharose 4B (Pharmacia) which furnishes galactose residues for which discoidin has affinity. (Simpson et al.1974). The pure lectin can be resolved into two molecular species, discoidin I and II (Frazier et al, 1975) each of which is a tetramer of molecular weight 100,000 - 110,000 (Reitherman et al, 1975). Each of the 4 subunits has a molecular weight of 26,000 (I) or 24,000 (II). The carbohydrate-binding specificity is slightly different between discoidins I and II, and discoidin I is present in much larger quantities (10-fold) than discoidin II at 12 hours of development. (Frazier et al, 1975). Pallidin is purified by elution from erythrocytes by a specific monosaccharide (Simpson et al, 1975). The molecular weight of pallidin is 250,000, and that of each subunit is 25,000.

The lectins from each of six slime mould species, D.discoideum, D.purpureum, D.mucoroides, D.rosarium, P.violaceum and P.pallidum, have different carbohydrate-binding specificities, which can be used to discriminate between these proteins. For example, pallidin is best inhibited by lactose, discoidin by N-acetylgalactosamine, and the lectin from D.purpureum, purpurin, is maximally

inhibited by several sugars including me-β-D-galactose (Rosen, Reitherman and Barondes, 1975). The inhibitory potency of a range of simple sugars was established for each lectin, and Rosen's results indicate that this is a discriminable basis for identification of the lectins. In some cases, however, only slight differences were found between several species, most especially between D.discoideum and P.violaceum.

Several pieces of evidence indicate that the lectins are located on the cell surface. Firstly, intact amoebae can form rosettes with fixed erythrocytes, and this behaviour can be inhibited by the sugars specific for the appropriate lectin (Rosen, et al, 1973; Rosen et al, 1974; Chang et al,1977). Interestingly, this property was not observed with feeding vegetative cells, but the appearance of rosette formation occurred with the onset of aggregation-competence. Chang et al(1975), using immunofluorescence and immuno-ferritin labelling, showed that anti-discoidin antibodies bind to the surface of aggregating cells. Using similar methods, the cell surface location of pallidin has also been demonstrated (Chang et al, 1977). In this latter case, there was detectable lectin on the surface of vegetative cells, and these were also able to cohere to some degree in the presence of EDTA. However, as the cells became aggregation-competent both their cohesiveness and the detectable amount of lectin increased in parallel.

Lactoperoxidase iodination of intact D.discoideum cells (Siu, et al.1976) and subsequent electrophoresis of

cell extracts revealed that discoidin appeared on the cell surface at about 5 hours post-starvation and increased until around 9 hours of development. Aggregateless mutants tested showed either a reduced level or no lectin at all. These workers also found discoidin present on the surface of log phase axenic strain (A3) cells, which have also been shown to possess discoidin by Rosen, et al.(1973). They exhibit some degree of EDTA-insensitive cohesion. Hoffman and McMahon (1977) found discoidin in purified membranes from log phase and aggregating A3 cells, and showed that in the latter case the lectin was more pronase-sensitive. This suggests that in growing cells the lectin is less exposed on the cell surface.

Paralleling the appearance of the lectin is an increase in lectin receptors on the cell surface. Intact aggregation-competent P.pallidum cells could be agglutinated by pallidin and intact, fixed D.discoideum cells could be agglutinated by discoidin (Rosen et al.1974; Reitherman et al, 1975; Chang et al.1977). This agglutinability also increased from 0 to 9 hours of starvation, suggesting that either the discoidin receptors increased in number or were exteriorised during that time. (ConA and WGA were more effective in agglutinating vegetative cells than aggregation-competent cells.) Chang, et al.(1977) used ferritin-conjugated pallidin to demonstrate its binding to receptors on the surface of fixed cells. The staining was evenly distributed. These workers have also calculated the number of molecules of lectin bound per cell: in the case of discoidin,

4-5 x 10^5 molecules (9-hour cells) and pallidin, 4 x 10^9 molecules per cell. Although there is some interspecific agglutination by these lectins, each shows an association constant for the native species at least an order of magnitude higher than for the alien species.

Since these lectins are implicated to play a role in cell cohesion, inhibition of lectin activity should prevent cohesion. Rosen, et al. (1974) had found that simple sugar inhibitors had this effect, but only at very high concentrations, (0.1M). They employed an immunological approach by raising antibodies to the lectins and investigating the inhibitory effect of these univalent Fab fragments (Rosen, et al.1976; 1977). The results are somewhat difficult to interpret: Fab did inhibit cohesion of the cells at low levels, but only in the presence of high salt or sugar concentrations. Possibly another cohesion system is inhibited under these conditions, which works in conjunction with the lectin system. This suggests that the lectin system alone is not solely responsible for cell cohesion during aggregation.

The possibility that contact sites A and discoidin (or the discoidin/receptor complex) are contiguous is at present under investigation. The molecular weights are almost equal and the distribution of these two entities is identical. In addition, contact sites A have been shown to be associated with carbohydrate residues. Huesgen and Gerisch (1975) have partially purified CSA and found that this does not agglutinate formalinised sheep erythrocytes, as discoidin I does.

However, discoidin II will not agglutinate these cells either, so the possibility still remains that CSA and discoidin II are one and the same.

Sussman and Boschwitz (1975a,b) have demonstrated that the adhesive properties of D.discoideum cells are reflected in cell ghosts and using this system have shown a 15-20 fold increase in calcium-binding or manganese-binding sites as the cells progress from the vegetative to aggregation stage. The cations are highly resistant to removal by EDTA and since the cohesion observed under these conditions is ATP-independent and not metabolically-driven, Sussman has argued that cell cohesion in D.discoideum operates through a simple interaction between plasma membranes. The possibility that the binding component for Ca^{2+} or Mn^{2+} may be identical to discoidin (or presumably to CSA) is at present being investigated by these workers.

1.2.6. Differentiation, Pattern formation and sorting-out; their relation to cell cohesion in the cellular slime mould.

Differentiation in the slime moulds results in just two cell types — the spores and the stalk cells. When the fruiting body is fully-formed the spore mass is held aloft on a narrow conical stalk. This is preceded by the grex stage in which the prestalk and prespore cells are located in the anterior and posterior parts of the grex respectively (Bonner 1967; Garrod and Ashworth, 1973; Loomis 1975;

Hayashi and Takeuchi 1976; Forman and Garrod, 1977a). Two schools of thought prevail as to the mechanism of formation of this pattern. The first is that the cells differentiate in position in response to some 'positional information' (Wolpert, 1969, 1971). The grex tip is usually assigned the role of 'organiser' which signals by some means to the rest of the cells (Rubin and Robertson, 1975; McMahon, 1973; Pan et al, 1974). The second theory is that each cell has a predisposition at an early stage of development to become either a spore or stalk cell. Each cell then sorts out to its correct position by the grex stage (Takeuchi 1969; Bonner et al, 1971; Leach et al, 1973; Garrod and Ashworth, 1973; Garrod, 1974; Maeda and Maeda,1974). Recent evidence from Forman and Garrod (1977a/b) and Garrod and Forman (1977) supports the latter theory. Vegetative cells (Ax-2) were shaken in suspension and the resulting aggregates sectioned and stained with fluorescent anti-spore cell antibody at various times. Any prespore cells present contain prespore vesicles which fluoresce using this method. The important point here is that as far as can be determined the aggregates formed are non-polarised, i.e. they do not possess a region analogous to the grex tip. Nevertheless, differentiation does occur. Early on (12 hours) the prespore cells are randomly distributed throughout the cell mass. By 18-24 hours the prespore cells were localised in one region of the aggregate, which was still overtly non-polar. The authors interpret these results as indicating cell sorting out within the aggregate with respect to cell type.

This result has been subsequently supported in principle by Sternfield and Bonner,(1977) and by Takeuchi (1977). Two points here are of prime interest in relation to cell cohesion. First, the mechanism by which the differentiated cells become grouped within a cell mass has been suggested to depend on the different cohesive properties of two cell types (Steinberg, 1964). Since the prespore and prestalk cells do not separate completely, it must be assumed that sufficient cohesive properties are common to both types to maintain the integrity of the aggregate. According to Steinberg's "Differential Adhesion Hypothesis" (1964) the simplest explanation is that the cohesive sites on the cell surfaces are quantitatively different. Thus heterotypic cohesions could be intermediate in strength between the two kinds of homotypic cohesion. Clearly cell-cell recognition is of prime importance during pattern formation.
Second, if cell contact is blocked so that aggregates cannot form, differentiation does not ensue (Garrod and Forman, 1977; Forman and Garrod, 1977b). The cells do, however, become aggregation-competent. Furthermore, cohesion must be maintained if the cells are to retain their differentiated state. If prespore cells from the grex are dissociated they lose their prespore vesicles (Gregg and Radman, 1970; Gregg, 1971; Sakai and Takeuchi, 1971). Cell contact at aggregation also results in the accumulation of certain enzymes (Loomis, 1977). Several proteins cease to be synthesised, while production of many novel proteins then begins (Lodish, 1977). Dissociation of cells at any time

up to spore wall formation results in recapitulation of normal development from the aggregation stage, including accumulation of enzymes. If the cells are repeatedly dissociated, these enzymes will accumulate to two or three times the normal level following new RNA synthesis (Newell et al, 1971; 1972). Gross et al. (1977) has also provided evidence that formation of end-to-end contacts at aggregation stimulates UDPG pyrophosphorylase accumulation and accumulation of cell-associated cAMP phosphodiesterase ceases. However, the production of at least one developmentally regulated enzyme, alkaline phosphatase, can be initiated by pulsing cells lacking cell contact with cAMP (Rickenberg, et al, 1977). This may, therefore, be an alternative (or complementary) mechanism by which differentiation is regulated.

Sorting-out in slime moulds has also been observed on another level — species-specific patterning. Nicol and Garrod (1977) investigated species-specific cohesion in aggregates formed by suspensions of mixed slime mould cell types. It had previously been shown that cells of certain species, when mixed on a substratum, would either aggregate separately, or would separate at the grex stage. (Bonner and Adams, 1958; Raper and Thom, 1941; Shaffer, 1957a,b). The basis for this may be in different chemotactic guidance systems. Agglomeration in suspension precludes this possibility. Early agglomerates always contained both cell types, whatever the combinations of species used. In most cases, sorting-out within the agglomerates was

observed after some hours and in the case of D.mucoroides/ P.violaceum mixtures, aggregates of each cell type alone also formed. (Nicol and Garrod, 1977). This suggests that the cellular slime moulds as a group share at least some cohesive sites while differing in such a way that species-specific recognition and sorting-out is possible. This is supported by Rosen's work on native lectins (section 1.2.5.iii)which shows that although it is possible to discriminate between the lectins from different species there is some degree of overlap. Lectins can agglutinate cells of alien species albeit at a lower level. Thus cohesion in the cellular slime moulds is not entirely species-specific.

1.2.7. The cohesion inhibitor.

The work reported in this thesis consists of an investigation of an inhibitor of cell cohesion produced during growth of a population of axenic (Ax-2) cells. The biochemical nature of this inhibitor and its effects on the cohesion, adhesion, acquisition of aggregation-competence, development and differentiation of Ax-2 and cells of other strains and species are reported. The relationship of the inhibitor to the known cohesive mechanisms of the slime moulds, to the cell surface and morphology, and to other inhibitors reported in the literature is also discussed, and a programme for future investigation in this area proposed.

SECTION TWO:

MATERIALS AND METHODS.

2.1. CULTURE OF SLIME MOULD CELLS.

2.1.1. Axenic growth.

Cells of D.discoideum can be grown both axenically and in association with bacteria on a solid surface. Axenically-grown cells may be cultured in a simple broth, their growth rates have been well defined, and they can be obtained in large numbers.

Axenic medium of pH6.1 was prepared using the following ingredients: (Watts and Ashworth, 1970).

Yeast extract (Oxoid,Basingstoke)	7.15g/l
Bacteriological peptone (Oxoid)	14.30g/l
$Na_2HPO_4.12H_2O$	1.28g/l
KH_2PO_4	0.491g/l

60ml or 700ml aliquots were pipetted into 250ml or 2000ml Erlenmeyer flasks respectively and autoclaved.

Cells of the axenic strain (Ax-2) of D.discoideum were inoculated into the medium under sterile conditions and the cultures shaken on an orbital shaker at 140rpm.(radius of rotation 1.5cm; temperature 22°C). The medium was routinely supplemented with glucose to a final concentration of 86mM. Under these conditions the cells (G-cells) attained a final concentration of $1\text{-}2 \times 10^7$ per ml, with a generation time of 8-9 hours. Cells in unsupplemented medium (NS.cells) divide every 12-14 hours and attain stationary phase when the cell density is approximately 4×10^6 per ml. Log phase G-cells were harvested from the growth medium when they reached a density of $2\text{-}3 \times 10^6$ per ml. Stationary phase cells were harvested when the cell count remained the same

(within 10%) for two consecutive counts taken 24 hours apart. Harvesting was by centrifugation at 350g. for 5 mins at 4°C (or in the case of 700 ml volumes, at 1300g for 7.5 mins at 4°C). The cells were then washed with cold distilled water and with cold 17mM phosphate buffer before resuspending in the required medium.

2.1.2. Bacterially-associated growth.

During the course of this work, cells of four species of cellular slime mould were grown in association with bacteria. These were; D.discoideum (strain NC-4)
D.mucoroides
D.purpureum
Polysphondylium violaceum

3-4 spore heads were spread together with 0.2ml of 1-2 day old bacterial broth (Escherichia coli B/r) onto sterile nutrient agar plates which contained the following ingredients (Sussman, 1966):

Agar (Oxoid)	20.0g/l
Glucose	10.0g/l
Bacteriological peptone (Oxoid)	10.0g/l
Yeast extract (Oxoid)	1.0g/l
$MgSO_4.7H_2O$	2.06g/l
K_2HPO_4	1.0g/l
KH_2PO_4	1.5g/l

The inoculated plates were cultured in the dark at 22°C under humid conditions. The feeding vegetative cells cleared the bacteria in about 40 hours (except D.mucoroides which was considerably quicker) and began to aggregate at

about 48 hours. Cells were harvested at the required stage by rubbing the agar surface gently with a glass rod after flooding with 10ml cold distilled water. The resulting cell suspension was centrifuged and washed 3 to 6 times with cold distilled water until contaminating bacteria had been removed.

2.1.3. Development of cells on Millipore filters.

Cells which have been removed from their nutrient source and plated onto Millipore filters will undergo synchronous development in a series of well-defined stages. The timing of these developmental stages is predictable, conveniently enabling the harvest of large numbers of cells at a required stage. The timing of the sequence of stages can be found in section 6.2.1. After washing with cold distilled water Ax-2 cells were resuspended at $1x10^8$ cells per ml. The filters (Millipore Inc.France; black, 47mm diameter, 0.8 µm pore size) were laid on absorbent pads saturated with 1.6ml of buffer in 50mm plastic petri dishes and were inoculated with 0.5ml of the cell suspension. The buffer solution (pH6.1) contained:

KCL	1.5 g/l
$MgCl_2 6H_2O$	1.0 g/l
Streptomycin sulphate	0.5 g/l
$Na_2HPO_4.12H_2O$	0.3 g/l
KH_2PO_4	1.2 g/l

The dishes were incubated at 22°C in the dark under conditions of high humidity. Cells were harvested by

simply washing them from the filters with cold distilled water and concentrated by centrifugation. When the effects of the inhibitor were to be tested, freeze-dried inhibitory extract was dissolved in the Millipore buffer solution to a concentration equivalent, per cell, to that found in stationary phase medium.

2.1.4. Acquisition of aggregation-competence in shaken suspension.

Cells on Millipore filters will become aggregation-competent approximately 8 hours after removal from their nutrient source. This differentiative change can also be brought about by shaking the cells in buffer; Ax-2 cells were harvested, washed and suspended at a density of $1x10^7$ cells per ml in 17mM phosphate buffer (pH 6.0). 4ml aliquots of this suspension were shaken in 25ml Erlenmeyer flasks on a New Brunswick G86 rotary shaker at 140 r.p.m.(22^oC; radius of rotation 0.7cm). After 8 hours, the cells had become aggregation-competent and cohered into small aggregates.

2.1.5. Assay for aggregation-competence.

After shaking, the cells were washed, dissociated in cold distilled water, and resuspended in phosphate buffer at a concentration of $5x10^5$ cells per ml. A drop of this suspension was allowed to settle on a coverslip for up to 30 minutes. The coverslip was then inverted onto a slide. Aggregation-competent cells form a chain-like pattern of end-to-end contacts as they aggregate on the glass coverslip. Photomicrographs of this stage can

be found in section 5.1.2.

2.2. COHESION ASSAYS.

2.2.1. Assessment of cohesiveness during axenic growth.

The most accurate method of assessing the cohesion level of cells in suspension is to count the number of particles (single cells and cell aggregates) for a given initial cell number over a unit period of time. The increase in cell number during growth, however, precludes this possibility. In this instance the level of cohesion was assessed by expressing the number of particles as a percentage of total cell number. Thus in a single-cell suspension the value is 100%; this percentage drops when the cells cohere.

2.2.2. Assessment of cohesiveness in test situations.

(i) With constant initial cell number.

After harvesting and washing cells were suspended at a known concentration in test flasks (cf.2.1.4.) This concentration was usually $1x10^6$ cells per ml. This gives a convenient haemocytometer count of 100. The flasks were then shaken and in the ensuing samples the particle count was expressed as an absolute number. As such, this may be compared with any other set of readings.

(ii) With different initial cell number.

Since in one or two instances the initial cell counts differed, the sample values were expressed as a percentage of the original cell number. This facilitates comparison between batches. To ensure that this procedure

does not introduce an unacceptable level of inaccuracy into the method, the effect of initial cell concentration on subsequent cohesion levels under standard buffer conditions (17mM phosphate; pH 6.0) was tested. The initial cell concentrations were varied between 0.4×10^6 and 4×10^6 cells per ml, and samples taken at set intervals. The results of this can be found in section 3.2.1.(ii).

2.2.3. Assessment of inhibitory activity in column chromatography fractions.

Column chromatography presented a problem in that the large numbers of fractions were collected each of which had to undergo biological assay to locate the position of the inhibitory activity. Throughout the course of this work one of the biggest problems has been of sterility, or at least the avoidance of heavy bacterial growth in the solutions during the purification procedure. In part, this necessitated maintaining the samples in the frozen state as much as possible, avoiding frequent thawing and re-freezing. Chromatography fractions, therefore, had to be assayed as rapidly as possible whilst retaining maximum material for the next purification step. Accurate cell counting in individual flasks, as in the test assays, was thus abandoned. Instead, Perspex plates with a series of small wells drilled into them were used as counting chambers. Each circular well was 7mm in diameter and 3mm deep, comfortably holding the assay mixture which consisted of 0.08ml of test solution plus 0.02ml of cell suspension (at a concentration to give a final solution containing

approximately $1x10^6$ cells per ml). Each plate contained 20 wells, thus allowing a large number of fractions to be assayed simultaneously. The plate was fixed to the top of a 250ml flask which was then placed on the rotary shaker and shaken under standard conditions. After 20 minutes the plate was removed and the degree of cohesion was assessed by eye using a low-power binocular microscope. Active chromatographic fractions were then pooled for further purification.

2.2.4. Assay for adhesion to glass.

Feeding (preaggregation) stage cells of D.mucoroides, D.purpureum, D.discoideum (NC-4) and P.violaceum were harvested from agar plates, washed and suspended in inhibitor solution, or in buffer. Aliquots of these suspensions were then injected into a chamber formed between two glass cover slips supported on a Perspex slide (Fig.2.1.) and left for 30 minutes allowing the cells to settle and attach to the glass. Twenty randomly selected cell counts were made using a standard squared graticule. The slide was then inverted, left for a further 30 minutes and a further twenty counts were made of cells adhering to the glass.

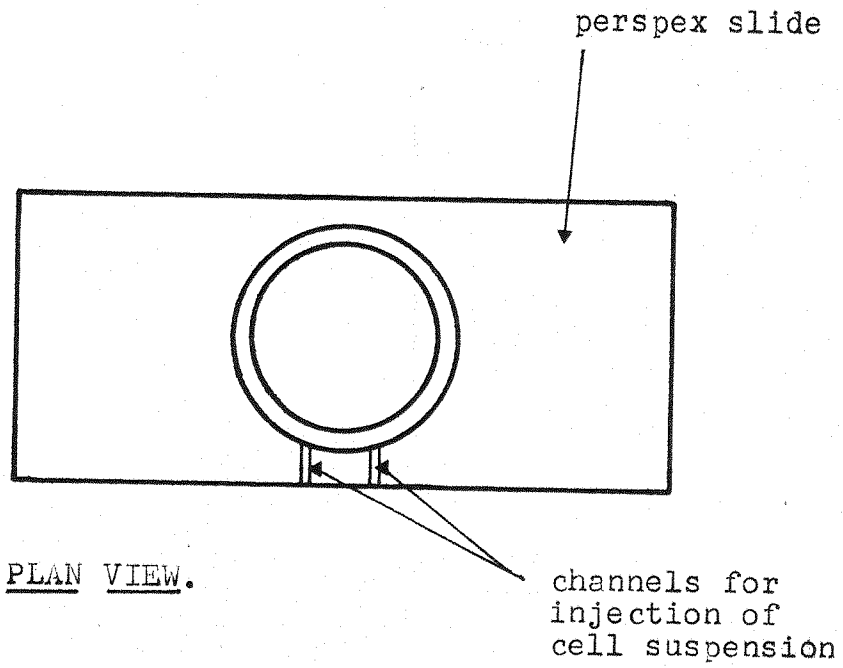

Fig.2.1. Chamber for assaying the adhesion of cells to glass

2.3. ISOLATION OF THE COHESION INHIBITOR FOR TESTS OF BIOLOGICAL ACTIVITY.

2.3.1. Dialysis.

Dialysis against four changes of distilled water at 4°C over 36h completely removes inhibitory activity from the medium. For routine preparation of partially-purified activity, 50ml or 200ml volumes of stationary phase medium were dialysed at 4°C for at least 18h against 500ml and 2000ml of distilled water respectively. The inhibitory activity was concentrated from the dialysate into a small volume (approx.5ml) by evaporation under reduced pressure using a Searle Rotary Evaporator.

2.3.2. Column Chromatography (Gel filtration)

Gel filtration provides a simple, quick and efficient method for separating mixtures of substances into fractions of similarly-sized molecules. Several gels are currently available; the type used in this work was the dextran gel Sephadex (Pharmacia, Uppsala, Sweden).

The gel is swollen in the eluting buffer and packed in a glass column.

The swollen Sephadex beads contain pores of a certain size depending upon the grade of Sephadex used. Sephadex itself is neither charged nor biologically reactive, therefore molecular size becomes the controlling factor in the separating process. The column is "loaded" by placing the mixture on the top of the gel bed and allowing it to soak into the gel bed. Eluant is then passed through the column.

Molecules larger than the pore size are unable to penetrate the gel particles and remain in the liquid phase between the beads. These molecules are, therefore, recovered first from the bottom of the column. Smaller molecules penetrate the gel particle to a greater or lesser degree depending on their relative size. The smallest molecules penetrate deepest and consequently are the slowest to be eluted from the column. A Sephadex column, therefore, behaves as a variable molecular sieve, with a distinct relationship between elution time (or volume if the elution rate remains constant) and molecular size. This attribute can be used to determine molecular weights (cf. section 2.4.1.)

The concentrated dialysis product was loaded on to a 100 x 2.6cm. chromatography column (Pharmacia) packed with Sephadex G-50 (Fine) and eluted with distilled water. The resulting fractions were assayed as described in section 2.2.3. and those containing activity were pooled, re-concentrated under vacuum to 3ml and applied to a 60x1.5cm column containing Sephadex G-10. This step effectively desalts the sample and removes contaminating small molecules. Active fractions were again pooled and reduced in volume to give a concentration of inhibitor equivalent to that in the original stationary phase medium. This was used for tests of biological activity. These steps were also performed during purification of the inhibitor. Further steps in this procedure follow in Sections 2.4.2. to 2.4.4.

2.4. PURIFICATION OF INHIBITOR FOR BIOCHEMICAL ANALYSIS.

2.4.1. Molecular weight estimation.

The method was essentially that of Andrews (1964). A 40x2.6cm column (Pharmacia) packed with Sephadex G-50 (Fine) was calibrated using a range of water-soluble substances of widely differing molecular weights. All were coloured enabling the peaks of the eluted substances to be assessed in the fractions by eye. The following substances were used:

Blue dextran	>2,000,000
Bovine serum albumin complexed with Evan's blue	60,000
Myoglobin	17,000
Cytochrome C	13,000
Vitamin B_{12}	1,350
Flavin Monophosphate	514
Phenol red	354

They were eluted with 10mM phosphate buffer pH7.0 in order of decreasing molecular weight, with a small volume of buffer between each to prevent mixing and possible interaction.

The elution volume of blue dextran (which is completely excluded from the gel) was taken as the void volume ($V_{\bar{o}}$). This was subtracted from the 'apparent' elution volumes ($V_{\bar{e}}$) of the standards to give the 'real' elution volume ($V_{\bar{e}} - V_{\bar{o}}$). These elution volumes were then plotted against the log molecular weight to give a calibration curve

for that column. Immediately the column had been calibrated, samples of partially-purified inhibitor (the concentrated dialysis product from 50mls of SPM) were applied to it. The fractions collected were assayed numerically under test conditions, and the fraction which contained the most activity was used for estimation of molecular weight from the calibration curve. This was repeated several times, as quickly as possible, and the mean of the elution volumes obtained was used for calculation. It was necessary to perform this operation quickly since it was found that if Sephadex G-50 is allowed to stand in a column for some time it will pack down and reduce the flow rate. Should this occur there is a high risk that elution volumes will be altered since a reduced flow rate introduces the possibility of inaccuracy due to diffusion.

2.4.2. Ion exchange chromatography.

Ion exchange matrices are composed of insoluble, non-reactive substances which bear charged groups and also mobile 'counter ions' of the opposite charge. Other ions of the opposite charge may be exchanged for the counter ions and become bound to the matrix as a result. Anion exchangers carry negative ions; cation exchangers exchange positive ions. The strength and capacity of an ion exchange matrix depends upon the type, number and accessibility of the charged groups. Separation can be effected by reversible adsorption. Different substances have differing ionic properties and by altering the pH

or the salt concentration of the eluting buffer it is possible to free a mixture of bound substances one at a time. At the end of the procedure the exchange matrix is 'regenerated' by treating with its normal counter ion.

In this work, two ion exchange matrices were used: QAE-Sephadex A-25 (amion exchanger) and SP-Sephadex C-25 (cation exchanger). These are prepared from Sephadex G-25 and are most suitable for small molecules (i.e. below 30,000 molecular weight). Such molecules are able to penetrate the gel particles where further charged groups are available to them. SP-Sephadex C-25 was swollen and equilibrated with 10mM acetate buffer (sodium acetate-acetic acid) at pH 5.0; QAE-Sephadex A-25 was equilibrated with 10mM Tris/Hydrochloric acid buffer at pH 8.0. Each ion exchange matrix was stirred into an excess of buffer and left to swell at room temperature for 2 days. The buffer was changed several times during this period. Ion exchange, however, facilitates separation of the inhibitor from any contaminating molecules bearing a charge (see section 4) and consequently it was decided to pass the extract through columns of both types of ion exchanger to remove negatively and positively charged contaminants. When swollen, each ion exchange matrix was packed into a 32cm x 1cm column. Samples of inhibitor were applied to the columns and eluted with the appropriate buffer. The fractions collected were assayed as described in section 2.2.3. Those containing activity were pooled and used for thin-layer chromatography.

2.4.3. Thin-layer chromatography.

Thin-layer chromatography (T.L.C.) using cellulose was employed to purify the cohesion inhibitor further from contaminants. Although paper chromatography (P.C.) enables larger quantities of test substances to be separated, TLC was chosen for the following reasons:

(i) Paper chromatography is a much lengthier process. The samples must be left to run for at least 24 hours, whereas development of ascending TLC plates can be completed in 3 - 6 hours.

(ii) Smaller samples can be used with TLC and, due to the shorter development time, the resulting spots are smaller and give a better reaction with locating reagents.

(iii) With the equipment available, more samples could be run concurrently. This is of particular importance when samples were run for comparative purposes.

(iv) Recovery of the sample by elution is less laborious. To retrieve the equivalent amount of sample from a paper system as from a single T.L.C. run, using the apparatus available, would require at least 4 runs of 24 hr. each.

(v) With such equipment limitations the use of T.L.C. represented a considerable saving in time and cost.

Separation was effected on aluminium plates, precoated with a 0.1mm cellulose layer (purchased in roll form from Merck, Darmstadt, Germany.). The separations were carried out in a sealed glass tank; the atmosphere in the tank

had previously been saturated with solvent by covering the bottom of the tank with solvent to a depth of 1.5 cm. for at least an hour. The evaporation of the solvent was aided by lining the tank with filter paper. The purpose of the saturated atmosphere is to minimise evaporation of solvent from the chromatogram surface which both slows the progress of the solvent front and distorts the separation of the components of the mixture being chromatographed.

The solvent system used was n-butanol: acetic acid: water in the proportions 12: 3: 5. (A sample was also separated using descending paper chromatography on Whatman No.1 chromatography paper using an ethyl acetate: pyridine: water (12: 5: 4) solvent system).

Samples were either spotted evenly or streaked from micro-pipettes along a line 2 cm. from the bottom of the plate. These spots were dried in a warm air draught from a hairdryer to prevent excessive spreading. The loaded plates were then clipped upright in the tank with the lower edge of the plate just immersed in the solvent. The plates were allowed to 'run' until the solvent front reached 5 cm. from the top of the plate.

The position of the cohesion inhibitor was located in the following way:

The inhibitor-containing fraction was streaked along the bottom of a TLC plate and run as previously described. When complete, the plate was dried and divided horizontally into a series of bands 1 cm. high. The cellulose thin-layer from each of these bands was then scraped off the

aluminium backing into a 15 ml centrifuge tube. Sufficient distilled water was added to the tubes to suspend the cellulose particles, and these mixtures were stirred vigorously and left for an hour for any separated substance to be eluted from the cellulose particles. The tubes were then centrifuged to clear the suspension of any light particles. The supernatant liquid was decanted off into freeze-drier tubes and lyophilised. In this form it can be stored indefinitely. The sample tubes were then individually assayed for inhibitory activity using the titre plate technique described in section 2.2.3 thus locating the position (band) the inhibitor had reached on the TLC plate.

In the experiments reported in section 4.2.3.(iii). designed to determine whether the cohesion inhibitor is a carbohydrate, the plates were calibrated by running known carbohydrates in this system. The separated carbohydrate spots were then located using a silver nitrate reagent.

On completion of a run, the TLC plates were allowed to dry in air at room temperature. They were then dipped into the silver nitrate reagent, quickly removed, and hung in the dark to dry. When dry, they were immersed in the methanolic potassium hydroxide solution until a satisfactory colour development occurred, then rinsed in tapwater, and immersed in sodium thiosulphate solution to clear the background. Finally, the plates were rewashed with tapwater and allowed to dry vertically at room temperature. The position of each reducing sugar was

indicated by brown/black spots.

The reagent and developing solutions consisted of the following:

(a) Silver nitrate reagent

1 volume of saturated silver nitrate solution was added to 200 volumes of acetone to form a white precipitate. Distilled water was added dropwise whilst stirring until the precipitate redissolved. This reagent must be prepared fresh before use, but the silver nitrate solution may be stored in the dark.

(b) Methanolic potassium hydroxide solution.

A 0.5% w/v solution was prepared by dissolving the appropriate amount of potassium hydroxide in a minimal quantity of distilled water, and diluting to the required volume with methanol. This must be prepared fresh before use.

(c) 2.5% w/v aqueous solution of sodium thiosulphate

2.4.4. Nuclear Magnetic Resonance; Mass Spectroscopy Gas/liquid Chromatography; Amino Acid Analysis.

A discussion of these techniques and their merits is included with the results in Section 4.2.3.

I am indebted to the following persons for their expertise in performing these tests:

Dr. D. Morris (Dept. of Physiology and Biochemistry) for the amino acid analysis.

Dr. D. Corina (Dept. of Physiology and Biochemistry) for the mass spectroscopy and gas/liquid chromatography.

Dr. D. Evans (Dept. of Chemistry) for the nuclear magnetic resonance studies.

2.5 SCANNING ELECTRON MICROSCOPY. (S.E.M.)

S.E.M. permits direct observation of the cell surface features. Cells were prepared by one of two methods, either by chemical fixation of cells in suspension, or by the freezing of cells on a solid substratum. Chemical fixation is marginally slower than fixation by freezing, and this may permit alteration of the surface features.

2.5.1 Glutaraldehyde/ethanol fixation.

This method was essentially that of Rossomando *et al*. (1974). Log and stationary phase cells were harvested, washed twice in lower pad solution (LPS) of the following composition:

0.04M phosphate buffer pH 6.4
plus 1.5 g/l KCl
0.5 g/l $MgCl_2 . 6H_2O$
0.5 g/l streptomycin sulphate

The cells were then suspended at $1x10^6$ per millilitre in LPS, or in LPS containing inhibitor at the concentration found in stationary phase. 50% glutaraldehyde was added to give a final concentration of 2.5% and the cells stored at 4^oC for 12 - 15 hours. They were then washed twice in LPS, resuspended in 50% ethanol (Analar), left for 3 hours at room temperature and subsequently resuspended in 70% ethanol (indefinite period). When required, the cells were taken through a graded alcohol series to 100% and then through a graded alcohol/amyl acetate series to 100% amyl

acetate. They were then dried by the critical point method and coated with palladium-gold on SEM stubs in a SEMPREP Vacuum Sputter Coater (Emscope Laboratories)

2.5.2. Rapid Freeze preparation.

This was a modification of the method of George, et al (1970), developed by Barlow (pers.comm.1977). Log phase axenic cells were harvested, washed and plated onto millipore filters (either with or without inhibitor) as described in section 2.1.3. After 4 hours' development the filters were cut into pieces about 5 mm square, placed in small chambers and plunged into liquid propane, pre-cooled to -196°C in a liquid nitrogen bath. The frozen filters were placed on dry ice blocks which lay on a bed of silica gel and transferred to the drying chamber of an Edwards EF03 freeze-drier. When drying was complete, the filters were attached to S.E.M. stubs by double-sided Sellotape and coated with gold-palladium as before. All stubs were examined on a Jeol (15 KV) P15 scanning Electron Microscope.

SECTION THREE:

CELL COHESION AND ITS INHIBITION IN AXENICALLY-GROWN *D. DISCOIDEUM* CELLS.

3.1. INTRODUCTION.

Ax-2 cells, grown in medium supplemented with 86mM glucose, divide on average every 8 hours and attain a final stationary phase concentration of $1\text{-}2\times10^7$ cells per ml (Fig.3.3.). When cells from log and stationary phase populations are mixed and allowed to develop on Millipore filters, they sort out so that log phase cells predominate in the spore heads, and stationary phase cells predominate in the stalks, of the final fruiting body (Leach, Ashworth and Garrod, 1973). This sorting out of mixtures of many cell types has been attributed to differences in cohesive properties between the cell types involved (Steinberg, 1964; Curtis, 1961). This stimulated the investigation by Swan and Garrod (1975) into the cohesiveness of the log and stationary cell populations. In this section, the results of this investigation are reported. The preliminary work suggested that the cohesiveness of log phase cells could be altered by stationary phase medium. The second part of this section, therefore, describes the examination of the properties of stationary phase medium and its effects on cell cohesion and the elimination of various factors which were possibly responsible.

Finally, during the course of this investigation, reports were published presenting evidence for the presence in stationary phase medium of one or more unidentified substances which inhibit cell division (Yarger, Stults and Soll, 1974; Hanish, 1975) and transcription (Yarger and Soll, 1975). The possibility that the described cohesion inhibitor and these division and transcription inhibitors were identical molecules was, therefore, tested.

3.2. RESULTS.

3.2.1. Cohesion of axenically-grown cells.

(i) The cohesive properties of log and stationary phase cells in buffer.

Log and stationary phase Ax-2 cells were suspended in 17mM phosphate buffer (pH 6.0) and shaken under standard assay conditions (cf. section 2.2.2.(i)). Particle counts were made at 10-minute intervals.

Log cells were found to be extremely cohesive. Fig.3.1 shows that the particle count for these cells decreased rapidly within the first 10 minutes. The cells aggregated into large clumps, and maximum aggregation had occurred within 20 minutes. After this time the cells either remained coherent to the same extent or disaggregated very slightly.

Stationary phase cells remained non-cohesive under the same assay conditions for at least the first 20-30 minutes of assay. Subsequently, they began to aggregate slowly (Fig.3.1). They finally attained a level of cohesion equal to that of log cells after approximately three hours.

(ii) The effect of initial cell density on subsequent cohesion levels.

Cohesion may be measured as the number of particles present after a unit period of time expressed as a percentage of the original cell number. This method has been described in Section 2.2.2 (ii). The use of percentage values can be considered unsound statistically because

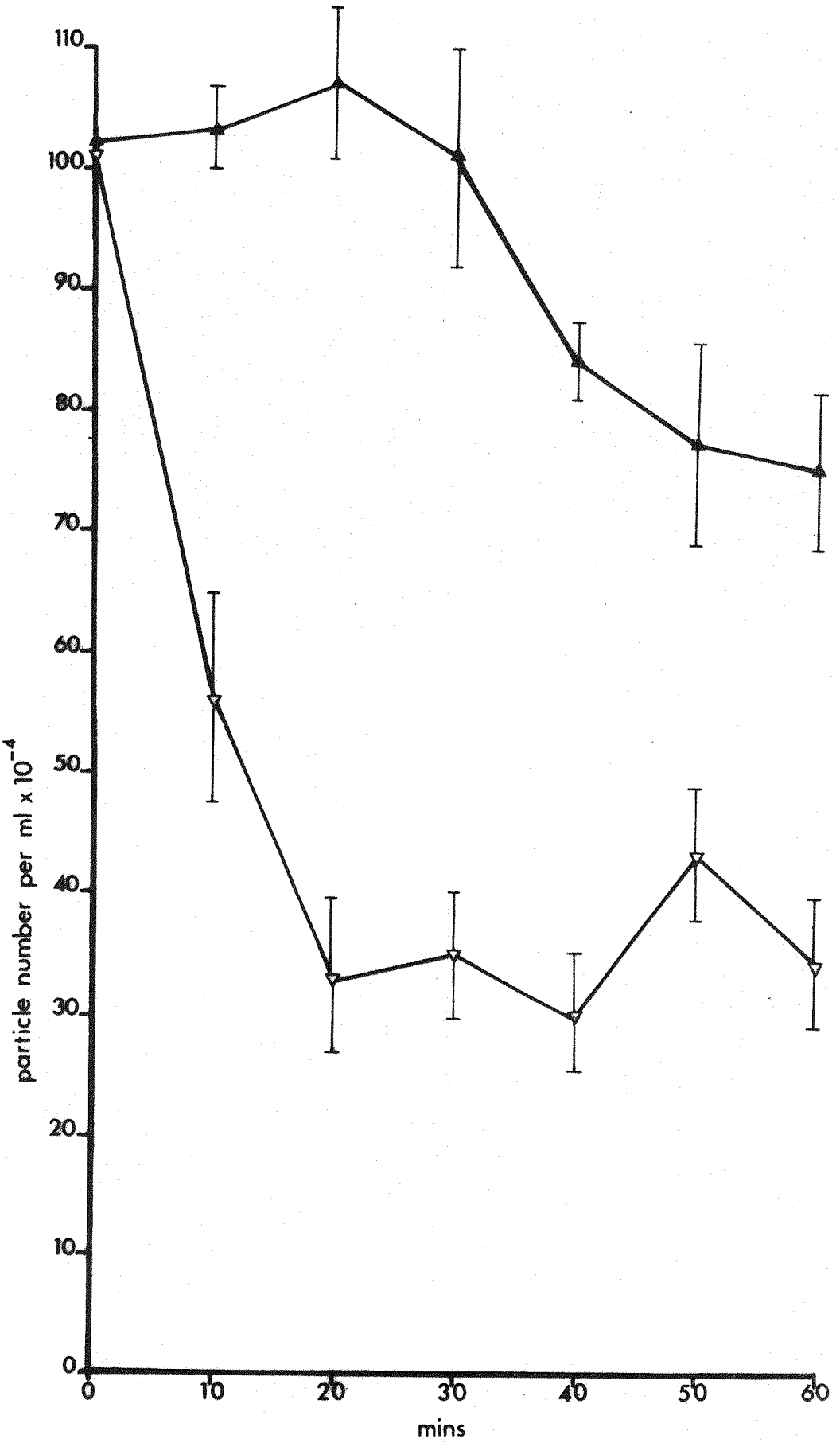

Fig.3.1. Cohesion of stationary (▲—▲) and log (▽—▽) phase cells in 17mM phosphate buffer

it represents a transformation and consequently skews the data. The data are no longer empirical but relative, making no allowance for the potential variable of initial cell number. For practical reasons the initial cell number cannot always be precisely adjusted to the standard concentration of $1x10^6$. It was, therefore, necessary to determine whether the initial cell number had any real effect on subsequent cohesion within the small range of initial cell densities employed throughout this work. The initial cell numbers used varied between $0.4x10^6$ and $4x10^6$ cells per ml. Particle counts were made after 10 minutes' shaking and plotted against initial cell density (Fig.3.2.) The result showed a linear relationship between the two parameters. Deviations from this line were small. Variations in initial cell density and the subsequent expression of cohesion in percentage terms are, therefore, acceptable.

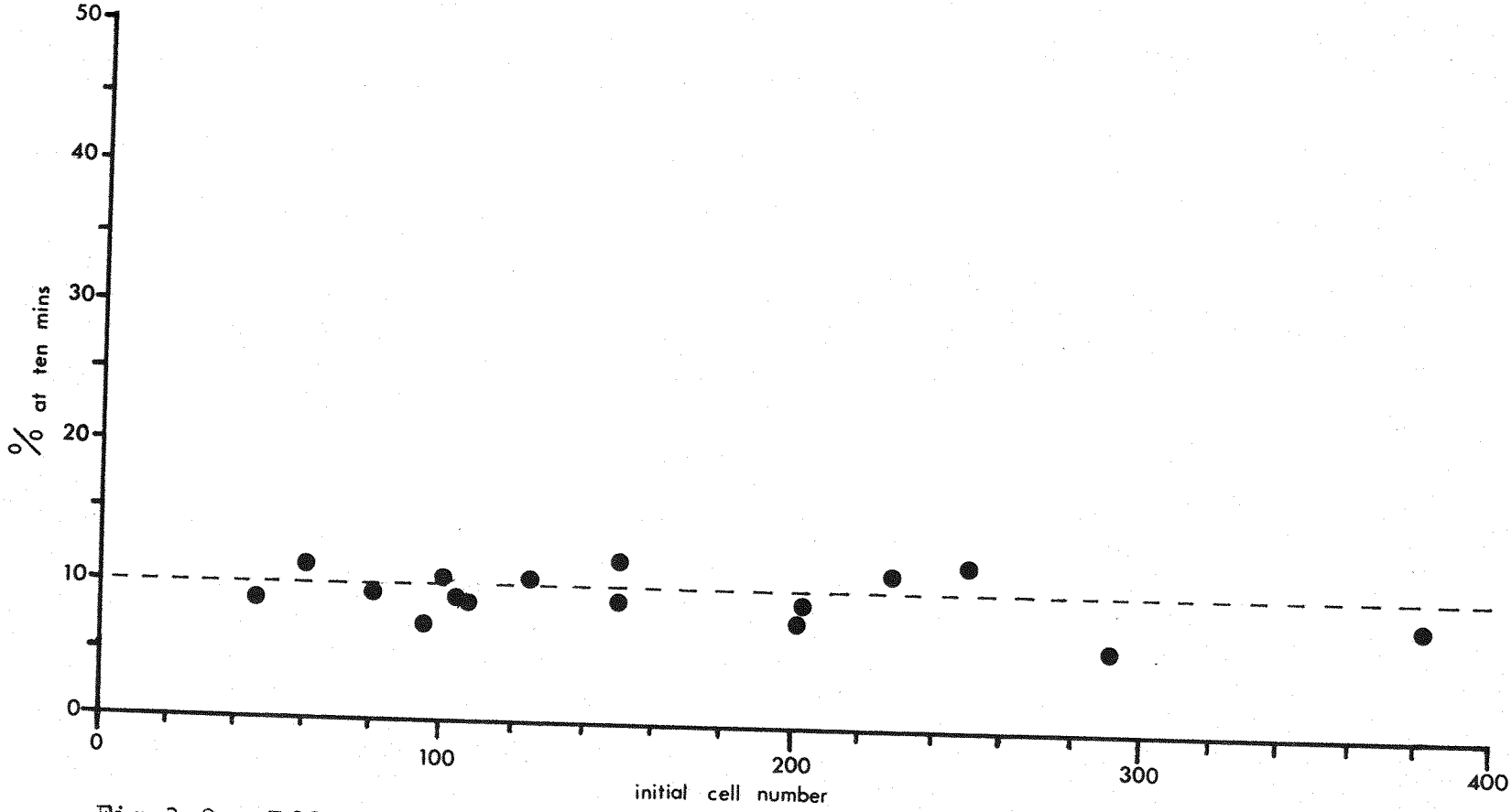

Fig.3.2. Effect of initial cell number on subsequent cohesion levels

(iii) Alterations in cohesiveness during axenic growth of a cell population.

Log and stationary phase cells exhibited different cohesive properties when shaken in buffer (3.2.1 (i)). Moreover, it was observed that during growth of a cell population, log phase cells tend to be aggregated in small groups whereas stationary cells exist entirely singly. To investigate this more closely, the growth curve and simultaneous cohesion curve were plotted for Ax-2 cell populations (Fig.3.3.). For the cohesion curve the number of particles is expressed as a percentage of total cell number.

Fig.3.3. shows that early- and mid-log phase cells tend to be cohesive. They may form clumps of up to 6 or 7 cells. This cohesiveness decreases through log phase and as a cell population approaches the stationary phase of growth the cells lose this property completely. By the onset of stationary phase they exist entirely singly in the medium, the particle count rising to 100% of the cell count. They remain in this non-cohesive state for as long as the culture is maintained.

(iv) Reversibility of the loss of cohesiveness in stationary phase cells.

Non-cohesive stationary phase cells will resume cell division when inoculated into fresh nutrient axenic medium. It was, therefore, interesting to determine whether the cohesive properties of these cells were also regained as they resumed division. The growth, and simultaneous

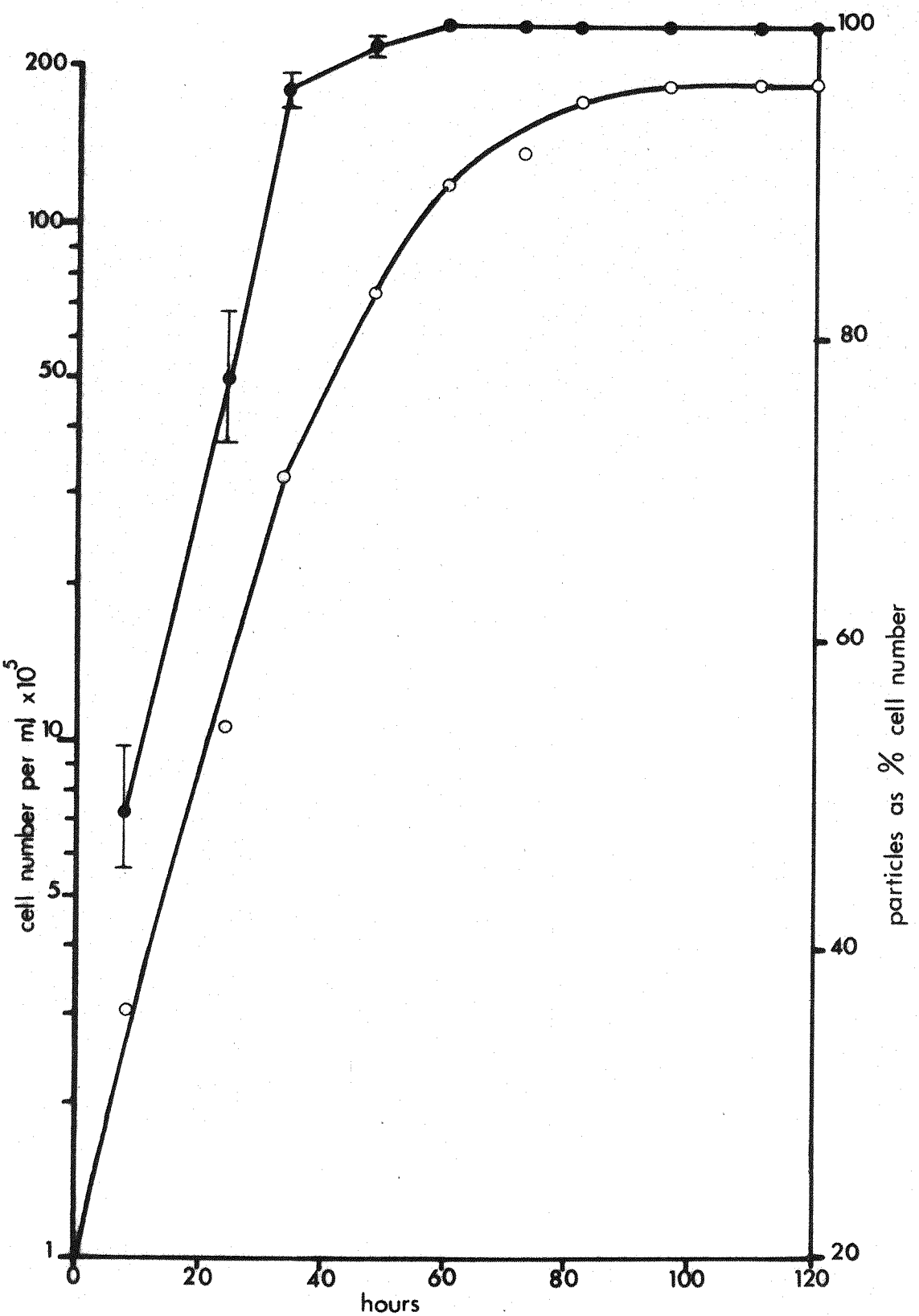

Fig. 3.3. Cohesion levels (●—●) during growth (○—○) of Ax-2 cell populations

cohesive properties of populations of stationary cells inoculated into fresh medium were monitored over the first 15 hours of shaking culture. The results are shown in Fig.3.4. The development of cohesiveness becomes evident after a lag period of about 3 hours. The particle number as a percentage of the total cell number continues to decrease and by 7-8 hours the population has attained a level of cohesiveness comparable to that of normal log phase cells. Since division also suffers a lag period the cell number has not doubled by this time. Cohesive properties are, therefore, regained well within one generation time. Thus, in addition to the resumption of growth, the loss of cohesiveness exhibited by stationary phase cells appears to be readily reversible.

3.2.2. Alterations in the cohesiveness of log cells: evidence for a cohesion inhibitor.

(i) Cohesion of log cells in buffer: a baseline.

It was necessary to establish a baseline for log cell cohesion in phosphate buffer. This would then allow the comparison of test solutions in future assays.

Log cell cohesion was monitored in 17mM phosphate buffer (pH 6.0) under standard assay conditions. The baseline produced is shown in Fig.3.5. The error bars represent ± 1 standard error. Each mean value comprises data from 37 independent observations obtained over a period of several months. This curve is used throughout the text as a control curve.

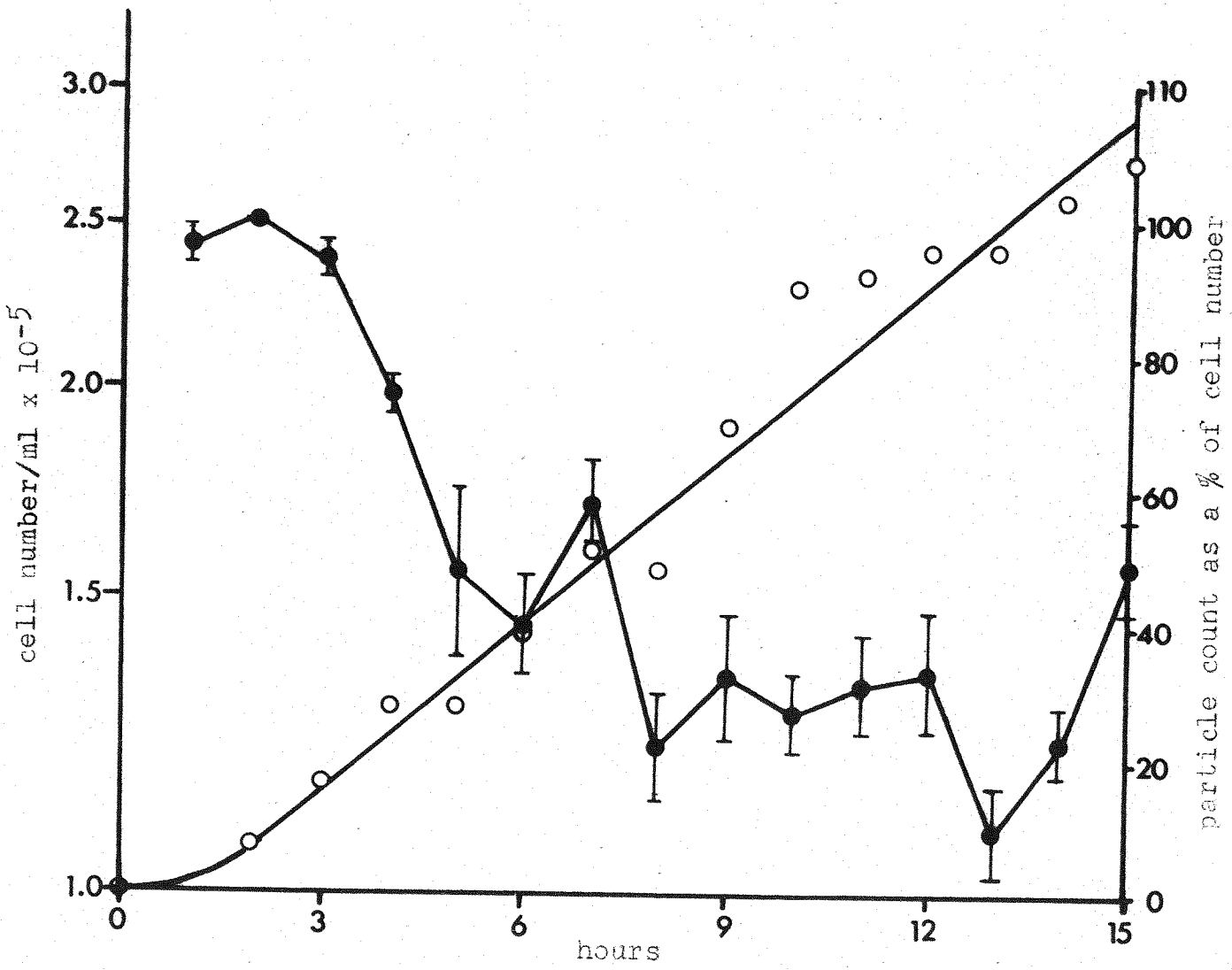

Fig.3.4. Growth (○—○) and cohesion (●—●) of stationary cells reinoculated into fresh axenic medium.

Log cells cohere almost immediately upon shaking to form large loosely-packed clumps (comprising up to 30 cells). A plateau level of cohesion was attained after only 10-20 minutes.

(ii) Cohesion of log cells in stationary phase medium.

In order to determine the effect of stationary phase medium (SPM) on the cohesion of log phase cells, freshly-harvested cells were suspended in cell-free SPM. The suspensions were placed on the rotary shaker and cohesion was monitored as described in section 2.2.2. The results are given in Fig.3.5.

Log phase cells cohered to the normal degree in the phosphate buffer control. Before harvesting, the log cells had been cohesive whilst in the growth medium (the particle count had averaged 70% of the total cell count). Stationary phase medium, however, rendered these cells completely non-cohesive. The particle count throughout the course of the assay remained at 100% of the total cell count. No other effects on the log cells were apparent: cell lysis did not occur to any detectable extent since the cell count remained steady over 60 minutes, and there was no observable atrophy or hypertrophy of individual cells.

(iii) Cohesion of log cells in fresh axenic medium.

As a control for the previous experiment, log phase cell cohesion was assessed in freshly-prepared autoclaved axenic medium. The result is presented graphically in Fig.3.6. Cohesion levels in axenic medium differed almost negligibly from those in buffer alone. The inhibitory activity apparent in stationary phase medium, therefore, appeared to accumulate in the medium during the growth of a cell population. By 24 hours into stationary phase

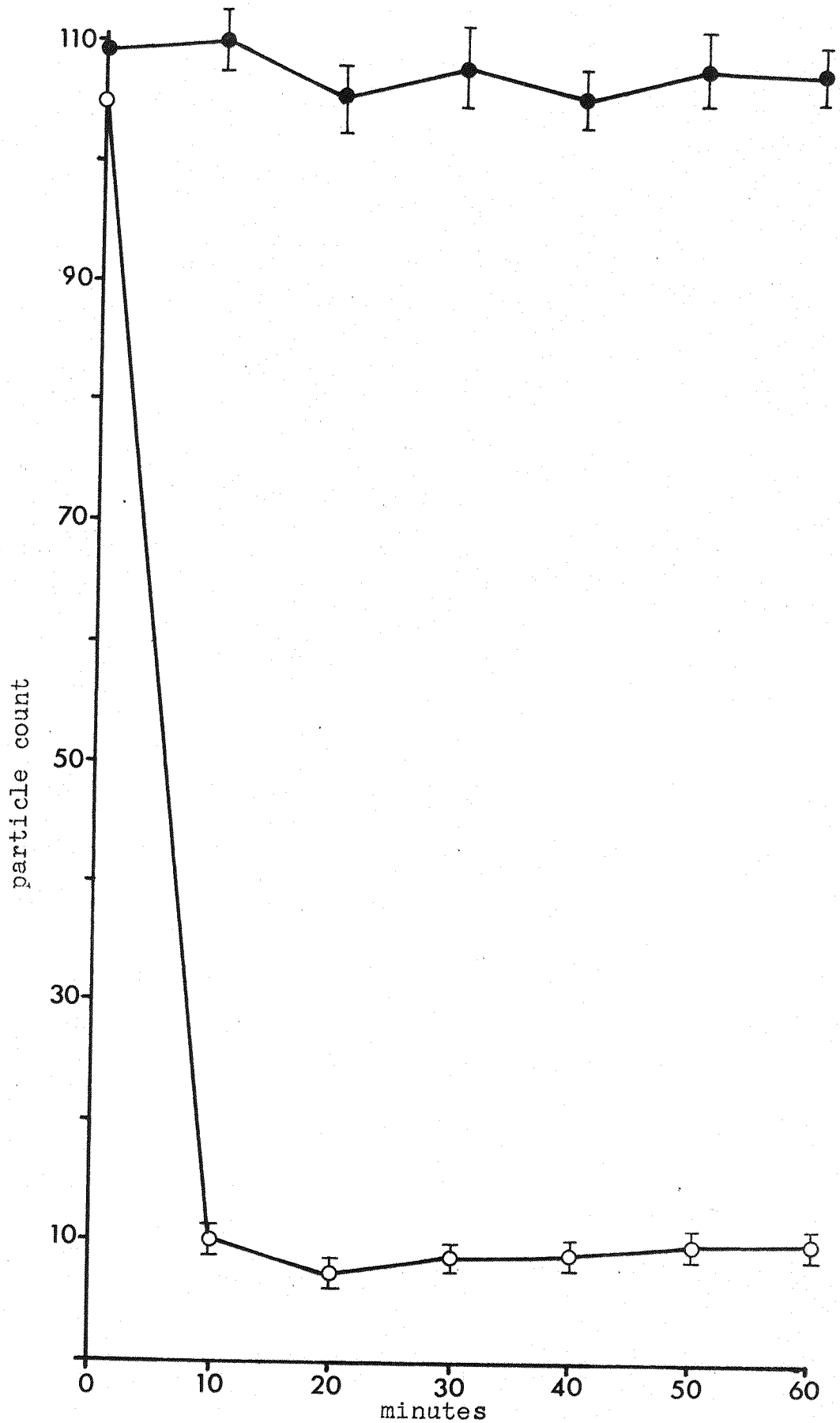

Fig.3.5. Cohesion of log phase cells in SPM(●—●) and in phosphate buffer(○—○).

(routine harvesting point) the level of inhibitory activity had reached a concentration sufficient to completely prevent cohesion between normally cohesive log phase cells.

(iv) Cohesion of log cells in axenic medium plus glucose.

To facilitate a faster growth rate, cultures of axenically-grown cells are routinely supplemented with D-glucose to a final concentration of 86mM. Simple sugars have been reported to inhibit cell cohesion in suspension (Weeks and Weeks, 1975) and block development on Millipore filters (Rickenberg, et al.1975) as well as inhibiting the activity of native slime mould lectins (Rosen, et al.1975; cf. 1.2.5.(iii). This added glucose may, therefore, have been responsible for the inhibitory effects of SPM. The investigation of the effect of glucose on Ax-2 cells was approached in three ways: first, cohesion of log phase cells was assessed in axenic medium plus the normal concentration (86 mM) glucose. The results of this assay are shown in Fig.3.6. In comparison with axenic medium alone, the supplemented medium was able to inhibit log cell cohesion to an appreciable extent. This level of inhibition is similar to that observed during the early log phase of growth. It does not, however, approach the complete inhibition afforded by stationary phase medium. 86mM can reasonably be expected to be the highest concentration of glucose found in the medium during growth of a cell population, since this hexose is gradually metabolised by the dividing cells,

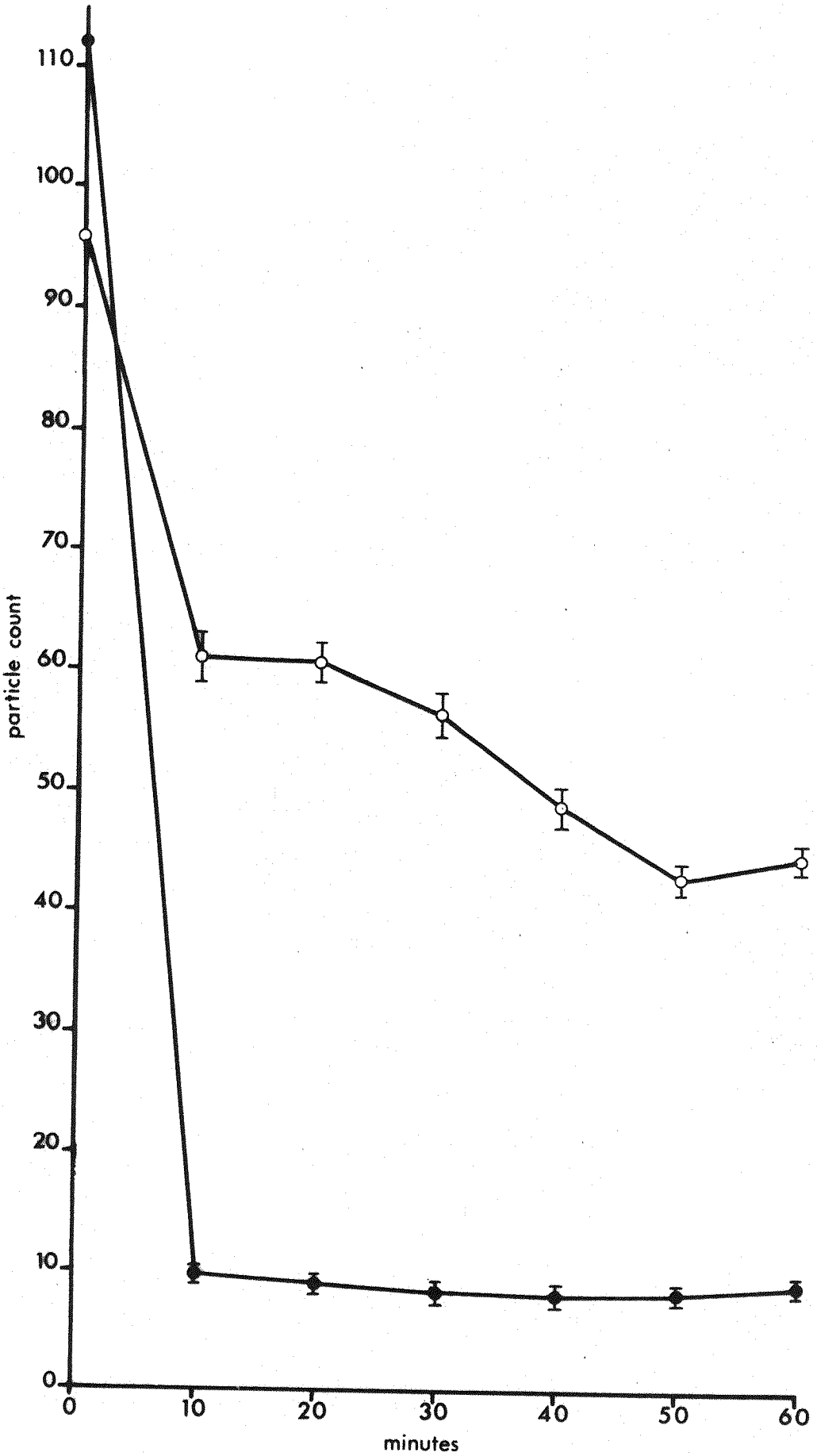

Fig. 3.6. Cohesion of log cells in axenic medium with (○—○) and without (●—●) glucose (86mM)

facilitating a faster division rate. It is unlikely, therefore, that glucose is responsible for the inhibition of cell cohesion by stationary phase medium.

The second approach in the investigation of the effect of glucose on Ax-2 cells was to examine log cell cohesion in a solution of glucose in phosphate buffer; and third, the effect of glucose on the development of D.discoideum cells on Millipore filters was examined. Glucose did not mimic the inhibitory effect of SPM in either of these two systems. The full results from these two approaches are reported in sections 4.2.2. and 6.2.1.(iii) respectively.

(v) The effect of stationary phase medium on log cell cohesion in phosphate buffer.

The previous results strongly suggest the presence of an inhibitor of cell cohesion in stationary phase medium. The ability of this medium to inhibit the cohesion of log phase cells in phosphate buffer was therefore tested.

Cells were suspended in 17mM phosphate buffer to which varying amounts of SPM had been added and cohesion assessed. The results are presented in Fig.3.7.

It was found that the addition of stationary phase medium could inhibit the agglomeration of log phase cells in this buffer. Increasing the proportion of SPM resulted in decreased cohesion. Even at only 10% strength, SPM was able to cause a small amount of inhibition of cohesion.

In a control experiment, log cell cohesion was assayed in phosphate buffer to which had been added fresh axenic medium plus 86mM glucose, (75% medium: 25% buffer). In this instance the degree of inhibition of cohesion was negligible (Fig.3.8).

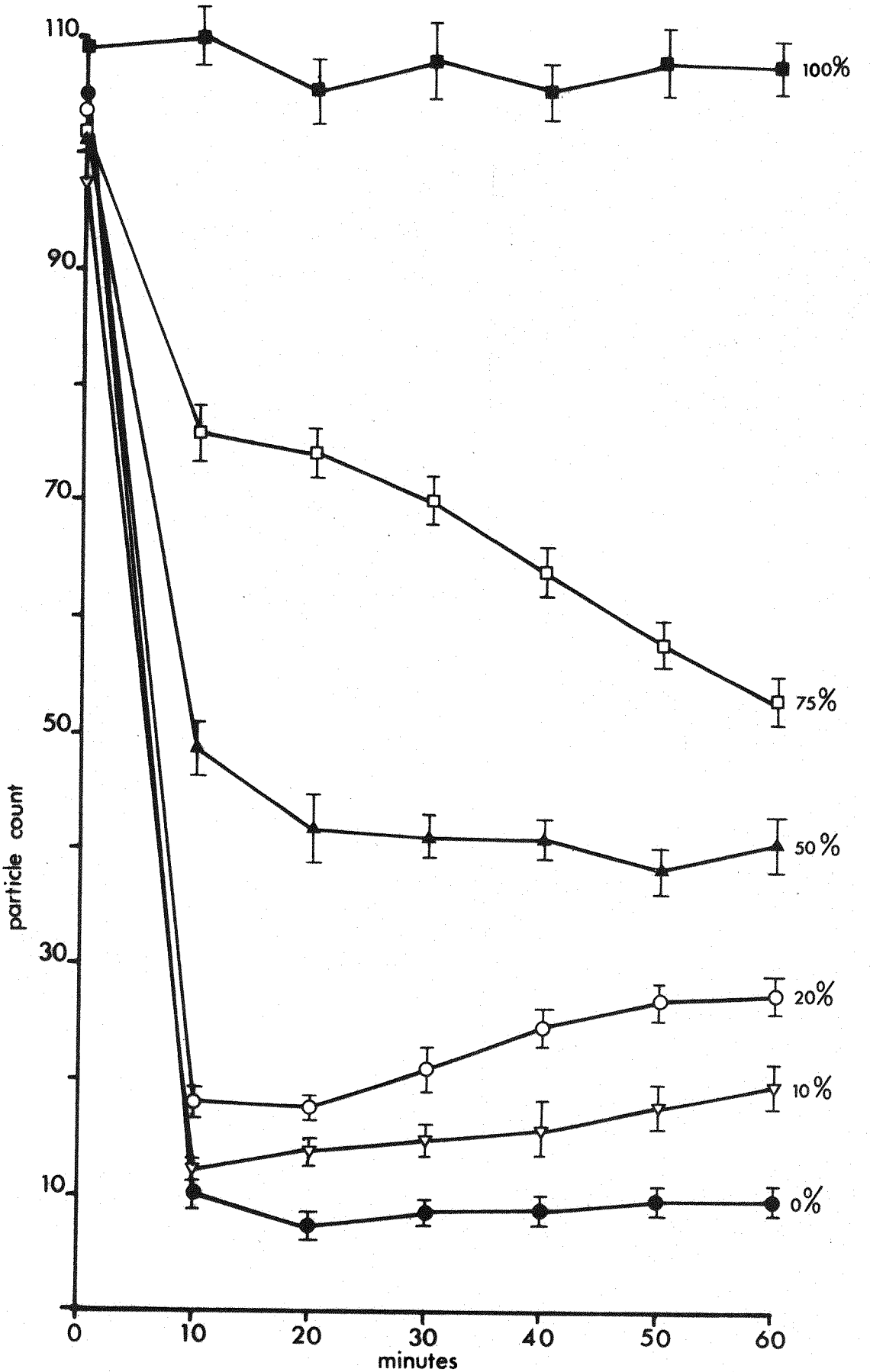

Fig.3.7 Cohesion of log cells in a range of concentrations of SPM diluted with phosphate buffer

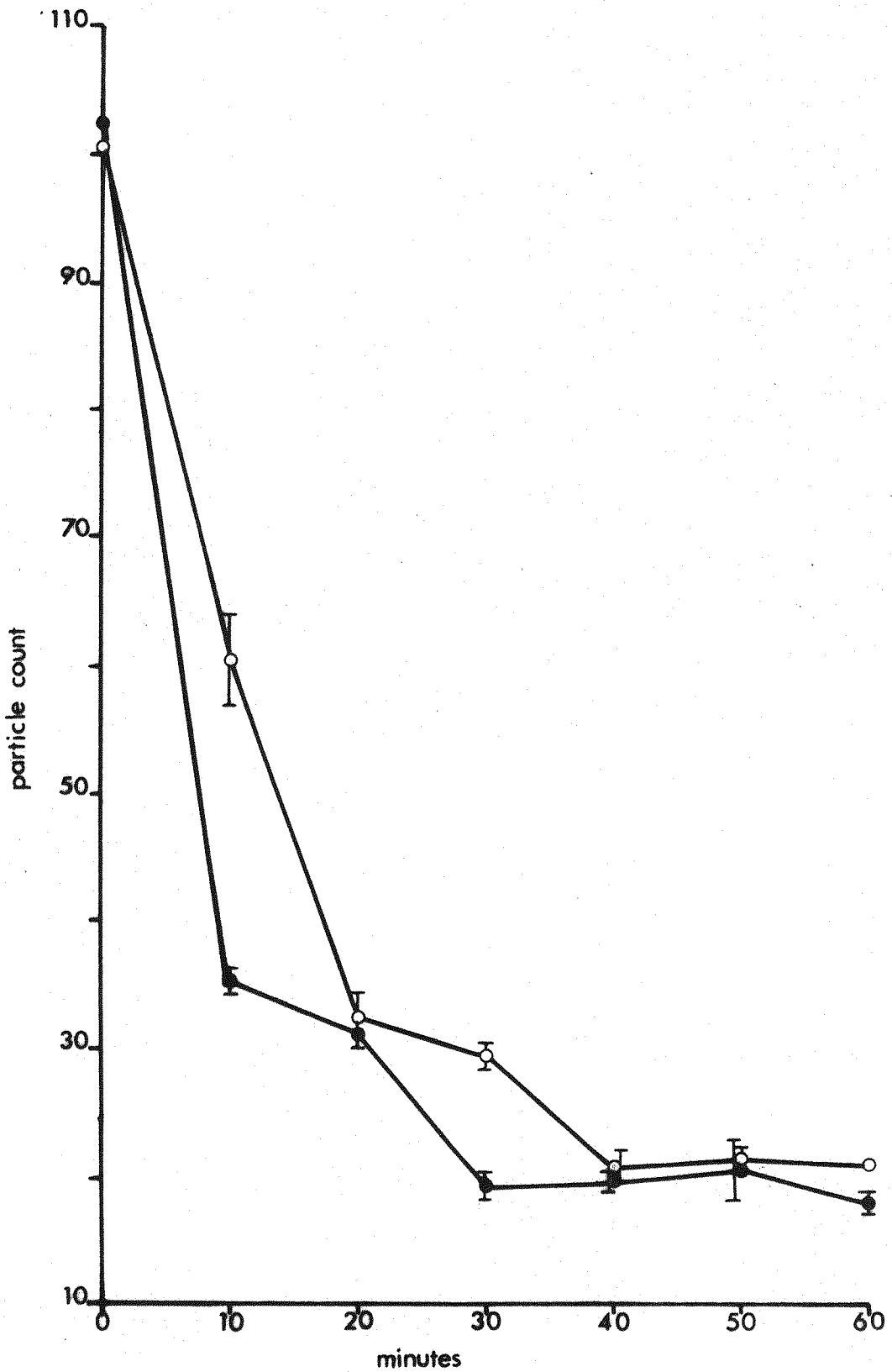

Fig. 3.8. Cohesion of log cells in 75% axenic medium (o—o) and 17mM phosphate buffer (●—●)

Normal growth medium does not, therefore, affect the cohesion of log cells in phosphate buffer. The addition of even small quantities of SPM, however, to buffer, will inhibit the cohesion of log cells in this buffer.

(vi) The effect of pH on log cell cohesion.

Yanagida and Noda (1967) had previously reported that extremes of pH diminished the degree of cohesion of vegetative, bacterially-grown slime mould cells. In buffers of pH below 5.0 and above 8.0, cohesion levels decreased dramatically. Maximal cohesion occurred in phosphate buffer at a pH of about 6.5.

When the pH of freshly prepared axenic medium and stationary phase medium were tested, a large discrepancy was discovered. The pH of freshly-prepared, autoclaved axenic medium was 6.1, whilst that of stationary phase medium was around 8.7. It was, therefore, necessary to eliminate the possibility that this pH difference alone could account for the inhibitory effect of SPM. In order to do this, the extent to which pH affected the cohesion levels of Ax-2 cells was determined.

The cohesion of log cells was, therefore, monitored in buffers of different pH values, varying between 6.0 and 9.0. The results are shown in Fig.3.9.

The differences in the degree of inhibition of cohesion in buffers of pH6.0 to pH8.5 were very small. At pH9.0 there was an increase in inhibition. This increase, though, was in no way comparable with the degree of inhibition afforded by stationary phase medium at a pH of 8.7.

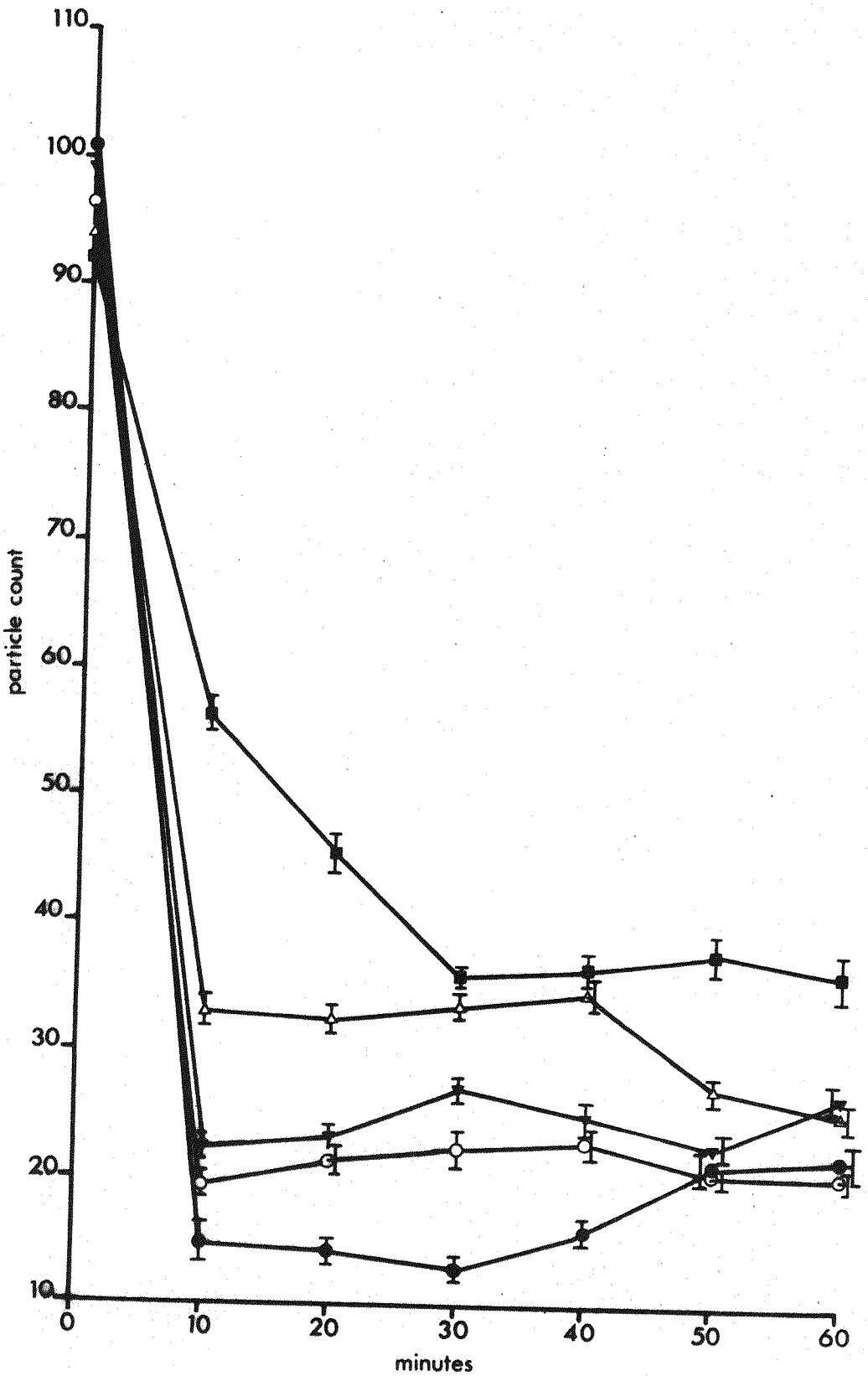

Fig. 3.9 Cohesion of log cells in buffers of varying pH values: 7.0 (●—●); 7.5 (○—○); 8.0 (▼—▼); 8.5 (△—△) 9.0 (■—■)

It must be concluded, therefore, that the inhibitory effect of SPM was not directly due to the increase in pH which occurs in the medium during growth.

(vii) The reversibility of inhibition.

It was necessary to determine whether the inhibitory effect of SPM on log cell cohesion could be reversed. This was important in order to answer certain questions.

First, was the inhibition causing permanent cell damage or even cell death? Conceivably, the observed effects could be the result of cell damage caused by an entirely separate agent. An alteration of cell cohesion could simply be a symptom of such an agent's action. Second, if inhibition is due to a substance(s) in the medium, is this responsible for reducing the cohesiveness of stationary phase cells, i.e. can this 'inhibitor' alter log cells into stationary cells?

Log cells were shaken in stationary phase medium for varying lengths of time and then removed and suspended in phosphate buffer. Their cohesion in this buffer was assessed.

It was found that their subsequent cohesion in buffer was unaffected, in rate or degree, by pretreatments of up to 8 hours in SPM. As soon as the log cells were placed in buffer, normal cohesive behaviour was resumed. Therefore, in whichever way the 'inhibitor' was affecting the cohesive system of these cells it was doing so completely reversibly. It did not have any long-term effect on the cells' cohesive properties. It also appeared to have no detrimental effects on the cells although the ability of

these cells to resume division was not tested. After 8 hours' incubation (and indeed a lot longer: cf.6.2.4.) the cells in SPM still appeared healthy and cell lysis was minimal. A Trypan blue exclusion test revealed only 2% of the cell population were dead after 8 hours in SPM.

(viii) The accumulation of inhibitory activity during axenic growth.

Inhibitory activity is not present in freshly-prepared axenic medium (cf.3.2.2.(iii)). Medium which has supported a population of stationary cells for 24 hours will completely inhibit log cell cohesion (cf. 3.2.2.(v)). It, therefore, seemed likely that this activity was accumulating in the medium during the growth of a cell population. The point at which the cohesion inhibitor began to accumulate was not known. To determine this, samples of medium were taken at intervals during the growth of populations of axenic cells. The cells were removed from these samples by centrifugation, and the cell-free medium frozen and stored until required. The ability of each of these samples to inhibit log cell cohesion was assayed. The results are presented in Fig.3.10. It can be seen that medium from early log phase can inhibit cohesion to some degree, (particle count approximately 50% of total cell number). This can probably be ascribed to the glucose present at this stage (compare with Fig.3.6.) Towards the mid- and late-log phases the level of inhibition decreases somewhat. This is probably due to depletion of the glucose by cellular metabolism. As the cell population begins to approach stationary phase the level of inhibition starts to rise slowly. This probably indicates the onset of production of the inhibitor. As the length

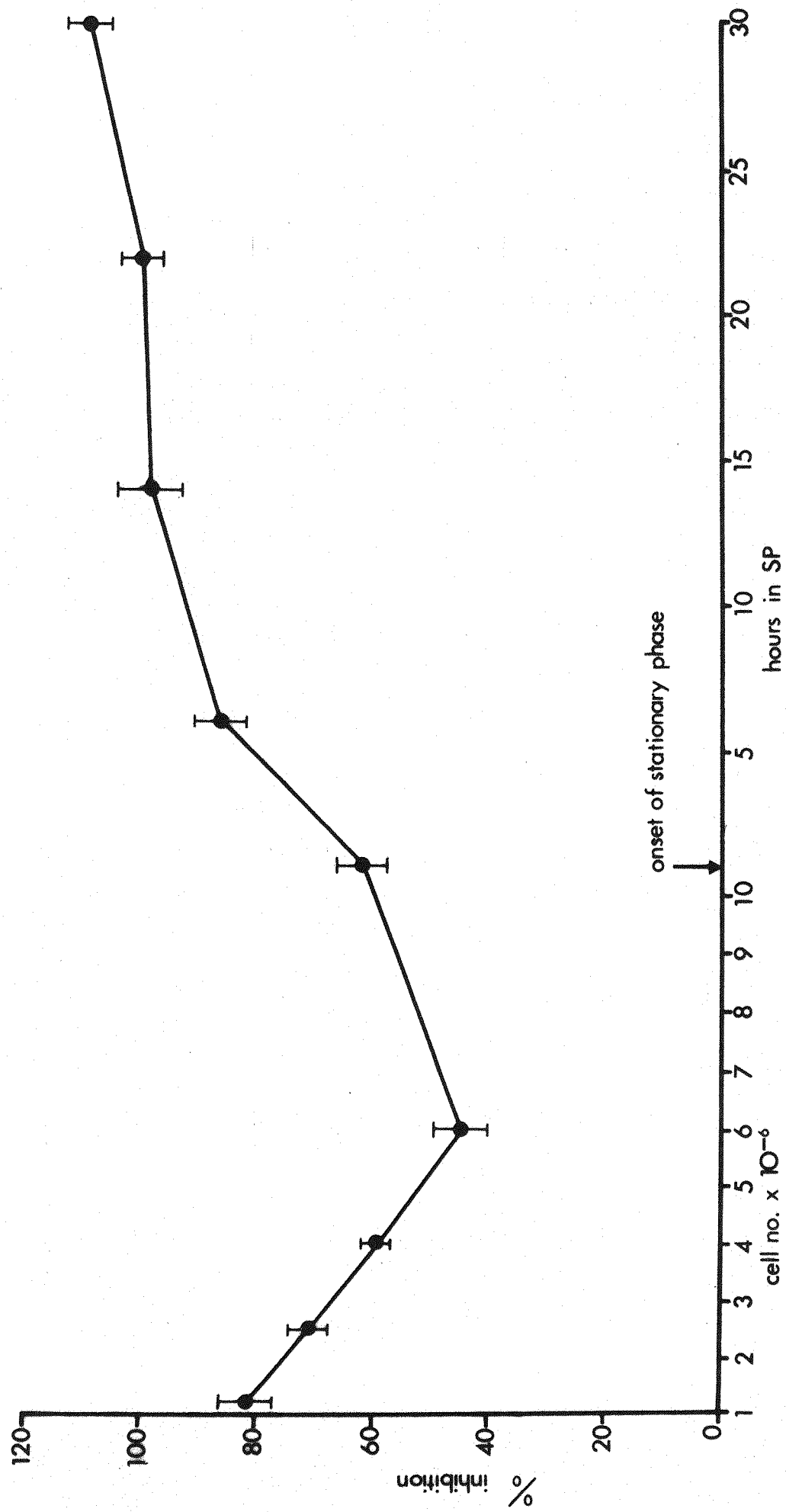

Fig. 3.10 Accumulation of inhibitor during population growth

of time in stationary phase increases the ability of the medium to inhibit log cell cohesion also increases. After 20-24 hours in stationary phase, 100% level of inhibition is attained. In the light of these findings it was somewhat fortuitous that the criterion of a stationary phase population was initially established as one in which the cell count had remained steady for 24 hours. The accumulation of inhibitor earlier in stationary phase is very low and, as such, less easily detectable.

(ix) The effect of the cohesion inhibitor on population growth.

Reports in the literature had presented evidence for inhibitors of cell division (Yarger, Stults and Soll, 1974; Hanish, 1975) and of transcription (Yarger and Soll, 1975), produced by D.discoideum Ax-3 cells. These cells began production of the inhibitors as they entered stationary phase. Yarger and Soll (1975) had not distinguished whether the division and transcription inhibitors were separate molecules or identical substances.

The cohesion inhibitor resembles these other inhibitors in its onset of production and its build-up during stationary phase. It was, therefore, important to try to distinguish between it and the division/transcription inhibitors.

In order to do this, freeze-dried inhibitory extract from SPM (see section 2.3.) was redissolved in a small quantity of sterile distilled water. The solution was then sterilised by exposure to ultraviolet irradiation for one hour.

Sufficient amounts of this sterile extract, along with 500 units/ml penicillin/streptomycin (Flow Laboratories,

Scotland) were added to 4ml volumes of log phase cell cultures to bring the final concentration of inhibitor in the cultures to the level found in stationary phase. The growth curves of the cultures were plotted both before and after addition of the inhibitor. The results are shown in Fig.3.11.

Clearly, addition of the cohesion inhibitor had no effect on cell division (and presumably transcription). The 'inhibited' cultures show no deviation from the growth rate of the control cultures after addition of the cohesion inhibitor. However, inhibition of cohesion in these cultures was noticeable. It thus appears that the cohesion inhibitor does not affect the rate of cell division and, therefore, that the cohesion and division/transcription inhibitors are separate molecules. In addition, it also appears that Ax-2 cells can continue to grow and divide normally despite the fact that they are non-cohesive (i.e. that with regard to their cohesive properties they are now equivalent to stationary cells).

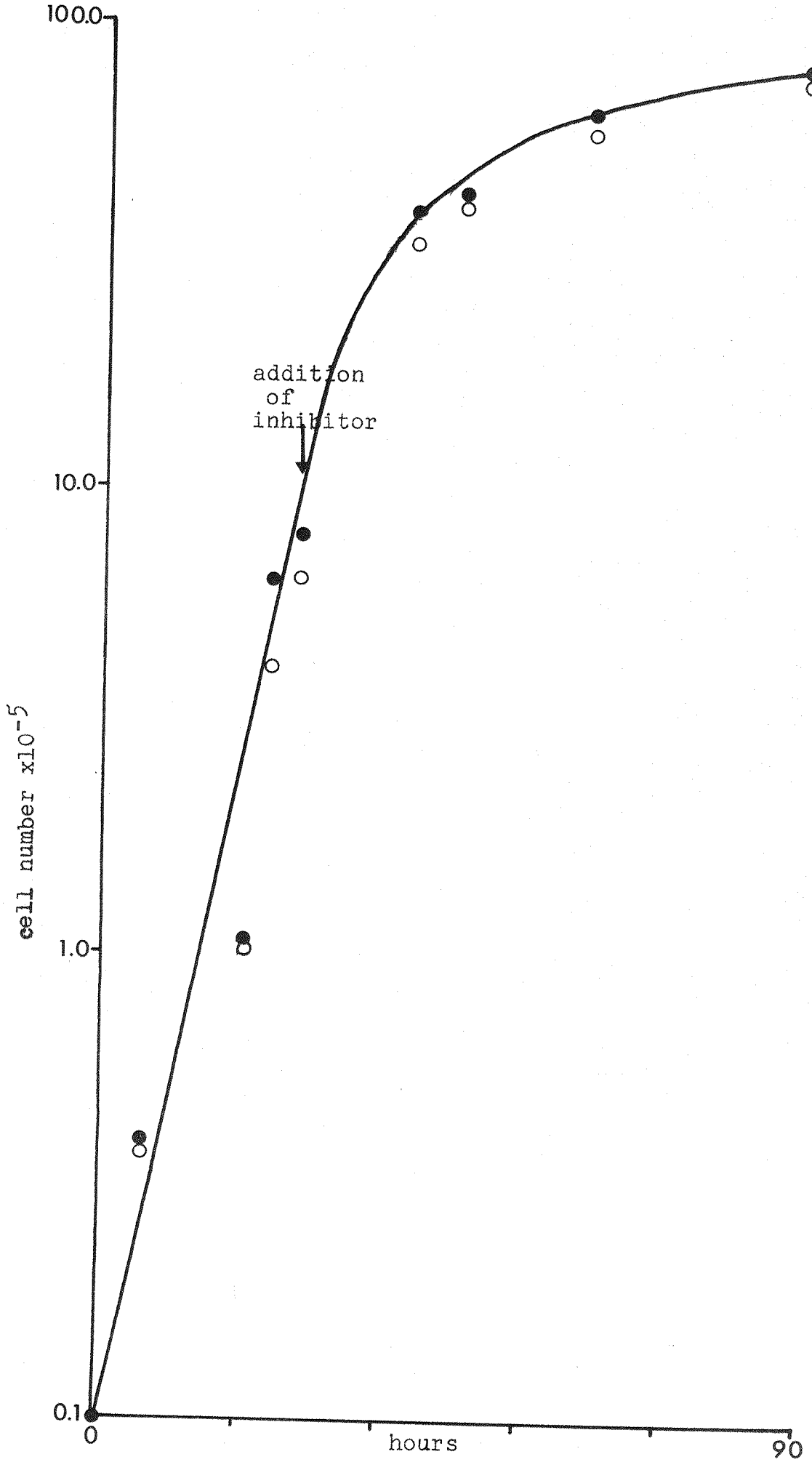

Fig.3.11. Effect of the inhibitor on the growth of Ax-2 cells (O—O). Control population (●—●).

3.3. DISCUSSION.

This preliminary investigation revealed information on two main fronts: first, the cohesive properties of log phase and stationary phase cells, their differences, and how the cohesive and non-cohesive phases can be manipulated. Second, the overwhelming weight of evidence at the end of this investigation points to the presence in stationary phase medium of an inhibitor of cell cohesion. The factors thought to be possibly responsible at this stage have been eliminated by experiment.

Log phase and stationary phase cells have vastly different cohesion levels in phosphate buffer. Log cells cohere immediately but stationary phase cells are at first completely non-cohesive. After a lag period they then begin to cohere but only very slowly, and it is at least 2-3 hours before their level of cohesion is equivalent to that of log phase cells. They exhibit this same lag period when inoculated back into fresh growth medium. In both cases, however, the cells regain their cohesive properties without undergoing cell division. In buffer they will not divide at all; in new medium they attain the normal cohesion level of log cells well within the first eight hours. This is the normal doubling time for log cells, and with re-inoculated stationary cells this is usually extended anyway, since they exhibit a short lag period before resuming division (Yarger, Stults and Soll, 1974; Yarger and Soll, 1975). The loss of cohesiveness seen in stationary phase is, therefore, readily reversible once the cells are placed in

new growth medium.

The difference in cohesive properties between log and stationary cells is reflected in their cohesiveness during growth of a cell population. By the onset of stationary phase the cells are completely non-cohesive. Although, however, the stationary phase cells do exhibit this property, the data shows that the medium from a stationary phase culture can completely inhibit the cohesion of log cells. Fresh axenic medium does not affect log cell cohesion and neither of the possible factors (the difference in pH between log and stationary media and the added glucose) mimic the inhibitory activity of stationary phase medium. The evidence, therefore, suggests that this medium contains a substance(s) which has a potent inhibitory effect on the cohesion of log phase cells and appears to accumulate in the medium during the growth of a cell population.

The inhibitory effect which this stationary medium has on log phase cells is immediately reversible when the cells are returned to buffer, even after long incubations in the SPM. There is no lag period in buffer by these log cells before cohesive behaviour is resumed, unlike the case of stationary phase cells. It would appear, then, that this inhibitor is not responsible for the loss of cohesiveness of stationary cells, and it does not 'alter' log cells to stationary cells in respect of their cohesive properties. The increase in pH from log to stationary phase, however, could conceivably have an effect on the cohesiveness of stationary cells. Alternatively, this

property may simply be a reflection of cell surface alterations associated with the non-dividing state. Stationary phase cells differ from log cells upon comparison of a variety of parameters: they show a significant increase in mean cell volume, average dry weight, average protein content, and average DNA content (Soll, Yarger and Mirick, 1976). At the same time there is an increase in intracellular cAMP, a decrease in the stability of ribosomal RNA, a decrease in the ratio of polysomes to monosomes and in the production of red pigment (Yarger and Soll, 1975).

The accumulation pattern of the cohesion inhibitor parallels that of the other inhibitors detected in SPM which inhibit division (Yarger, Stults and Soll, 1974; Hanish, 1975) and transcription (Yarger and Soll, 1975). The onset of stationary phase marks the start of production of these inhibitors, which then accumulate steadily in the medium. Yarger and Soll have not yet managed to distinguish between, or identify fully, these inhibitors. The results presented in this section, however, show that the cohesion inhibitor, at least, is a separate entity. Addition of the inhibitor to log phase cultures had no detectable effect on the subsequent growth rate, which was indistinguishable from that of the control populations. It would seem, then, that whatever the identity of these other inhibitors, the cohesion inhibitor is a different molecular species, exerting entirely different effects. The possible importance of this inhibitor for use in cell cohesion and adhesion studies was great. For this reason, further investigations into its biological properties and effects,

and into its molecular nature were initiated.

SECTION FOUR:

PROPERTIES AND CHARACTERISATION OF THE COHESION INHIBITOR.

4.1. INTRODUCTION

The results reported in Section 3 point to the existence of an inhibitor of cell cohesion in stationary phase medium. It was important to attempt to characterise this molecule(s) and to determine its mode of action. This information would contribute a little more to the knowledge about the mechanism of cohesion and adhesion in slime mould cells and possibly add more to our understanding of cell adhesion in general.

The literature contains numerous reports of both 'aggregation factors', such as those in sponges (Henkart et al.1973; Burger et al.1975; Zahn et al.1976), in sea urchin embryos (Kondo, 1974), and chick embryonic cells (McClay and Moscona, 1974; Hausmann and Moscona, 1975), and of 'inhibition factors' (Curtis and Greaves, 1965; Merrell et al, 1975). These latter two inhibitory agents are both proteins. This is not the only class of molecules known to exert effects on cell cohesion. From studies using haemagglutinins (Lectins) it is abundantly clear that even simple sugars could play a major role in cell cohesion and its inhibition. (Kemp, 1968; McGuire, 1972; Oppenheimer, 1975).

A number of possibilities have also been suggested by previous work with slime moulds, as follows.

(a) Cyclic AMP has an important function in the life-cycle of D.discoideum, where it functions as the chemical attractant, "acrasin", during aggregation (Konijn, et al 1968). Moreover, cAMP has recently been suggested to be important

in cell adhesion and cohesion in vertebrate systems (Johnson and Pastan, 1972; Willingham and Pastan, 1975; Storrie, 1975). It has also been shown to accumulate in the growth medium of a population of D.discoideum Ax-2 cells, reaching a final concentration in stationary phase of 1mM (Malkinson and Ashworth, 1972; 1973).

(b) Incubation of log phase cells in 3mM glucose for 10 minutes prior to assay will inhibit subsequent cohesion in buffer (Weeks and Weeks, 1975).

(c) Other simple sugars which will enhance vegetative growth have been shown to prevent the acquisition of aggregation-competence (Rickenberg, et al 1975).

(d) Several glycosidase species have been isolated from the spent growth medium (stationary phase) of a population of Ax-2, in high yield. These include β-N-acetylglucosaminidase, β-N-acetyl-galactosaminidase, ∝-glucosidase, β-glucosidase and ∝-mannosidase (Every and Ashworth, 1973; 1975). These enzymes remove specific residues from the carbohydrate chains on the cell surface. They, therefore, have a potential role as inhibitors of slime mould cell cohesion, especially since they accumulate in stationary phase medium.

It was essential, therefore, to test each of these possibilities: cAMP and the sugars were tested for inhibitory activity under standard assay conditions. In addition to cAMP, its breakdown products, 5'AMP and 5'GMP were assayed.

Biochemical methods for progressive characterisation of the inhibitor eliminated the possibility of a molecule of a protein nature being responsible.

Routine biochemical procedures, the effect of heat, freezing and thawing, the solubility in organic solvents, amino acid analysis and molecular weight estimation by column chromatography were carried out.

Subsequently, the inhibitory fraction (purified by ion-exchange and thin-layer chromatography) was analysed by nuclear magnetic resonance, gas liquid chromatography and mass spectroscopy.

4.2. RESULTS.

4.2.1. Properties of the inhibitor.

(i) The effect of dialysis.

The initial step in characterising the inhibitory molecule(s) was to determine the effect of dialysis upon stationary phase medium. A 50ml sample of SPM was dialysed exhaustively (four changes of buffer over 36 hours) at 4°C against 17mM phosphate buffer, pH6.0. The result- and dialysate was then assayed for its ability to inhibit cohesion of log phase cells. The data obtained is present- ed graphically in Fig.4.1. The cells cohered to the normal degree in the buffer control, and were completely inhibited from cohering in stationary phase medium. In the case of dialysed SPM, however, the level of cohesion was almost equivalent to that in the buffer control. The inhibitor is removed completely by this process. Since the exclusion limit of the dialysis tubing is 20,000 MW, the inhibitory activity must be due to a substance of relatively low molecular weight. The enzymes considered as possibilities, therefore, could now be discounted.

The inhibitory activity could be recovered by dialysing SPM against one volume of distilled water overnight (distilled water was used to avoid increasing the salt content of the fraction containing inhibitor) and evaporat- ing this under reduced pressure to a volume equivalent to that of the original SPM sample. When log cell cohesion was assayed in this solution, cohesion was completely inhibited. The dialysate could also inhibit cohesion in buffer (Fig.4.1.b)

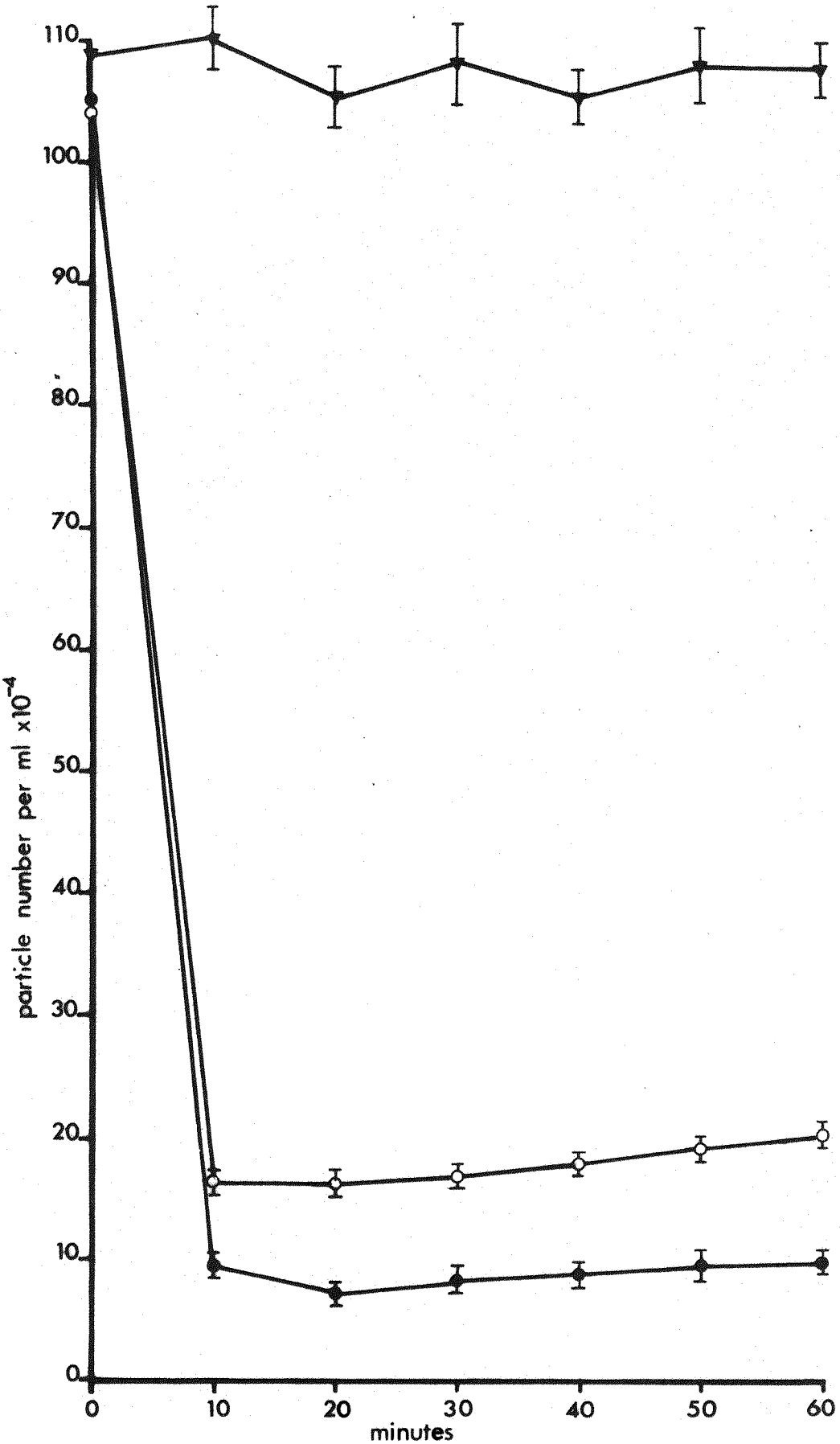

Fig. 4.1.a. Cohesion of log cells in buffer (●—●), undialysed SPM (▼—▼) and dialysed SPM (○—○)

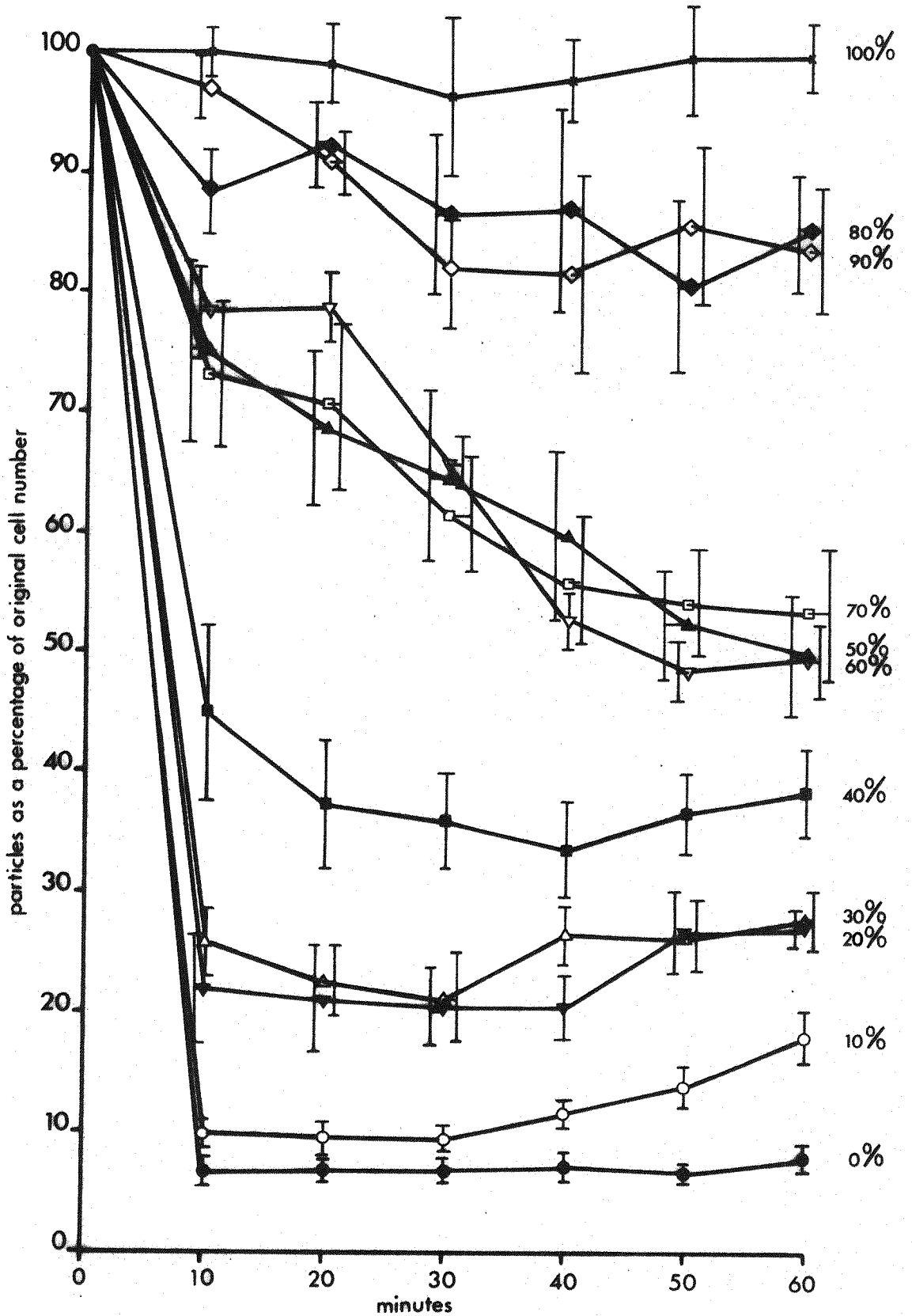

Fig. 4.1.b. Cohesion of log cells in a series of dilutions of dialysate containing inhibitor.

If the inhibitory molecule was a protein, then it was clear from this result that it must be restricted to an oligopeptide of not more than about 20 amino acid residues. If carbohydrate residues were incorporated in the molecule, the number of possible amino acids was correspondingly reduced.

To determine whether or not a peptide was responsible for the inhibitory activity, the effects of trypsin and heat were tested.

(ii) The effect of trypsin.

Stationary phase medium (4ml volumes) was incubated with trypsin (Sigma) at 20°C for 4.5 hours. The trypsin concentrations used were 0.1%, 0.5%, 1.0% and 2.0%. At the end of the incubation period, trypsin inhibitor (Sigma) was added to the solutions to the appropriate concentrations to terminate the reaction and inactivate any trypsin which may subsequently affect the cells during assay.

Cohesion of log phase cells in these solutions was then monitored. Controls used were as follows:

(a) Trypsin inhibitor.

(b) Stationary phase medium (untreated)

(c) Inactivated trypsin (100°C for 20 minutes) plus trypsin inhibitor.

(d) 17mM phosphate buffer.

The results are presented in Fig.4.2. Control cells in buffer cohered normally.

Mins	Mean particle count (10 observations)			
	0.1% trypsin	0.5% trypsin	1.0% trypsin	2.0% trypsin
0	137±8.91	127±6.41	142±3.14	126±7.19
10	125 7.63	112 4.52	128 7.89	110 13.36
20	123 9.32	111 4.78	139 5.42	118 9.47
30	118 6.74	127 3.13	129 7.97	114 4.51
40	115 8.11	118 8.98	130 8.13	125 8.57
50	128 6.40	119 7.65	127 9.11	129 11.91
60	126 6.54	125 7.97	136 6.42	119 2.23
	Trypsin inhibitor (10mg/ml)	SPM	Heat inactivated trypsin	Phosphate buffer
0	120±8.13	120±3.41	116±2.57	115±1.55
10	116 11.97	121 1.03	111 1.53	17 0.91
20	116 4.84	114 1.71	118 4.86	20 1.03
30	118 6.06	120 1.64	116 4.32	21 0.70
40	117 2.51	122 1.98	110 2.09	15 1.23
50	123 7.01	118 2.07	115 1.49	20 1.79
60	116 9.09	110 1.83	112 1.13	28 1.91

Fig.4.2. The effect of trypsin on the inhibitory activity.
(Values are shown ±1 standard error)

SPM completely inhibited cohesion: this complete inhibition was undiminished in the trypsin-treated SPM, and in the other controls. The activity was, therefore, not susceptible to the proteolytic activity of trypsin.

(iii) The effect of heat.

The heat-stability of the inhibitor was tested in the three following ways:

(a) Heating SPM at 90°C for up to 2 hours.

(b) Immersion of SPM in boiling water for 10 minutes.

(c) Brief direct boiling followed by aeration.

Log cell cohesion was assayed in the cooled media, and the results are shown in figure 4.3. Again, there was no diminution of inhibitory activity after any of these treatments. The activity, therefore, appeared to be heat-stable.

The trypsin-resistance and heat-stability of the inhibitor in SPM indicated that it was extremely unlikely to be a peptide molecule. One further test was carried out to confirm this.

(iv) The effect of freezing and thawing.

Peptide molecules are often especially sensitive to freezing and thawing. Samples of stationary phase medium, and of reconcentrated dialysate, were frozen to -20°C for 18 hours and then thawed at room temperature. Cohesion of log cells was assayed in both once-frozen and twice-frozen samples. The results for SPM and for the dialysate were similar; the numerical results for the SPM only are, therefore, presented (Fig.4.4.)

Mins	Mean particle count (8 observations)									
	SPM		Buffer		Boiled SPM		SPM heated 90°C for 2h		Immersion in boiling H_2O	
0	141	± 2.5	102	± 2.33	136	± 10.91	150	± 6.16	109	± 4.59
10	143	1.5	11	1.76	134	11.61	134	11.56	86	2.84
20	142	2.5	9	1.23	127	6.73	144	3.53	81	5.98
30	146	22.5	7	1.11	134	7.89	138	2.61	80	3.40
40	139	8.0	9	2.21	142	9.45	126	4.21	96	2.78
50	150	1.2	10	1.91	126	12.32	125	5.62	86	1.56
60	143	7.5	9	1.11	126	13.91	124	5.07	81	6.41

Fig.4.3. Effect of heat on the inhibitory activity

(Values are shown ± 1 standard error)

Mins	Mean particle count (8 observations)		
	Phosphate buffer	Once-frozen SPM	Twice-frozen SPM
0	123 ± 3.12	106 ± 5.72	105 ± 5.76
10	12 1.76	102 4.09	82 11.94
20	17 2.13	94 2.97	75 13.36
30	22 2.11	97 4.63	99 7.97
40	20 1.97	95 5.13	95 7.98
50	18 2.04	97 4.21	90 1029
60	16 1.07	103 5.85	97 6.51

Fig.4.4. Effect of freezing and thawing on the inhibitory activity
(Values are shown ±1 standard error)

Clearly in each case, freezing and thawing, even more than once, had no detectable diminishing effect on the inhibitory activity.

On the basis of evidence reported so far it was finally concluded that the molecule(s) responsible for inhibition of cohesion was not a peptide.

(v) Solubility in organic solvents.

Samples of stationary phase medium were extracted with several common organic solvents, simply by shaking the two liquids together. When they had separated completely, the aqueous layer in each instance was removed, and the organic fraction was freeze-dried. Solid matter resulting from this was taken up in the appropriate volume of 17mM phosphate buffer and assayed for inhibition of cohesion of log cells. Fractions extracted with chloroform and acetone exhibited no inhibition of cohesion; The fraction extracted with ethyl acetate exhibited a very slight degree of inhibition. The inhibitor was, therefore, insoluble in the two former solvents and only marginally soluble in ethyl acetate.

(vi) Estimation of molecular weight.

A much better idea of the nature of the inhibitor might be gained by the knowledge of its approximate molecular weight. So far, the only information known about this was that the molecular weight was less than 20,000. The molecular weight was estimated by column chromatography using Sephadex G-50 as the filtration gel (see section 2.3.2.). This method was chosen because a value for the molecular weight can be obtained using impure samples.

Molecular weights can be derived by other methods, but these normally require extensive purification to prepare the substance free of contaminants. At this stage the only purification step performed had been dialysis. This had removed all the macromolecules present at stationary phase, but in addition to the inhibitor the dialysate still contained all the unmetabolised low molecular weight components of the yeast extract present in growth medium, plus any other small molecular species produced by the slime moulds during their growth phase.

Even the approximate molecular weight of the inhibitor was at this stage unknown. It was, therefore, decided to use Sephadex G-50 (Fine): this grade is claimed by Pharmacia to have a fractionation range of 1500 - 30,000MW for proteins and peptides, and of 500 - 10,000MW for dextrans. This was the most adequate for the separation of the 0 - 20,000MW fraction from dialysis.

The method used was essentially that of Andrews (1964) and is described in detail in section 2.4.1. The substances used to calibrate the column, and their elution volumes, are shown in Fig.4.5. The calibration curve for the column is presented in Fig.4.6.

Samples of concentrated dialysate were applied to the column and the resulting fractions assayed for inhibitory activity. The elution volumes for each sample's peak of activity were determined and the range of these was plotted on the calibration curve. The mean value is indicated by the arrow. The 'log molecular weight' at this point was transformed to give a real molecular weight range of

Substance	Mol.wt.	V_e	V_e-V_o	Log MW
Blue dextran	$1x10^6$	56	-	-
Phenol red	354	215	159	2.549
Flavin mono-phosphate	514	169	113	2.711
Vitamin B_{12}	1357	152	96	3.133
Cytochrome C	13,000	96	40	4.114
Myoglobin	17,000	80	34	4.230
Bovine serum albumin/Evan's blue	60,000	58	2	4.778

Fig.4.5. Column calibration data for estimation of molecular weight.

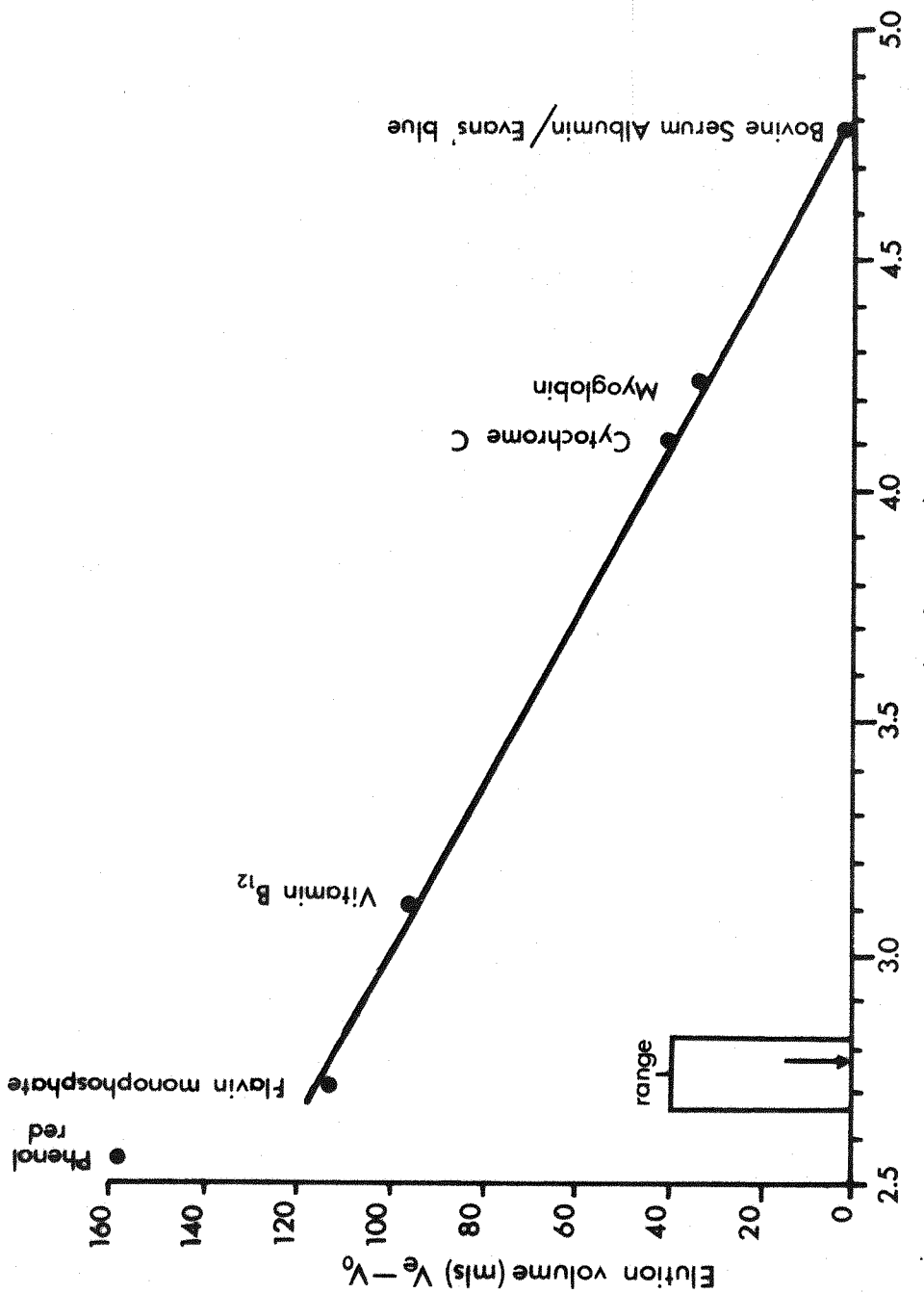

Fig. 4.6. Calibration curve for molecular weight estimation

500 - 700. The mean is at about 540. The inhibitory activity was, therefore, eluted at the lower end of the resolution range for this gel. Its elution volume was, however, similar to that of flavin monophosphate (which has a molecular weight of 514). The calibration curve at this point is still straight; it deviates only at lower molecular weights. The inhibitor is, therefore, eluted at a point still within limits of reasonable accuracy, and this estimation of its molecular weight can be taken to be correspondingly accurate. Only one peak of activity was detected in the chromatography fraction. It is, therefore, almost certain that the inhibitory activity was restricted to only one molecular species. The likelihood that two such species with almost exactly the same molecular weights were responsible was remote.

The inhibitor is thus an extremely small molecule. The possibility of its having a peptide nature has been eliminated by previous tests, but with a molecular weight of only 500 - 600 the remaining possibilities were still numerous. The most likely candidates at this stage were cyclic AMP (or cyclic GMP) and one or more of the simple sugars known to block development on Millipore filters. It was of prime importance to assay these and eliminate them. First, however, it was desirable to construct a standard curve for the inhibitory activity of concentrated dialysate. Levels of inhibition obtained in future assays could then be compared with the level in SPM.

(vII) Standard curve for the inhibitory activity.

The dialysate was reconcentrated under vacuum to the appropriate volume (i.e. to give a concentration of inhibitor equal to that of SPM) Samples were then diluted with 17mM phosphate buffer to different concentrations, and used to assay log cell cohesion in the standard way. The values obtained were expressed in percentage form because of the difficulty in adjusting initial cell numbers in each flask to the same figure (the procedure involved over 50 separate experiments). The mean value for each dilution after 10 minutes' shaking was plotted against concentration, and the regression line plotted through these points gives the initial rate curve. This is presented in Fig.4.7.

4.2.2. Possible candidates for the role of inhibitor.

(i) The cyclic nucleotides.

The cyclic nucleotides and their derivatives already described were dissolved in 17mM phosphate buffer to final concentrations of 10^{-3}M, 10^{-6}M and 10^{-9}M. Their ability to inhibit log cell cohesion was determined. The results are tabulated in Fig.4.8.

The log cells cohered to the normal degree in the phosphate buffer controls, and the cohesion levels in the test solutions were indistinguishable from the control values. Clearly, none of these nucleotides confers any degree of inhibition on log cell cohesion.

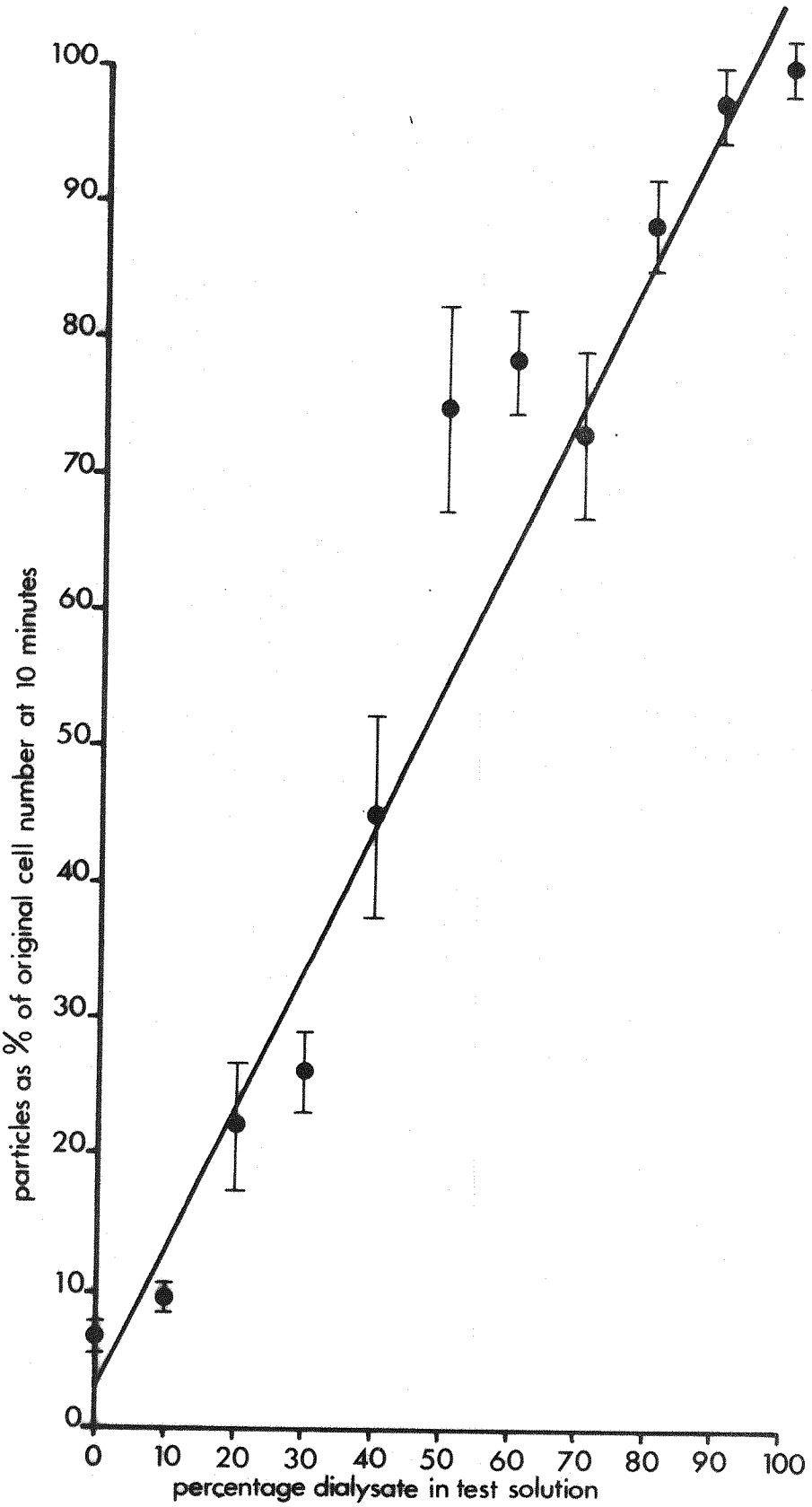

Fig.4.7. Initial rate curve for inhibitor concentrations

MEAN PARTICLE COUNT				
Mins	Cohesion in cAMP (26 observations)			
	Buffer	10^{-3}M	10^{-6}M	10^{-9}M
0	100 ± 4.10	101 ± 2.47	102 ± 3.80	106 ± 3.17
10	13 1.71	12 1.01	14 0.60	20 0.62
20	16 1.09	14 1.11	12 0.52	21 0.76
Mins	Cohesion in cGMP (18 observations)			
	Buffer	10-3M	10^{-6}M	10^{-9}M
0	107 ± 3.93	98 ± 3.74	104 ± 4.21	115 ± 3.73
10	15 1.05	17 1.71	16 0.81	18 0.65
20	14 1.31	15 1.45	16 1.20	22 0.96
Mins	Cohesion in 5'AMP and 5'GMP(16 observations)			
	Buffer	10^{-3}M 5'AMP	10^{-3}M 5'GMP	
0	103 ± 2.71	95 ± 2.21	101 ± 3.53	
10	10 0.90	9 1.08	10 0.81	
20	9 1.21	10 1.38	11 0.76	

Fig.4.8. Effects of cyclic nucleotides on cell cohesion.

(Values are shown ±1 standard error)

(ii) The simple sugars.

The following sugars were assayed for their ability to inhibit cohesion: 100mM solutions in phosphate buffer were used in each case.

Trehalose
Galactose
Fucose
Maltose
Fructose
N-acetyl-galactosamine
Cellobiose
Mannose
∝-D-galacturonic acid
D-glucuronic acid
∝-me-D-glucopyranoside
N-acetyl-glucosamine

The results are tabulated in Fig.4.9. Significant inhibition was absent in all cases except trehalose, ∝-D-galacturonic acid and ∝-methyl-D-glucopyranoside. By comparison of the initial rates in these instances, however, it is seen that the degree of inhibition afforded by these sugars is equivalent to less than 70% SPM. It was reasonable to expect that if any of these sugars were identical to the inhibitor then they should have completely inhibited cohesion at a concentration of 100mM. Since this was not the case, it was concluded that none of these sugars were responsible for the inhibitory effects described in this work. This point was confirmed by

Carbohydrate	Mean particle count (12 observs.)					
	0 mins		10 mins		20 mins	
Galactose	118	± 3.43	16	± 1.14	22	± 1.24
Fucose	102	3.08	17	0.85	23	1.31
Trehalose	114	4.80	72	4.09	76	2.72
Maltose	102	2.61	31	2.33	30	0.96
Fructose	100	4.21	25	1.73	22	1.28
Cellobiose	99	6.21	34	2.11	39	1.58
Mannose	106	2.52	26	2.37	36	3.39
N-acetyl-galactosamine	103	4.60	15	1.15	23	2.01
N-acetyl-glucosamine	98	4.16	44	3.48	56	3.65
α-D-galacturonic acid	102	8.87	74	4.04	79	3.34
D-glucuronic acid	100	8.72	40	7.02	39	5.03
α-methyl-D-gluco-pyranoside	96	4.27	35	3.01	43	2.18
D-glucose	104	3.87	39	2.98	35	2.06
Phosphate buffer	100	8.50	21	3.53	18	4.61

Fig.4.9. Effect of simple carbohydrates on log cell cohesion

later studies using thin-layer chromatography, nuclear magnetic resonance and gas chromatography.

4.2.3. Characterisation of the molecule.

Several biochemical procedures were undertaken in order to further purify the inhibitor, and to enable further characterisation of the molecule.

(i) Amino acid analysis.

Earlier in this work, when the possibility that the inhibitor was a protein had not been eliminated, the impure fraction had been sprayed with ninhydrin, which had given a positive reaction. Ninhydrin is generally used as a locating agent for proteins, since it reacts with molecules furnishing free amino groups. These groups are commonly found in amino acid residues. A ninhydrin-positive reaction, however, can also occur with other molecule containing free amino groups. It does not, therefore, necessarily mean that amino acids are present. The impure inhibitory fraction, however, gave a strong reaction with this locating agent and as a result of this, an amino acid analysis was carried out.

An amino acid analyser functions, in essence, as two ion exchange columns, through which the sample is run in buffer. The amino acids present are eluted separately, and in an order dependent upon their molecular charge. As the fractions reach the end of the process they are reacted with ninhydrin. A positive reaction is recorded on the print-out as a peak, and each peak can then be located to a certain fraction collected.

When the inhibitory fraction was analysed it was found to contain a large range of amino acids which were present as impurities. Four ninhydrin-positive peaks, however, could not be ascribed to any known amino acids. They occurred at the beginning of the trace, at the 'basic' end, and before the first known amino acid had been eluted.

When the corresponding fractions were assayed one was found to possess some inhibitory activity. The amount of activity recovered from this procedure was small, and was required *in toto* for the assay. The amino acid analysis did, however, suggest that the inhibitory molecule might possess an amino group somewhere in its structure. Alternatively, it was being eluted at precisely the same time as another molecular species with a ninhydrin-positive group.

The degree of impurity revealed by the analysis necessitated further steps to purify the inhibitor. Ion exchange chromatography was chosen for this next step. This method should enable separation of the inhibitor from both the contaminating amino acids and any other charged molecules in the dialysate which are not adequately removed during the column chromatography procedure.

(ii) *Ion exchange chromatography: the molecular charge.*

Pilot experiments to determine whether or not the inhibitor was charged were performed as follows.

10 gram quantities of cation and anion exchangers were swollen and equilibrated in buffers of pH values 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. These ion exchangers

were then agitated briefly with small amounts of inhibitory extract dissolved in buffer of the corresponding pH. The ion exchangers were then filtered off and the pH of the filtrates adjusted to between 5.0 and 7.0 where necessary. Normal cohesion levels are maintained within this range. The inhibitory activity of each buffer sample was then assayed. None of the samples showed any appreciable decrease in activity compared to the inhibitor control. The inhibitory molecule, therefore, appeared uncharged.

Ion exchange chromatography was, however, routinely employed in the purification of inhibitory extract to remove contaminating molecules of both positive and negative charge. This procedure is described in section 2.4.2.

(iii) Thin-layer chromatography.

This technique provided both an additional purification step for the inhibitory extract, and a possible diagnostic tool in its characterisation. The procedure for locating the position of the inhibitor was carried out as described in section 2.4.3. This purified inhibitor was then used for the tests reported in section 4.2.3.(iv).

Some TLC plates were sprayed with a silver nitrate reagent to locate the positions of any reducing sugars present. When this was done, one large brown/black spot was located at almost the same distance from the origin as the inhibitor. This was subsequently identified as D-glucose: it ran fractionally faster than the inhibitor under the same conditions. Various other sugars were

subjected to T.L.C. and their Rg values were calculated. The mobilities of sugars in TLC are conventionally expressed in these terms, and signify the distance they travel relative to glucose.

$$Rg = \frac{\text{distance travelled from origin}}{\text{distance glucose travelled from origin}} \times 100$$

galactose	= 94.2
Mannose	= 98.4
n-acetyl-glucosamine	= 136.7
n-acetyl-galactosamine	= (i) 131.7 (ii) 101.5
cellobiose	= (i) 62.1 (ii) 97.2
INHIBITOR	= 92.2

The inhibitor, therefore, runs at a very similar speed to several monosaccharides and it was impossible to make a complete characterisation on the basis of this TLC data. (Cellobiose and n-acetyl-galactosamine give two spots under the TLC conditions used. This is probably due to partial hydrolysis). Since the spot formed by glucose was fairly large, it was impossible to deduce whether or not the inhibitor was also reacting with the silver nitrate reagent.

Paper chromatography, using a different solvent system was employed to attempt to resolve this problem. Certainly again the 'glucose spot' was elongated, but whether this was really due to a reduction reaction by the inhibitor or to 'tailing' by the glucose, could not be determined conclusively.

(iv) Nuclear magnetic resonance; gas/liquid chromatography.

(a) Nuclear magnetic resonance.

An atomic nucleus is a charged, spinning body which tends, in a uniform magnetic field, to behave as a bar magnet and orientate itself such that its magnetic axis lies parallel to the direction of the applied field. Energy is required to deflect a nucleus from this low energy (ground) state to the high energy (excited) state where the magnetic axis lies anti-parallel to that of the field.

When such transitions occur, there is in fact a net absorption of energy, since the nuclei in the ground state can absorb energy just in excess of that liberated by excited nuclei. When nuclei are subjected to radiation of a certain frequency the absorption is detected as a resonance phenomenon (i.e. when the energy difference during transition exactly balances the quantum of radiation absorbed).

In practice, the radiation frequency is kept constant, the strength of the magnetic field is varied, and the intensity of absorption is measured.

The absorption spectrum of an isolated nucleus takes the form of a sharp peak. However, in solid crystal samples, nuclei interact to some degree with neighbouring nuclei; they generate a weak magnetic field (H_N) so that neighbouring nuclei experience a field $H \pm H_N$. Pairs of nuclei, therefore, give a resonance spectrum in the form of a doublet (such as CH groupings) or a triplet CH2 groupings).

Many samples, however, are in gaseous or liquid form (often dissolved in D_2O to avoid the interference by protons of H_2O) where the individual molecules are effectively independent of one another; each nucleus is magnetically shielded to some extent, though, by its orbiting electrons. Since chemical bonding with another atom alters the share and distribution of these electrons about the nucleus, the resonance line for a certain applied field strength shifts. These chemical shifts are related to the functional groups in which an atom occurs, and have characteristic positions dependent upon this. For instance, the proton resonance lines of ethanol have intensities 3:2:1 arising from the protons in the methyl, methylene and hydroxyl groups. The intensities of the lines are dependent upon the number of nuclei in any one group.

These chemically-shifted resonance lines still depend, in their fine structure, on the interactions of neighbouring nuclei, presenting as doublets and triplets as a consequence.

Experienced NMR operators can compare the resonance spectrum of an unknown sample with those of known standards and determine the following information about gaseous or liquid samples.

(i) Identify the presence of particular functional groups from the chemically shifted NMR lines.

(ii) Give the relative numbers of nuclei in these groups from the intensities of these lines.

(iii) Determine the relative positions of these groups from the multiplicity of these lines.

A sample of the purified inhibitor (from TLC) was dissolved in D_2O, and subjected to NMR analysis. Spectra of the carbon and hydrogen profiles were obtained. The number of hydrogen atoms was difficult to determine due to too much 'noise' in the system. The carbon resonance spectrum was more profitable. Initial runs produced resonance lines similar to glucose, but contained two additional prominent peaks and one smaller one: when this spectrum was expanded and decoupled from the 'noise', doublets and triplets could be discerned (see Figs.4.10. a, b, c).

The 'new' peaks (i.e. those additional to the ones in glucose) are labelled X, Y and Z. Accompanying peaks forming doublets and triplets are indicated by X_1, Y_1, Z_1, etc. In addition to a basic glucose ring structure, then, the inhibitor appears to possess the following chemical groupings:

The $X_1 - X - X_2$ triplet, probably CH_2

The $Y_1 - Y - Y_2$ triplet, probably CH_2

The $Z - Z_1$ doublet, probably CH

From the spectra it is not possible to determine the precise molecular arrangement of these groupings, nor whether other atoms, e.g. oxygen and phosphorus are also involved.

b) Gas/liquid chromatography.(GLC)

This technique is used for accurate analysis of any volatile substance. Most often employed in the

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Fig.4.10. Nuclear magnetic resonance spectra of a sample of the inhibitor dissolved in D_2O.

(a) Carbon resonance spectrum (noise decoupled). 'New' peaks additional to the glucose control are arrowed X,Y, and Z.

(b) 'Off resonance' (noise decoupled) spectrum showing carbon atoms and interacting protons (^1H). New peaks are labelled. The spectrum is expanded and shown in Fig.4.10.c. (overleaf). Two clear couplets are labelled 'CH'.

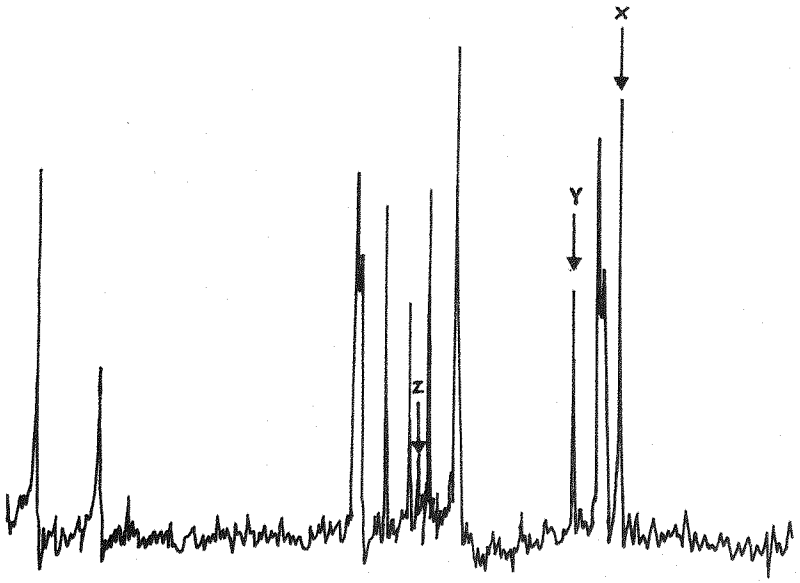

Fig.4.10.a.

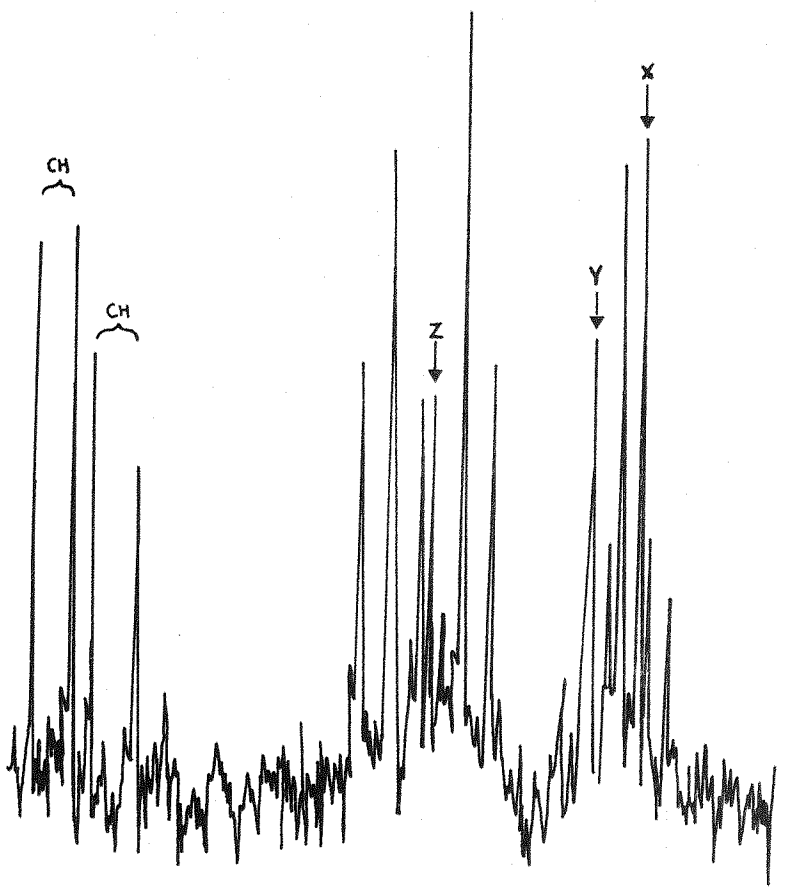

Fig.4.10.b.

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Fig.4.10.c. 'Off resonance' (noise decoupled) spectrum as in Fig.4.10.b., expanded x4.5. The first two peaks shown in the previous spectrum (labelled 'CH') are not shown here. The new peaks, X, Y, and Z, are arrowed, along with accompanying peaks forming doublets or triplets. The triplet O_1-O-O_2 (also present in the glucose control) is indicated at the more electropositive end of the spectrum.

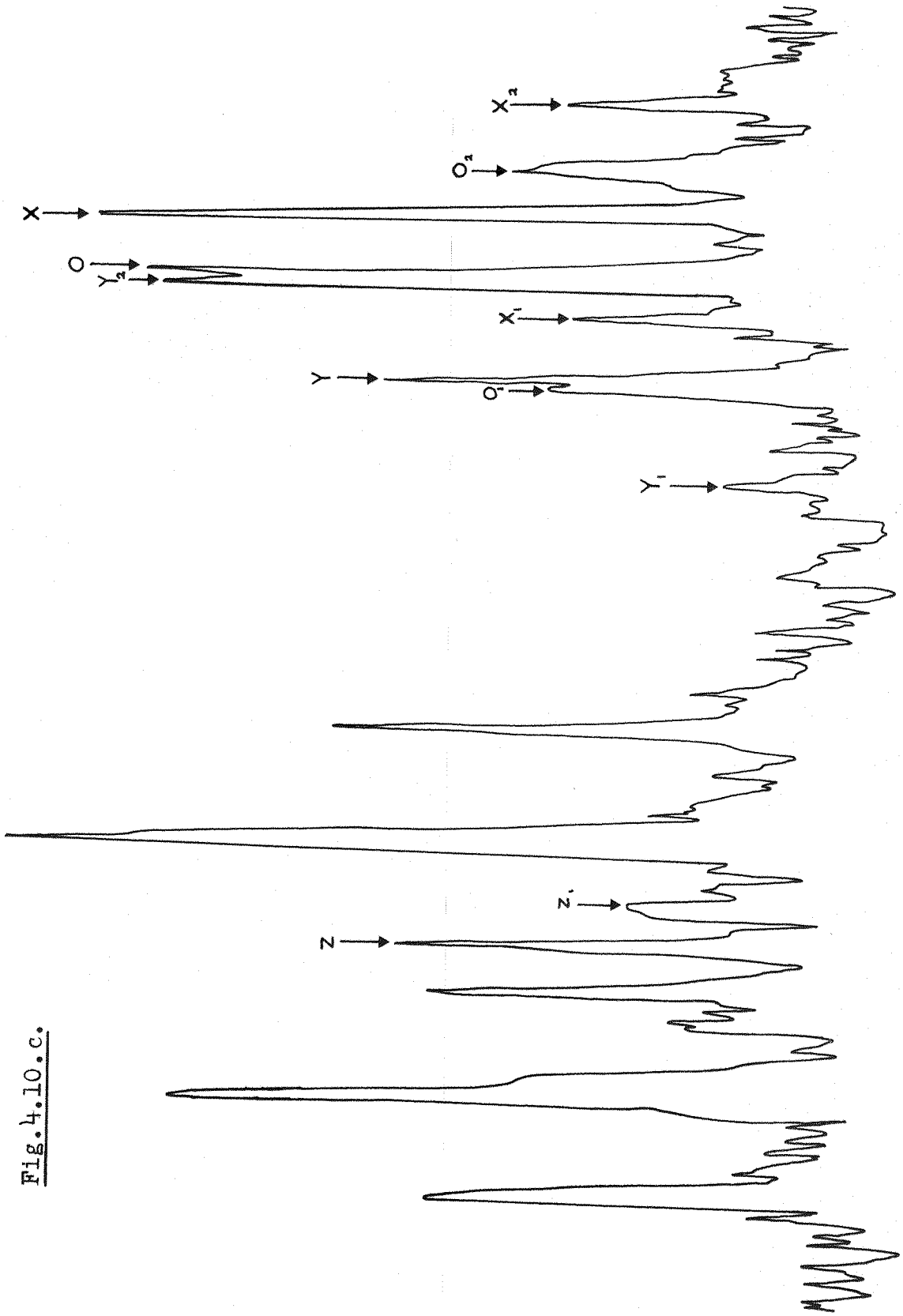

Fig.4.10.c.

analysis of lipids and gaseous mixtures, the process can also be used with a high measure of success with non-volatile compounds which have been converted to volatile derivatives. This latter group includes both the non-volatile amino acids and carbohydrates.

The volatile sample is injected into a column on a stream of inert gas (e.g.helium). A liquid absorbant is present within the column and the sample components partition into the liquid absorbant. The differences in their partition coefficients enables separation. The fractions ultimately pass through a 'detecting' unit which signals to a recorder. This converts the signals into a series of peaks on the print-out trace.

When the inhibitor was analysed by this technique peaks corresponding to D-glucose and D-fructose were detected, plus one thought to represent dihydroxyacetone and another small peak which was unidentifiable by its position. This peak could possibly represent the position of the inhibitor. Glucose and fructose were already known to have no inhibitory activity. The possibility that they were acting in combination, or in combination with the dihydroxyacetone remained. To determine whether this possibility was a fact, all three substances were checked again for their ability to inhibit cohesion. They were then combined in all possible combinations, including all three together in different ratios. The concentration used for each was 100mM. In none of the cases was there any appreciable degree of inhibition.

4.3. DISCUSSION.

In an attempt to isolate and characterise the cohesion inhibitor several techniques were employed to obtain information about the molecule. Initial evidence, from dialysis, trypsinisation, action of heat and freezing refuted the likelihood of the inhibitory molecule being protein in nature. The apparently very low molecular weight suggested two main possibilities, namely the cyclic nucleotides or the simple sugars. None of the members of these two groups which were tested showed a degree of inhibition of cohesion comparable to that of the inhibitor itself, even at very high concentrations. The molecule appeared to be uncharged, or at least so weakly charged that it failed to bind to the Sephadex ion exchangers used. The amino acid analysis indicated that the molecule may possess a grouping somewhere in its structure which reacted with ninhydrin, suggesting the possibility of its being an amino-sugar. The NMR and gas chromatography studies were more informative. From the resonance spectra, the inhibitor appeared to have three additional carbon atoms to that of the glucose control. These appeared to be in the form of one CH group, and two CH_2 groups, probably linked as a 3-carbon chain. The presence or absence of phosphorus, nitrogen or oxygen could not be determined by this method. No methyl groups were detected. The suggested structure, therefore, was one of a glucose

ring onto which the additional 3-carbon chain was attached, with the likely possibility of exygen and/or phosphorus atoms on the terminal carbon. The gas/liquid chromatography data could be interpreted to agree with this proposed structure. Before this process is carried out, the sample is derivatised to form a volatile compound; in this case derivatisation was by a process termed silylation. This involves the treatment of sugars with hydroxylamine hydrochloride. The oximes resulting from this procedure are converted to trimethylsilyl (TMS) esters by reaction with a silylating agent such as hexamethyldisilazane (HMDS) in the presence of pyridine or trifluoracetic acid. During this process, it is possible that a molecule such as the proposed structure for the inhibitor could be cleaved into two smaller units. In this case the most likely cleavage point is between the hexose ring and the 3-carbon chain, resulting in glucose and a 3-carbon molecule. The glucose-plus-dihydroyacetone pattern detected by this method may, therefore, be the result of such a process. The presence of fructose is more difficult to explain. Again, one likely possibility is that during silylation the hexose (glucose) formed mutarotated during the derivatisation procedure to produce some fructose.

Although speculation as to the possible identity of the inhibitory molecule is not really profitable at present, the suggested possible structure is that of a

a-carbon saccharide unit, probably composed of a hexose ring attached to a tri-carbon chain. This chain may also possess hydroxyl, phosphate or amino groups which have not as yet been detected.

It has, therefore, been possible to proceed at least some way toward characterisation of this molecule. Further investigation may reveal its precise molecular structure which would be invaluable in determining such information as the site of action of the inhibitor, its binding kinetics (cf.section 5) and how it is produced.

SECTION FIVE:

EFFECTS OF THE INHIBITOR ON CELL COHESION AND ADHESION.

5.1. INTRODUCTION

The effect of the inhibitor on the cohesion of Ax-2 log cells has already been described (section 3.2.6.). Since this effect was so complete, it was important to determine to what extent the inhibitor was able to impede the cohesion of aggregation-competent cells and of cells of later developmental stages. With the development of aggregation-competence new cell surface moieties appear, the most important of which are the contact sites A and the lectins, both of which were described in full in section 1.

Cells of the slime mould grex cohere very tightly, and to effect complete and rapid disaggregation proteolytic procedures are usually employed (cf. section 1.2.4.) At this stage many new proteins and glycoproteins have appeared on the surface which may yet be shown to be involved in cell cohesion. It was, therefore, pertinent to investigate the ability of this inhibitor to affect cohesion in these cells.

Anti-aggregation-cell-homogenate Fab renders cells non-adhesive to glass (Beug, et al.1970). Although it is not clear which of the target cell surface sites for this Fab is responsible for the property, it was necessary to determine the inhibitor's ability to prevent cells from adhering to glass.

A further area of investigation was that of species-specificity. Do cells of other slime mould species respond in the same way or extent as those of D.discoideum, from which the inhibitor was isolated?

Finally, an attempt was made to determine whether the inhibitor actually binds to some component of the cell surface as it acts.

5.2. RESULTS.

5.2.1. The effect of the inhibitor on aggregation-competent cells.

(i) Cohesion in suspension.

Log phase Ax-2 cells were made aggregation-competent in suspension as described in Section 2.1.4 . The cells were then washed, disaggregated, and finally resuspended at approximately $1x10^6$ per ml in inhibitor solution and in phosphate buffer. Cohesion in these two media was then assessed. The results are depicted graphically in Fig 5.1. Clearly, these cells are extremely cohesive in phosphate buffer, but this cohesion is greatly reduced in the presence of inhibitor. Interestingly, though, this level of inhibition is significantly lower than that seen with normal log cells. In this concentration of inhibitor log cells would exist entirely singly. When the concentration of inhibitor was doubled, aggregation-competent cells were still partially cohesive. The level of inhibition increased only slightly over that for the 'single' concentration (Fig.5.1.). Moreover, at this double concentration the cells appeared to be somewhat shrunken, possibly due to an increased salt level in the medium. Because of this manifestly detrimental effect, further increases in inhibitor concentration were not tested. The possible reasons for the observed pattern of inhibition using aggregation-competent cells is discussed more fully in Section 5.3.

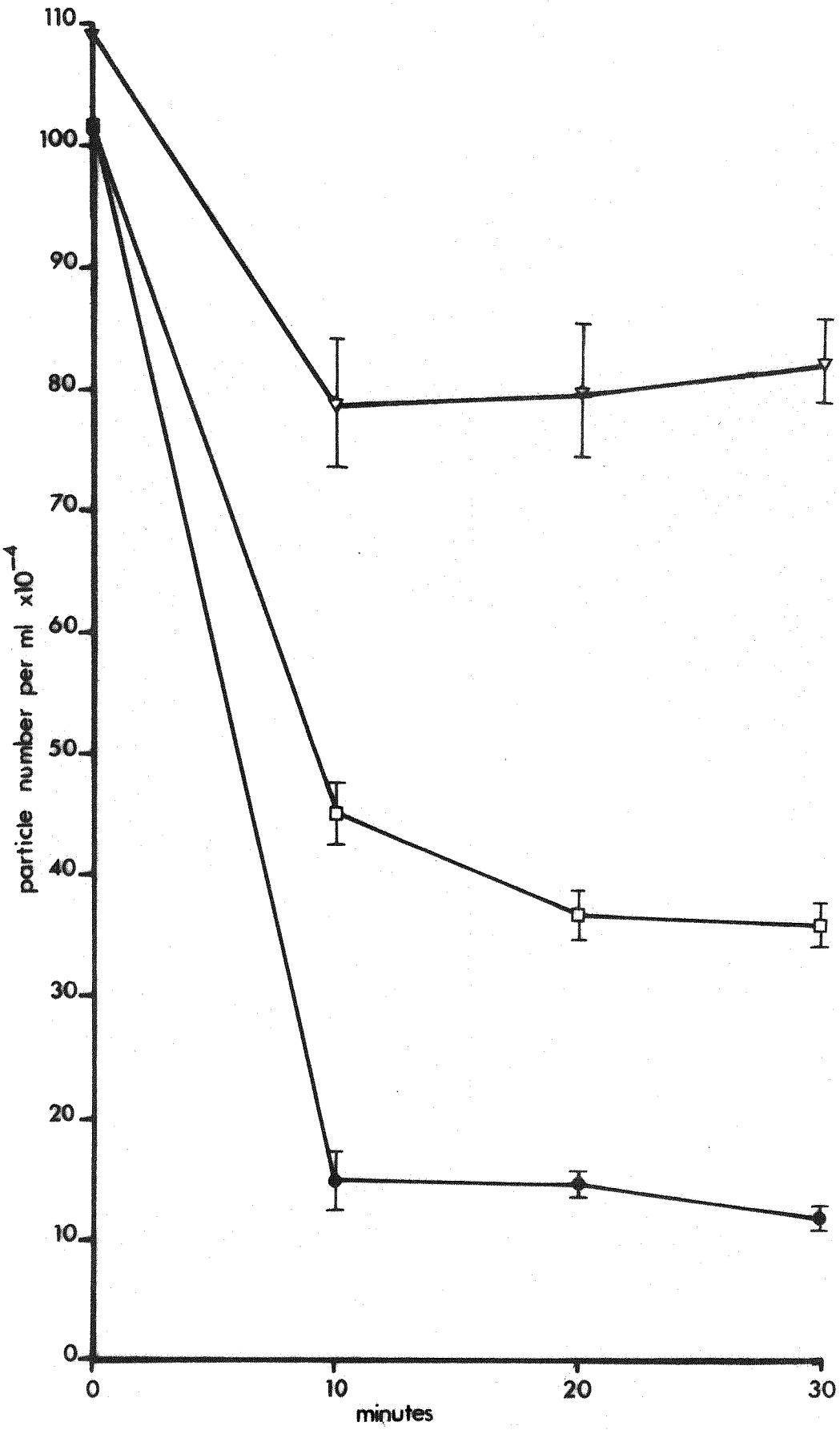

Fig.5.1. Cohesion of Ax-2 aggregation-competent cells in buffer (●—●) and inhibitor (□—□) single and (▽—▽) double strength

(ii) Adhesion to glass.

The effect of the inhibitor on the adhesion of aggregation-competent NC-4 cells to glass was examined in the way described in section 2.2.4. The cells, which had acquired aggregation-competence on plates, were suspended in either buffer or inhibitor solution in small chambers enclosed by glass cover slips. After settling for 30 minutes, the cells were counted over 20 unit areas. The slides were then inverted and a further 20 counts were made after another 30 minutes. The results are presented in Fig.5.3. together with the figures for other cell types. In buffer, there was no significant difference in the number of adherent cells between 0 minutes and 30 minutes. In inhibitor, however, the percentage decrease in these adherent cells after 30 minutes reached 55%. These results are discussed in section 5.3.

5.2.2. The effect of the inhibitor on cells of other slime mould species.

(i) Cohesion in suspension.

Cells of D.purpureum, D.mucoroides, D.discoideum (strain NC-4, wild type) and Polysphondylium violaceum were grown on agar in the presence of E.coli B/r (see section 2.1.2.). The cells were harvested from these culture plates whilst at the vegetative stage of growth. They were then washed thoroughly and suspended in either phosphate buffer or inhibitor solution. Cohesion was then monitored during a period of shaking

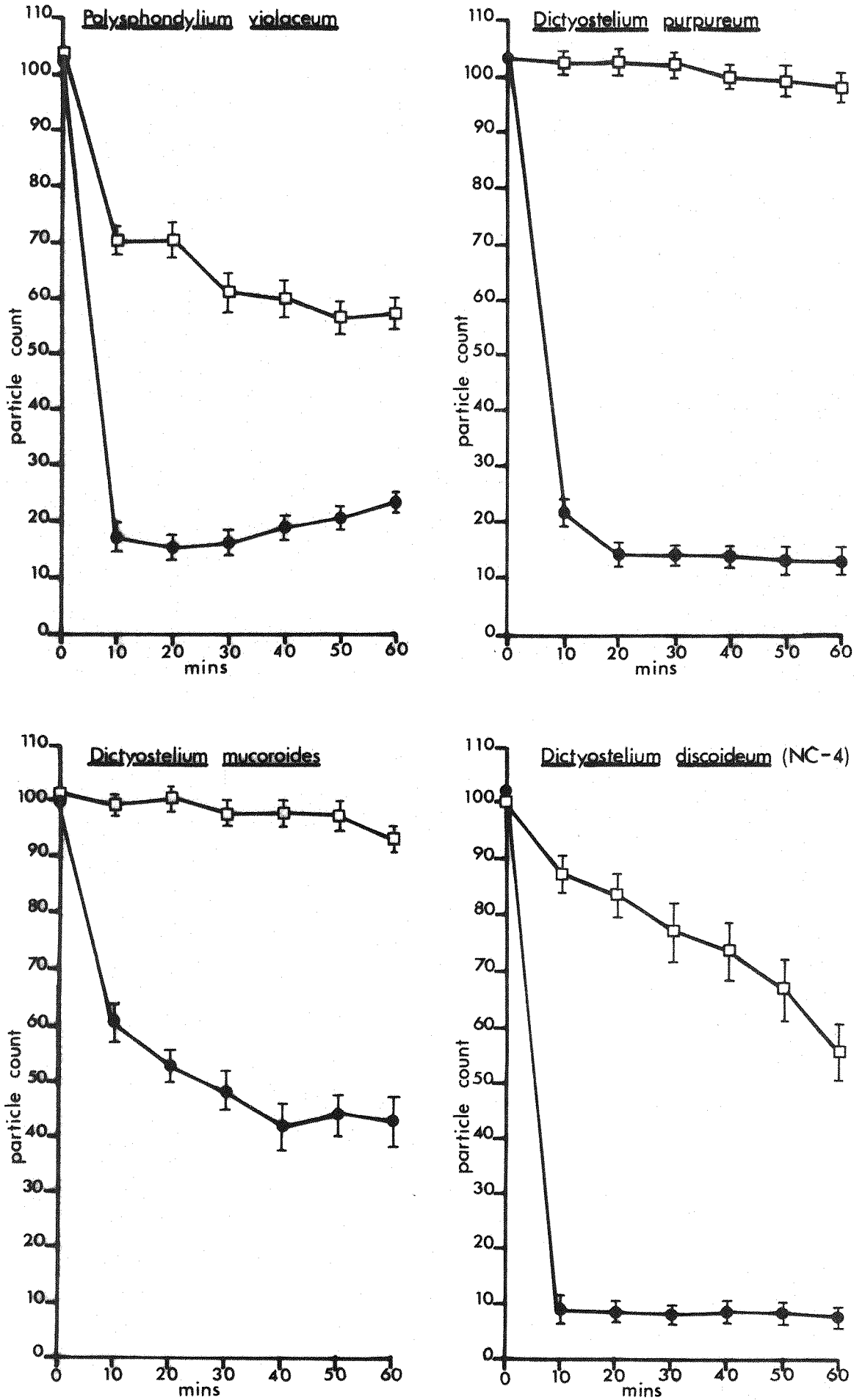

Fig. 5.2. Cell cohesion in buffer (●—●) and in inhibitor (□—□) of four slime mould species

incubation. The results are shown in Fig.5.2. The major observation is that all four species are clearly inhibited in their cohesion by the inhibitor produced by D.discoideum axenic cells. D.purpureum and D.mucoroides cells are completely inhibited from cohering by an inhibitor concentration sufficient to maintain Ax-2 cells in a dissociated state. The reason why D.mucoroides cells are not as cohesive in buffer as cells of the other three species is not understood at present. This inherent low level of cohesiveness is nevertheless susceptible to inhibitory effects. Another interesting point revealed by this experiment is that cells of P.violaceum exhibit a somewhat reduced susceptibility to the inhibitor. These cells maintain a very low level of cohesion (usually shown microscopically as cell couplets) in the presence of inhibitor at a level sufficient to keep Ax-2 cells completely dissociated.

(ii) Adhesion to glass.

Aggregation-competent cells can cohere to some degree when suspended in inhibitor although their ability to remain adherent to glass was markedly reduced in its presence. It was, therefore, apposite to determine whether feeding cells of various slime mould species, although prevented from cohering in suspension, could adhere to a glass substrate and remain attached in the presence of the inhibitor. This assay is described in section 2.2.4.
The following cell types were used:

D.discoideum (NC-4)

D.mucoroides (Dm)

D.purpureum (Dp)

P.violaceum (Pv)

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Fig.5.3. Effect of the inhibitor on the adhesion of slime mould cells to glass.

The mean % decrease in adhering cells after 30 minutes is shown (see text for details). The two treatments (buffer and inhibitor) for each species were compared by Student's t test.

Species	Test solution	Mean % decrease in adhering cells	S.D.	t	Probability P
D.discoideum(NC-4)	Buffer	2.58	2.01	3.94	0.02
	Inhibitor	19.34	6.90		
D.discoideum(NC-4) (aggregation-comp.)	Buffer	-1.03	7.34	8.73	0.02
	Inhibitor	45.44	1.12		
P.violaceum	Buffer	2.28	1.05	7.66	0.001
	Inhibitor	22.72	5.24		
D.purpureum	Buffer	2.75	0.60	2.81	0.05
	Inhibitor	16.89	10.06		
D.mucoroides	Buffer	2.85	4.04	3.37	0.02
	Inhibitor	22.52	10.97		

Fig.5.3.

The results are presented in Fig.5.3; the probabilities are derived by Student's 't' test. With every cell type used, the difference between the number of adherent cells at time 0 minutes and at time 30 minutes in buffer was non-significant. In all cases, however, in inhibitor the difference between these values was highly significant. After 30 minutes the percentage decrease in adherent cells in inhibitor was between 16% and 23%.

5.2.3. Effect of the inhibitor on cohesion of cells of a late developmental stage.

After Ax-2 cells have become aggregation-competent they are able to cohere to a limited degree in the presence of the inhibitor. If aggregation-competent cells continue to develop they pass through a series of different morphological stages. During development they also differentiate into two distinct cell types, prespore and prestalk. At the grex stage these differentiation products are arranged in a pattern, the front of the grex comprised of prestalk cells and the rear comprised of prespore cells. These cells cohere very tightly, the prestalk cells even more so than the prespore cells. The response of grex cells to the inhibitor was investigated in order to determine whether such cells still possess the ability to cohere to some extent, (as exhibited by aggregation-competent cells), whether they are completely inhibited or whether they have become non-susceptible to the inhibitor by this later developmental stage.

Ax-2 cells were plated onto Millipore filters and allowed to develop to the grex stage. The grexes were washed off the filters with cold distilled water, and disaggregated as far as possible by trituration using a drawn-out Pasteur pipette. By gently centrifuging down the cell clusters which could not be completely dissociated, it was possible to obtain a single-cell suspension. This complete operation must be carried out at low temperature (5°C). Complete dissociation can be effected by using proteolysis combined with EDTA treatment. Since these cells were to be used immediately, however, they would not have time to recover from this procedure. As the cohesive system is presumably disturbed by this treatment, mechanical dissociation was judged to be preferable. The separated cells were suspended in inhibitor solution and in buffer and cohesion assessed in the standard manner. The result is shown in Fig.5.4. Over the first 10 minutes of shaking the particle count decreased from around 100 to below 60 and thereafter very slightly decreased over the assay period. This level is comparable with that of aggregation-competent cells under the same conditions. The control cells in buffer cohered rapidly into large clumps and maintained this level throughout the 60 minutes of assay.

5.2.4. The site of action of the inhibitor.

The most likely site of action of the inhibitor is at the cell surface. It was, therefore, essential to

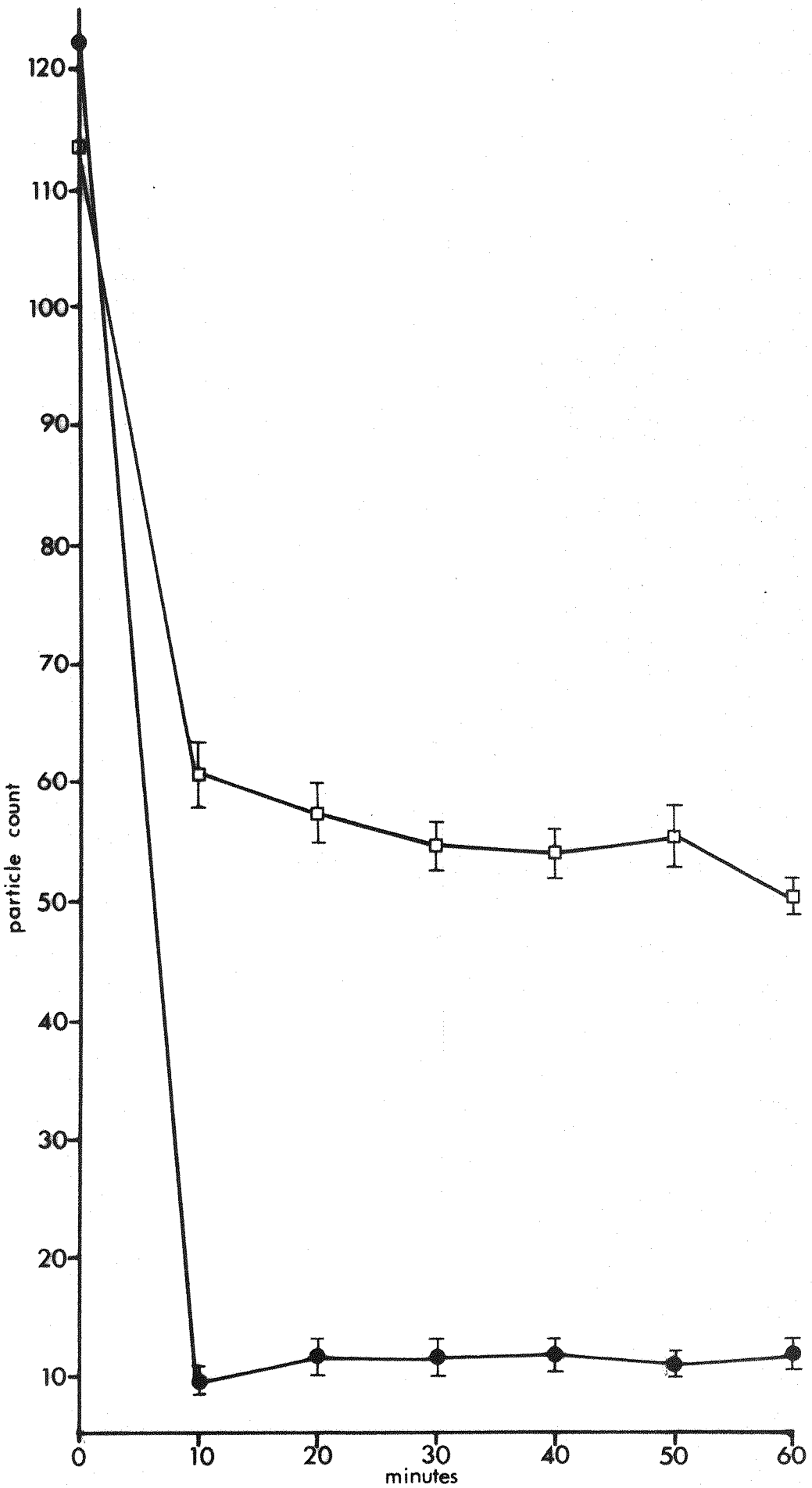

Fig. 5.4. Cohesion of dissociated grex cells in buffer (●—●) and in inhibitor (□—□).

try to assess whether the molecule attaches to the cell surface. If so, it is likely that it directly affects the adhesive sites of the surface in some way. If not, the inhibitor may act in an alternative manner similar to a chelating agent for example.

Since the identity of the molecule was still undetermined, labelling with radioisotopes or other markers was not possible. The simplest approach to the problem was to attempt to adsorb the inhibitory activity from the medium, using log cells as the receptor substrate. If medium which had been incubated with a large number of log cells still possessed undiminished inhibitory potency, it must be assumed that the inhibitory activity does not bind to the cell surface in order to exert its effect. If, however, the ability of the medium to inhibit cohesion is reduced after such incubation, then there are two likely explanations. First, that the inhibitor has been adsorbed onto the cells from the medium; or second, that the cells had metabolised the inhibitory activity in such a way as to render it inactive. To eliminate this latter possibility the cells used were fixed with glutaraldehyde (2% for 1.5h). This fixation procedure inactivates most but not all enzymic functions. A few, most particularly proteolysis, continue to act after this treatment. In addition, glutaraldehyde treatment has the immediate effect of cross-linking membrane proteins, thereby "freezing" many cell surface components in position. The flow of receptors within the plane of the membrane is therefore prevented.

The fixed log phase cells were washed three times in large volumes of cold distilled water in order to ensure complete removal of all traces of fixative. They were then suspended at $1x10^7$ cells per ml in stationary phase medium. This incubation mixture was shaken under standard conditions for a period of 1h. After this time the cells were spun down and a small sample of the medium was removed. This sample was used to assess the ability of the medium to inhibit cell cohesion. Meanwhile, a fresh batch of fixed cells was suspended at $1x10^7$/ml in the remaining SPM. This was shaken for a further period of 1h, after which a second sample was taken for analysis. With each successive incubation, the inhibitory activity in the SPM was diminished (see photographs in Fig.5.5.) After five such incubations, the level of inhibition had been reduced to that of fresh axenic medium plus glucose (cf.Fig 3. 6.). The dilution of the SPM with the addition of successive batches of cell suspension could have accounted for the apparent decrease in inhibitory activity. To avoid this possibility the cell suspensions used were extremely dense ($1x10^9$ cells/ml) and the volumes of SPM for incubation were comparatively large.

(To face page143)

Fig.5.5. Log cell cohesion in SPM after successive 60-minute incubations of the SPM with batches of fixed log phase cells at $1x10^7$ cells/ml.

×300

a) After 60 minutes incubation

b) " 120 " "

c) " 180 " "

d) " 240 " "

e) " 300 " "

a

b

c

d

e

5.3. DISCUSSION.

Several interesting pieces of information have arisen from this investigation of the effects of the inhibitor on cohesive and adhesive properties of slime mould cells.

The evidence obtained from studies using aggregation-competent cells may be explained by a simple hypothesis. During differentiation to the aggregation-competent state, the cells develop a new mechanism of cohesion additional to that found in log phase cells. By this new system, which is unaffected by the inhibitor, the cells are able to cohere, albeit at a reduced level, in the presence of inhibitory activity. The possibility that the new cohesive system is insensitive to the inhibitor is supported by the fact that even when the inhibitor concentration is doubled the cells are still able to cohere to almost the same degree. Thus the new cohesive sites are still functioning to the same extent. Increasing the concentration of inhibitor fails to break these cohesions. The cohesion of these cells is partially prevented, nevertheless, by the inhibitor. This strongly suggests that normal cohesion (at least under these conditions in suspension) of aggregation-competent cells is mediated in part by the same entities that are responsible for cohesion in vegetative cells. When the cells are suspended in inhibitor, this system ceases to function, thereby reducing the level of cohesion to that maintained

by the new cohesive sites alone. Thus aggregation-competent cells appear from this evidence to possess a "dual" system for cohesion; one part of this dual system is sensitive to the inhibitor, the other is not. The most attractive possibility with respect to the inhibitor-insensitive cohesion is that cohesion is mediated via the newly-formed contact sites A. These would remain functional and unblocked by the inhibitor. Further evidence for the inhibitor-insensitivity of contact sites A is discussed in section 6.3.

The vegetative cells of D.mucoroides and D.purpureum are completely prevented from cohering by the inhibitor. The pattern of cohesion which they show is comparable to that of Ax-2 cells, except that D.mucoroides cells in buffer do not cohere to the same extent. The reason for this is not known. This species of slime mould may simply have an inherently low level of cohesion. The reduced sensitivity of P.violaceum cells to the inhibitor may be due to one of two things; these cells could possibly be slightly more advanced than expected, i.e. they may not be a pure vegetative cell population, but some cells may be developing aggregation-competence. This point is discussed later with respect to NC-4 cells. The alternative explanation is that this reduced cohesiveness is a reflection of a genuine difference in cohesive properties of P.violaceum cells relative to Dictyostelium species. Small divergences may have occurred during the evolution of these two genera to account for this result. This would not be surprising in the light of information from

lectin studies (cf. section 1.2.5.(iii)). Monosaccharide inhibition tests showed that although all the slime mould species studied (from both Dictyostelium and Polysphondylium genera) had very similar lectin systems. It was possible to discriminate between these species by comparing the range and potencies of these simple sugars in their ability to inhibit cohesion (Rosen, Reitherman and Barondes, 1975). Thus it seems reasonable that P.violaceum might be expected to show reduced sensitivity to the effects of an inhibitor produced by a mutant strain of a Dictyostelium species.

D.discoideum NC-4 cells also exhibit a similarly reduced response to the inhibitor. This may again be a genuine reflection of new surface properties of this parent strain. These properties may have become lost in Ax-2 cells. In this case, though, a simpler explanation may be put forward, i.e. that the NC-4 cells used for the assay were not true vegetative cells but had progressed some way towards aggregation-competence. When the cells exhaust their bacterial nutrient supply, there is a lag period of several hours before they are seen to aggregate. During this lag period, the new surface components associated with aggregation-competence begin to develop. If these cells were inadvertently harvested during this period one may expect their cohesive behaviour to be at least partly that of aggregation-competent cells. An examination of the raw data for these tests confirms that this is the most likely possibility. Of the 24 individual observations which were used to compile each

mean, some showed complete inhibition at all time intervals. Some, however, showed a more marked aggregation. It would appear, then, that several of the cultures used were already in the post-feeding period. It is known that in this period susceptibility to inhibitory activity is markedly reduced (Section 5.2.1.) Values obtained using these cultures are, therefore, responsible for producing the slight degree of aggregation apparent in Fig.5.2. In fact, it is highly probable that the true feeding cells of strain NC-4 are, as with Ax-2 cells, completely prevented from cohering by the correct concentration of the inhibitor.

The most pertinent information to arise from these results is that the inhibitor is not solely specific to D.discoideum. Its effect is as potent on other Dictyostelium species as it is on Ax-2 cells, and at least one Polysphondylium species responds to its action. This suggests that the target sites of the inhibitor are common to all these species. Since vegetative cell cohesion is completely blocked by the activity of this inhibitor, it may be concluded that the cells of all slime mould species tested, when in the vegetative stage, cohere by a common mechanism when in the vegetative stage. A divergence in these mechanisms at the onset of aggregation-competence may subsequently ensure that there is no mixing of species in later development. Whether or not these 'common sites' are synonymous with Gerisch's contact sites B, present on vegetative D.discoideum cells, is at present

a matter for speculation. What is clear, however, is that the action of this inhibitor on a class of surface components is sufficient to completely block cell cohesion in several species. Cell adhesion to a glass substrate was also diminished by inhibitory activity. Vegetative cells of all four slime mould species again responded in a similar manner. In the presence of buffer alone, cells adhered to the glass and remained adherent when inverted. In inhibitor solution, the cells detached from the glass on inversion. The degree of spreading of these cells onto the glass was also less than that of cells in buffer. In fact, such cells may have only formed weak adhesions to the substrate initially. Not only was the adhesion to glass of these preaggregation cells sensitive to the inhibitor: D.discoideum NC-4 aggregation-competent cells were also affected. These cells exhibited an even greater tendency to detach from the glass than vegetative cells. Anti-contact-sites Fab fragments have a similar effect (Beug, Gerisch, Kempff,Riedel and Cremer, 1970). The simplest explanation for this phenomenon is that these cells adhere by their newly-developed CSA which would be blocked by the inhibitor. However, the weight of evidence is that these CSA are insensitive to the action of the inhibitor (see also section 6). The reason why these aggregation-competent cells then detach from glass more readily than vegetative cells remains unclear. One tentative argument can be put forward: at the onset of aggregation-competence, when CSA are developing on the cell surface, some of the CSB are lost, or at least redistributed, to the sides of the elongated cells.

They are then responsible for the side-to-side cohesion observed in cells actively aggregating. They may also be the mediators of the cells' adherence to glass. This reorganisation may result in a decrease in the ability of an aggregation-competent cell to attach to glass. The apparent increased susceptibility of these cells to the inhibitor may, therefore, be a reflection of this inability, the inhibitor still acting upon the adhesive sites remaining from the vegetative stage, causing the cells to detach from the glass. This, coupled with the already weak adhesion would be shown by an increased level of detachment relative to vegetative cells. It must be stressed that this argument is purely speculative. Examination of the raw data, however, does lend some support. The percentage of aggregation-competent cells detaching in buffer alone is higher than that found in vegetative cells. This would suggest that aggregation-competent cells have a lower intrinsic adhesiveness than vegetative cells. Nevertheless, the percentage detachment in inhibitor is probably still greater than would be expected when this is taken into account. The preliminary evidence for the binding of the inhibitor to the cell surface is encouraging. By incubating successive batches of cells with a volume of SPM, a definite depletion of inhibitory activity within that SPM was detected. This strongly suggests that the inhibitor was being bound, metabolised or internalised by the cells. The latter two possibilities are unlikely following fixation of the cells by glutaraldehyde. Although some enzymes do remain

functional after glutaraldehyde fixation, these are largely from one group — the proteolytic enzymes. The inhibitor is not of a protein nature, and would presumably be unaffected by these enzymes anyway. Internalisation is also dependent on the normal functioning of the plasma membrane. Glutaraldehyde fixation cross-links the membrane proteins, preventing lateral movement of proteins within the membrane, and inside/outside flipping of these proteins. Vesicle formation by membrane fixed in this manner would be impaired. In addition, if the cells were capable of removing the inhibitor at such a rate, it is surprising that it can build up to such an extent in the growth medium, unless the stationary phase cells lack the ability of log phase cells to "mop up" inhibitor after its production.

Thus, from evidence obtained in this work and from knowledge of other systems, it seems likely that this inhibitor binds to some type(s) of surface-located site in such a way as to prevent normal cohesive functioning. As yet, the exact target site(s) on the surface are unknown. The evidence presented both here and in section 6 would refute the hypothesis that contact sites A are involved. Far more likely, is that the inhibitor is acting on contact sites B, or another type of site present on the surfaces of vegetative cells. Once the inhibitory molecule is fully characterised it should be possible to label it (perhaps with a radioactive precursor) and thereby discern its exact surface location, the kinetics of its binding, and the direct effect of the acquisition of aggregation-competence upon these. This would prove an invaluable step

in the definition of the inhibitor's mode of action.

SECTION SIX:

EFFECTS OF THE INHIBITOR ON DIFFERENTIATION AND DEVELOPMENT.

6.1. INTRODUCTION.

The inhibitor has been found to partially block the cohesion of aggregation-competent cells and the cells of at least one later developmental stage. It also affected the adhesion of aggregation-competent cells to a glass substratum. Rickenberg, et al.(1975) showed that slime mould development could be blocked by a series of simple sugars. These sugars, which will enhance vegetative growth, prevented the acquisition of aggregation-competence on Millipore filters. There is also considerable evidence that cell contact is necessary for certain aspects of slime mould development (Newell, Longlands and Sussman, 1971; Newell, Franke and Sussman, 1972; Gregg and Badman, 1970; Gregg, 1971; Aldrich and Gregg, 1973; Yu and Gregg, 1975; Sakai and Takeuchi, 1971; Garrod and Forman, 1977; Forman and Garrod, 1977/b). This point is discussed in full in section 1.2.6. In particular, Garrod and Forman (1977) and Forman and Garrod (1977(b),) have shown that if Ax-2 cells are shaken in suspension under conditions which prevent the formation of aggregates, differentiation into prespore and prestalk cells does not ensue as it does within cell aggregates. These cells, however, do acquire aggregation-competence under these conditions.

Garrod and Forman's information, therefore, raises several questions. First, will the cohesion inhibitor block development on Millipore filters? Second, if so, will it do so by preventing the cells from adhering to the substrate? Third, will the inhibitor block the acquisition of aggregation-competence in the first instance in a manner presumably similar to that of sugars as described by

Rickenberg *et al.*(1975)? Fourth, will cells shaken in inhibitor, and thereby prevented from maintaining any contacts, differentiate in suspension to prespore and prestalk cells? Finally, it was important to try to investigate further whether the inhibitor affects contact sites A and at which point cells approaching aggregation-competence became insensitive to the inhibitor.

6.2. RESULTS.

6.2.1. Development on Millipore filters.

Slime mould cells will undergo synchronous development when washed free of nutrients and placed on a Millipore filter supported by a buffer-soaked pad. They proceed through the stages of aggregation, grex formation, migration, culmination and finally fruiting body construction (see Fig.1.1.) in 24 hours. The timing of each stage is constant and predictable (Fig.6.1.) It is possible, therefore, to prepare large populations of cells at the required stage of development at a predetermined time. This facilitates the study of the effects on slime mould development of certain conditions or factors, for example the point of action of blocking agents can be traced to a precise developmental stage, and alterations in the duration of any one stage in development can be finely monitored.

Ax-2 cells were plated out on filters as described in section 2.1.3. Control filters were placed on support pads soaked in buffer solution alone; test filters were supported on pads soaked in buffer to which inhibitor had been added to the required concentration per cell (generally that found in stationary phase medium unless otherwise stated). The cells were incubated in the dark.

Control populations developed normally and provided a reference with which the test filters were compared. The effects of the inhibitor were investigated in two ways: either the cells were exposed to inhibitor at time 0 and

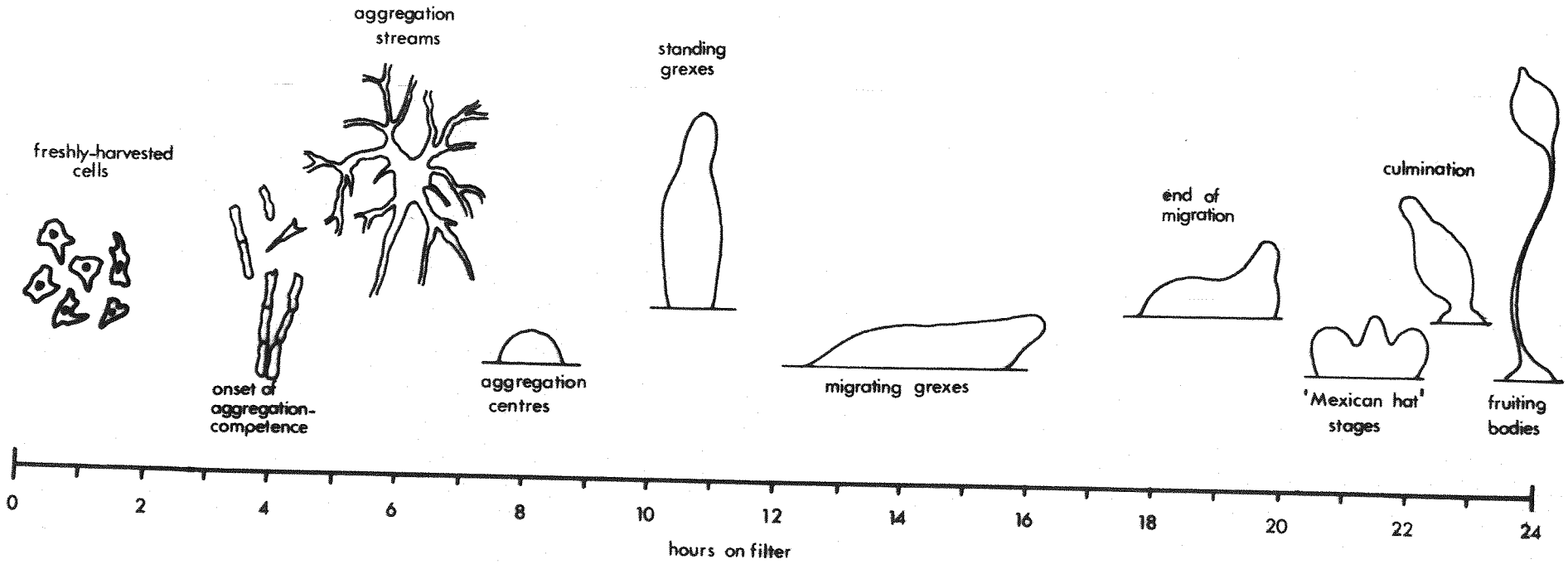

Fig. 6.1. Timing of development on Millipore filters

removed from its influence onto buffer-soaked pads at known intervals or populations of cells which had been allowed to develop normally for certain periods of time and were then exposed to inhibitor. This latter sequence enables the stages most susceptible to inhibitory action to be determined.

(i) Cells exposed to the inhibitor from beginning of development.

Cells exposed to the inhibitor from the outset do not show any signs of development even by 24 hours. If removed from the inhibitor at any point up until at least 24 hours, (longer exposures were not carried out) however, the cells will immediately begin to undergo synchronous development but the timing of this development is very much altered. The longer the period of inhibition, the shorter the time taken to reach culmination. Moreover, this "time-saving" seems to be in the period before firm aggregate formation. For example, cells which had been inhibited for 2 hours took 10 hours to begin streaming, and formed discrete aggregates after 11 hours. Those which had been inhibited for 12 hours, however, began to stream only 2 hours after removal from the inhibitory influence, and formed firm aggregates only 1 hour later. The timings, given in Fig.6.2., are the means of several different experiments (at least 4) and refer to the earliest appearance of the particular stage in a population of cells on a filter. It was also important to establish whether all

Length of inhibition (hours)	Time taken to reach specified stage after removal from inhibitor (hrs)	
	Streaming	Discrete aggregates
0 (control)	6	8
2	10	11
4	6	8
6	5	6
8	3.5	5
10	4	5
12	2	3
18	(not observed)	2
24	(not observed)	3

Fig.6.2. Effect of the inhibitor on development.

I. Cells inhibited from t_o.

developmental stages were sensitive to inhibitor or whether the effect was solely operative during the period up to aggregation.

(ii) Cells exposed to inhibitor during development.

Populations of cells were allowed to develop to various stages on filters under normal buffered conditions and when the required stage was reached, the filter was removed from the buffer pad and placed on one containing inhibitor. The effect of this action on the cells was carefully monitored and the results are presented in Fig.6.3. The most striking result was that filters which carried cells at any stage of aggregation up to the "firm aggregate" stage exhibited complete disaggregation when they were placed on inhibitor pads. 2-hour and 4-hour filters showed little or no signs of aggregation anyway, and when exposed to inhibitor the cells remained non-cohesive. Filters incubated for 6-hours and 8-hours, however, bore cells which were actively streaming and in some cases had almost completed aggregation. When these were exposed to inhibitor the cells almost immediately disaggregated and remained so for as long as the inhibitor was present. Again, this effect was reversible and once the cells were removed from inhibitory influence they began to aggregate as normal. In a very few cases, usually at the edges of the filters, some groups of cells had attained a later stage of development than the rest. Thus on the 8-hour filters, there were a few firm discrete aggregates situated around the circumference. These were not disaggregated by exposure to the inhibitor, and proceeded

Time placed onto inhibitor soaked pads (hours of development)	Time taken to reach specified stages after exposure to inhibitor (hours)			
	Aggregates	Standing grex	Migrating grex	Culmination
<8	Except in a very few cases, cells exposed to inhibitor before the formation of firm aggregates tend to disaggregate and cease development until removed from inhibitor.			
10		7.5	10-11	11.5
12		7.0	9	11.0
19				<8.0
Control (not exposed to inhibitor)	8	11.0	15-16	24.0

Fig.6.3. Effect of the inhibitor on development.

II. Cells inhibited at different developmental stages.

through the developmental pathway as usual, and with similar timing to the control filters. The later stages of development, i.e. standing and migrating grexes and the culmination stage (10-hour filters onwards) were unaffected by the inhibitor. These cells completed development in the usual way.

Inhibition of development in this manner appears to be an all-or-nothing phenomenon with regard to an aggregate. If 75% or 50% strength inhibitor is used, some aggregates are formed and complete normal development. However, large areas of the filter remain covered with cells which are inhibited and never show any sign of aggregation. When 25% strength inhibitor is used virtually all the cells undergo development as usual. An interesting observation with regard to differentiation in the presence of inhibitor was that the fruiting bodies when formed appeared to be of abnormal proportions; the spore: stalk ratio (Leach, Ashworth and Garrod 1973) appeared to be considerably less than that of control populations. This was marked in filters which were exposed to 50% or 75% inhibitor until fruiting body formation had occurred. If these filters were then placed on buffer-soaked pads, the remainder of the cells underwent development. The fruiting bodies formed by these latter cells bore much larger spore heads than those formed in the presence of inhibitor. This may bear some relation to the differentiation patterns obtained with cells in suspension (see Section 6.2.4.). The role of cell contact in differentiation is also discussed in Sections 1.2.6 and 6.3. It should be stressed that this

result is only tentative, relying solely on observations by eye. This phenomenon merits fuller investigation.

During the development of cell populations which continued to undergo development in the presence of inhibitor the migrating grex stage was often omitted completely. If present, the migratory period was very much shortened. This phenomenon is not unusual in slime moulds — migration may be omitted in adverse conditions such as abnormally high salt and sugar concentrations and low humidity.

(iii) Effect of glucose on development.

Glucose was dissolved in the Millipore buffer solution to a concentration of 100mM, and the support pads were saturated with this solution. Ax-2 cells were plated onto filters on top of these and incubated under standard conditions.

Development of these cells almost paralleled the development of cells on control filters. Some populations were slightly delayed, completing fruiting body formation some two or three hours after the control populations. There was no blocking of development analogous to that effected by the cohesion inhibitor. Under these conditions, therefore, glucose does not mimic the inhibitor in its effects upon development.

(iv) Effect of inhibitor on development of other slime mould species.

The effect of the inhibitor on the development of other

slime mould species on Millipore filters was also investigated. Vegetative cells were harvested from agar plates at the feeding stage, washed and plated out in the normal way. P.violaceum cells were completely inhibited at 100% normal inhibitor concentration and development at 75% strength was negligible. At 50% strength considerably more cells had undergone development, although about half the cells were still non-cohesive. D.mucoroides cells, at the 100% concentration, showed some development (equivalent to that of P.violaceum at 50%) and at the 75% and 50% strengths development was similar to the control populations. The inhibition of development observed in both P.violaceum and D.mucoroides was readily reversible when the filters were placed on buffer-soaked pads.

6.2.2. The development of aggregation-competence.

Aggregation-competent cells can cohere in the presence of inhibitor, albeit at a reduced level. They cannot, however, adhere well to glass when treated with inhibitor. Development on a solid substratum is blocked by the inhibitor during the aggregation stage but at the firm aggregate stage and thereafter development is unaffected. Does the inhibitor block the acquisition of aggregation competence?

(i) The effect of the inhibitor in suspension.

Log phase Ax-2 cells were suspended in buffer and in buffer plus inhibitor (sufficient to maintain a single cell suspension) at a concentration of $1x10^7$/ml and shaken for 8 hours. An assay of aggregation-competence was then performed (section 2.1.5.).

Cells in buffer had formed large, round aggregates which had to be dissociated mechanically in cold distilled water using a drawn-out Pasteur pipette. When placed on a cover-slip in salt solution they immediately flattened onto the glass, elongated and began to form the chains characteristic of aggregation-competence (see Fig.6.4). Cells which had been shaken in inhibitor cohered to a limited extent. They were washed thoroughly and dissociated into single cells and when placed on glass in a buffered salts solution they too flattened onto the glass and assumed the chain-like configuration of aggregation-competent cells. The time taken for this to occur was about 30 minutes; that for the control cells was 15-20 minutes. This delay is not of great significance, since the total time-span involved is so short anyway. It possibly constitutes the time taken for the cells to recover from the inhibitory influence. The inhibitor, therefore, does not appear to block the acquisition of aggregation-competence in suspension.

(ii) EDTA-insensitive and inhibitor-insensitive cohesion.

As post-feeding cells shaking in buffer begin to develop aggregation-competence, their cohesive properties alter. They become progressively insensitive to the effects of EDTA (Beug, et al.1973,a). This property is attributed to the gradual appearance on the cell surface of the new contact sites A which are unaffected by EDTA chelation, and are thus responsible for the increasing cohesiveness of these cells. Since fully aggregation-competent cells can cohere in the

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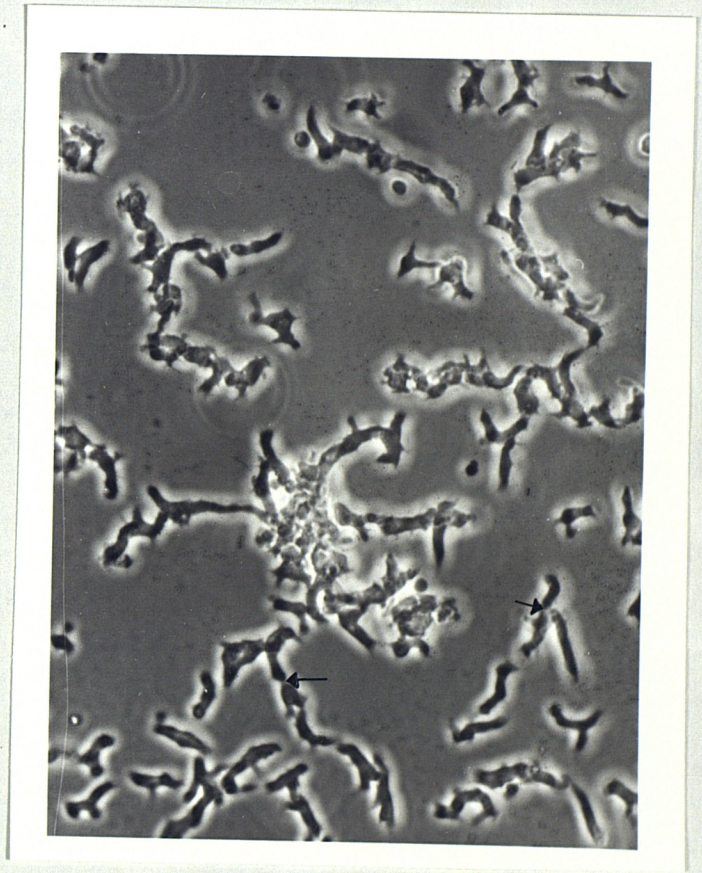
Fig. 6.4. Chain-like configurations of cells characteristic of aggregation-competence. Note the extensive formation of end-to-end (contact sites A-mediated) cohesions (arrowed), flattened, elongate morphology, and side-to-side cohesions where cell chains merge.
×300



presence of inhibitor, it was interesting to see whether the cells developed their EDTA-insensitive and inhibitor-insensitive cohesive properties in parallel. If so, the probability that contact sites A are unaffected by the inhibitor increases.

A population of Ax-2 cells were washed, suspended in buffer at $1x10^7$ cells/ml and shaken under standard conditions. Samples of these cells were taken at intervals, washed and suspended in either 0.01 M EDTA in phosphate buffer or in inhibitor solution. Their cohesiveness in these two solutions was assayed. The experiment was repeated several times with different batches of log phase cells. The pattern of cohesion was the same in each case; the means and standard errors (error bars indicate $\pm$ 1 s.e.) are shown in Fig.6.5. At time 0 hours, there was no cell agglomeration in either the inhibitor solution or in EDTA. This was maintained at 2 hours. After 4 hours' shaking in EDTA, however, permanent cohesive contacts were evident, although the overall level of cohesion was very low. Thereafter, the level of cohesion in both solutions increases rapidly and in parallel. By 8 hours' shaking the cells are fully aggregation-competent and are correspondingly cohesive in EDTA and in inhibitor. Both EDTA-insensitive and inhibitor-insensitive cohesions therefore, appear to begin to develop at about 4 hours' starvation. From then on both types of cohesion increase in parallel. EDTA-insensitive cohesion, though, is always greater than that of inhibitor. By 8 hours the cells cohere in EDTA

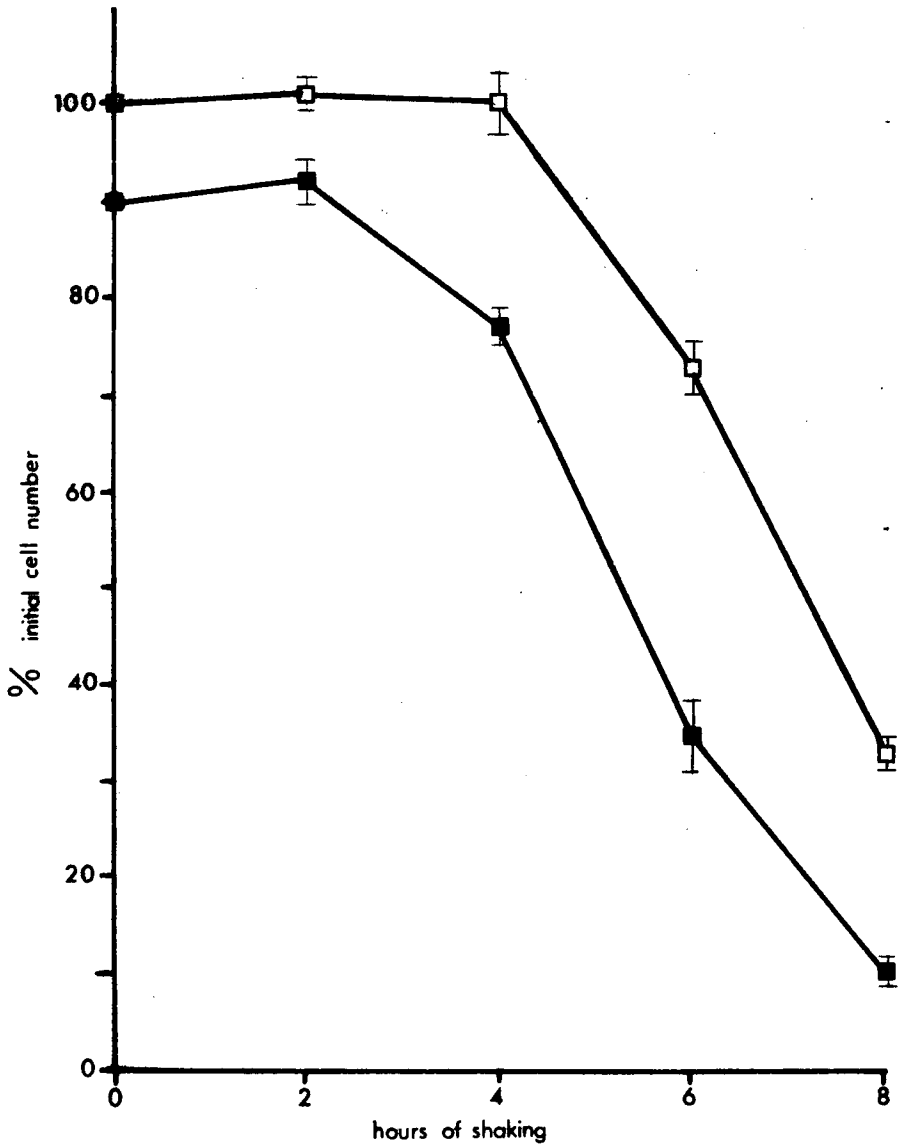


Fig.6.5. Cohesion of Ax-2 cells in inhibitor (□——□) and in EDTA (■——■) after shaking in phosphate buffer.

in large clumps, at a level indistinguishable from that normally observed in phosphate buffer alone. The cohesion of the same cells in inhibitor remains partially inhibited, at the level characteristic of aggregation-competent cells.

6.2.3. The effect of the inhibitor on aggregating cells.

The study of the effects of inhibitor treatment on cells already in the process of aggregating was approached microscopically using both the light and scanning electron microscope.

(i) Light microscope study.

Aggregation-competence was developed in a population of Ax-2 cells by shaking in buffer in the normal way. After 8 hours' shaking a sample of the cells was taken. The cell clumps were disaggregated in cold distilled water and the cells then suspended in buffer. Small amounts of this suspension were introduced into the chambers used previously for observing the effect of the inhibitor on the cells' adhesion to glass. The cells were allowed to settle and attach to the glass and then the chamber was gently inverted and placed on the microscope stage (Zeiss Universal, Germany). The cells rapidly developed characteristic end-to-end contacts and began to assume chain-like configurations (cf.Fig.6.4.). A representative field of these was photographed (Fig.6.6.) Double strength inhibitor was then introduced to the suspension in the chamber by means of a syringe. The inhibitor solution was exuded gently from the syringe and allowed to diffuse through the field of cells.

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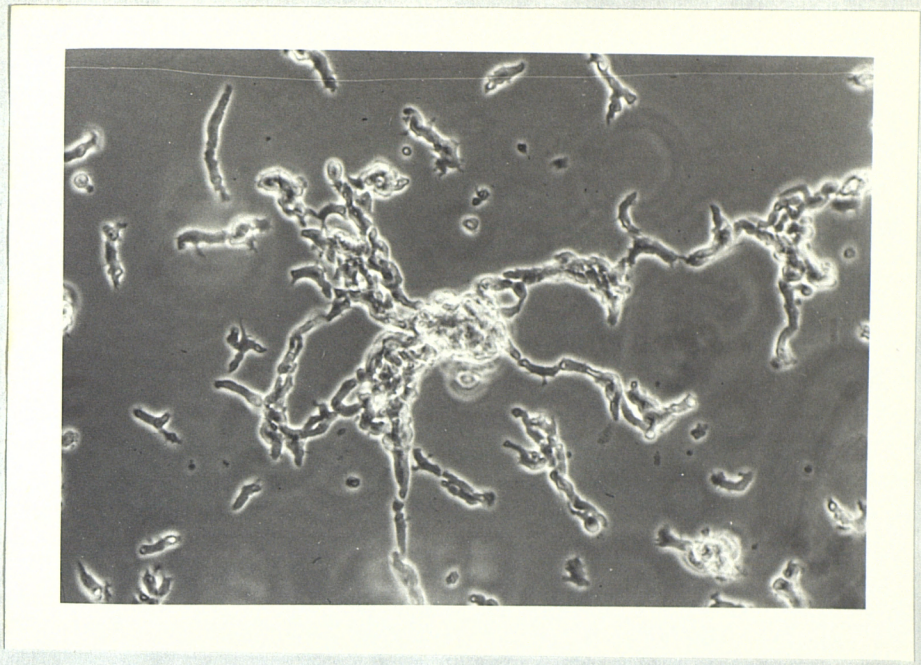
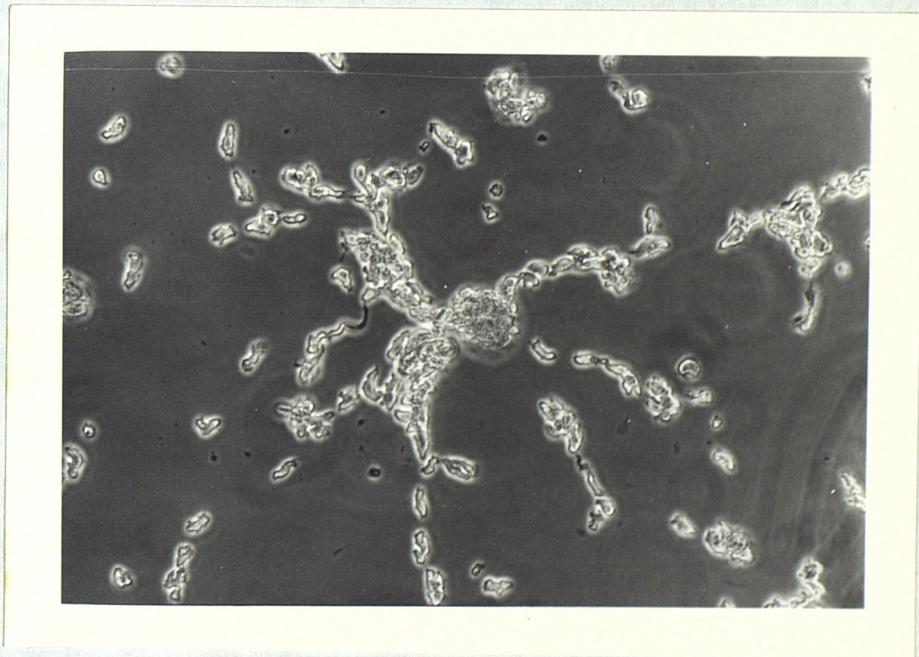
Fig.6.6. The effect of inhibitor on aggregation-competent cells attached to glass and already cohering by end-to-end contacts: (a) cells in buffer; (b) cells 5 minutes after addition of inhibitor, (see text for details).

×300



a



b

After 5 minutes the same field was re-photographed without altering the focus. The cells were then out of focus, but still visibly coherent by their end-to-end contacts. When inhibitor was added the cell chains always behaved consistently, rounding up very slightly, detaching from the substratum and floating through the medium. The side-to-side cohesions had not formed in sufficient numbers at this stage to judge the effect of the inhibitor on them. Since, however, the cells responded in the way described above over a course of several trials it may be concluded that the inhibitor does not, at least, disrupt the end-to-end contacts of aggregation-competent cells.

(ii) Scanning electron microscope study.

This study was initiated in order to try to answer two questions. First, does the inhibitor alter the adhesion of aggregation-competent cells to the surface of a Millipore filter as it does on glass? Second, is the gross morphology of these cells altered by the inhibitor in such a way as to make them unable to forge contacts with each other?

Ax-2 Cells were plated onto Millipore filters supported on buffer-soaked or inhibitor-soaked pads respectively. These were left to develop in the dark for 4 hours, removed and prepared for S.E.M. study by the rapid-freeze method described in section 2.5.2. The resulting specimens were viewed and photographed on a Jeol P15 (15Kv) Scanning Electron Microscope. The photomicrographs are shown in Fig. 6.7.

Photographs (a) and (b) show cells developing on control

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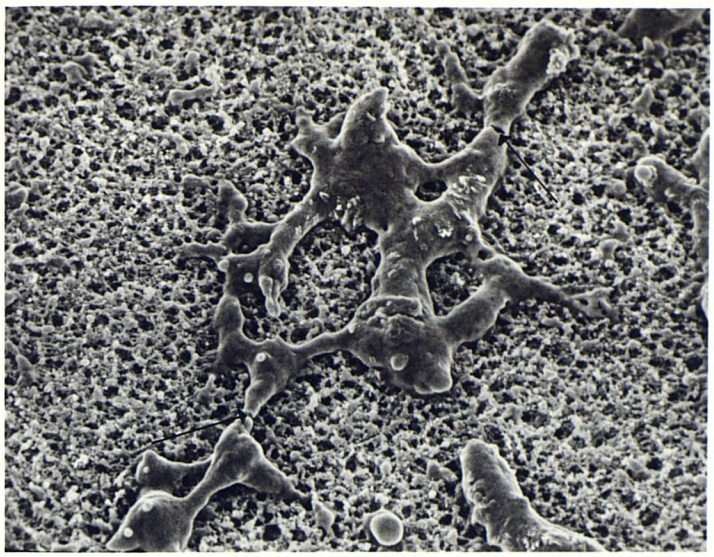
Fig.6.7. Scanning electron micrographs of Ax-2 cells on Millipore filters.

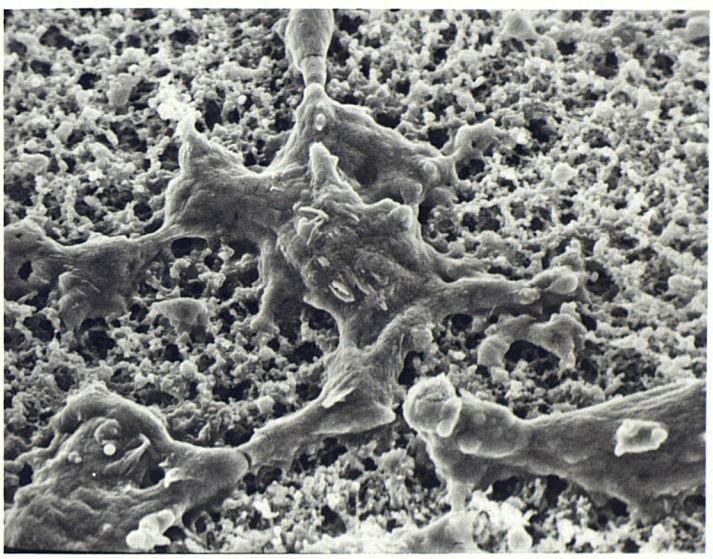
(a) Aggregating cells (no inhibitor) cohering by end-to-end contacts (arrowed) x 900

(b) As (a) x 1.8K

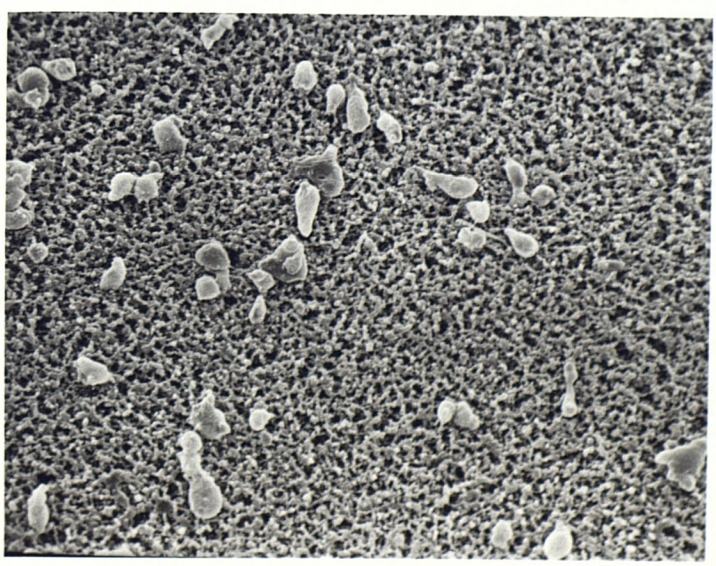
(c) Cells exposed to inhibitor x 900. Note back of orientation and absence of end-to-end contacts.



a



b



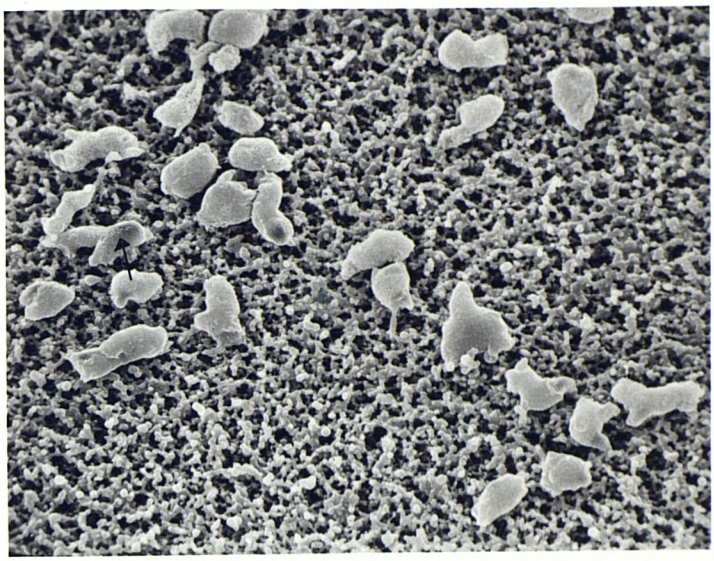
c

(To face page 171a)

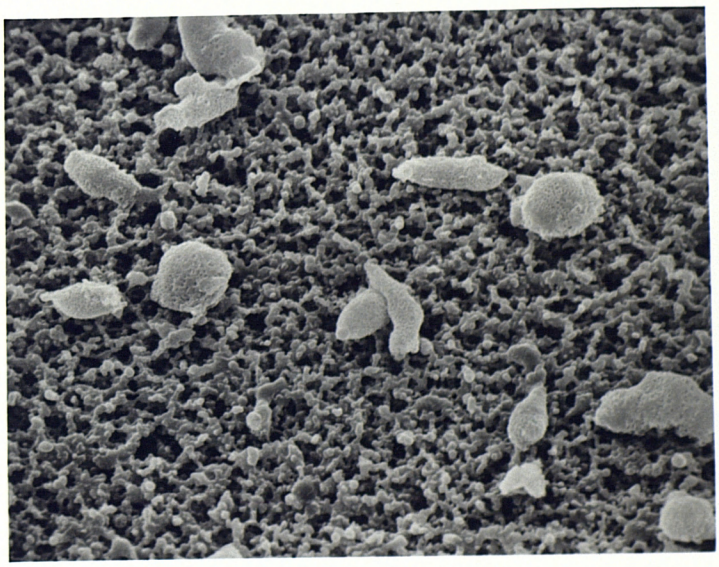
S.E.M. Contd.

(d) Cells exposed to inhibitor x 1680. Note cell on left (arrowed) which appears to be crawling over another cell.

(e) Cells exposed to inhibitor x 1.8K. Again note cell in centre which seems to be moving over its neighbour.



d



e

filters exposed only to buffer (4 hours' incubation). These cells are already showing the flattened alongated morphology characteristic of aggregation-competence, and are cohering by both end-to-end (arrowed) and side-to-side cohesions (in the aggregate masses).

Cells exposed to inhibitor (also 4 hours' incubation) are shown in (c), (d) and (e). Although the cells do not show the extremely flattened and elongated shape of the control cells, they do appear to possess polarity and to be migrating over the surface of the filter. When in suspension, before plating onto the filters, these cells are spherical. On the filter, however, they have flattened and elongated to some extent. The inhibitor, therefore, does not appear to prevent the cells adhering to the substrate and block their motility over it. The cells do not, though, exhibit end-to-end contacts as do control cells. Moreover, their side-to-side encounters do not appear to result in stable permanent cohesions. In (d) and (e) some cells seem to be actually crawling <u>over</u> other cells (arrowed). Whether this is a correct interpretation of these photographs is difficult to say at present and the situation merits further investigation.

6.2.4. <u>The effect of the inhibitor on differentiation in suspension: the role of cell contact</u>.

Cells shaken in suspension to form aggregates will undergo differentiation to become prestalk or prespore cells after 18 hours. Cells shaken so fast that they cannot cohere, become aggregation-competent but do not differentiate under

these conditions (Forman and Garrod, 1977/b)). Since the inhibitor will maintain a suspension of cells in a dissociated state, it was apposite to check whether this would also prevent differentiation.

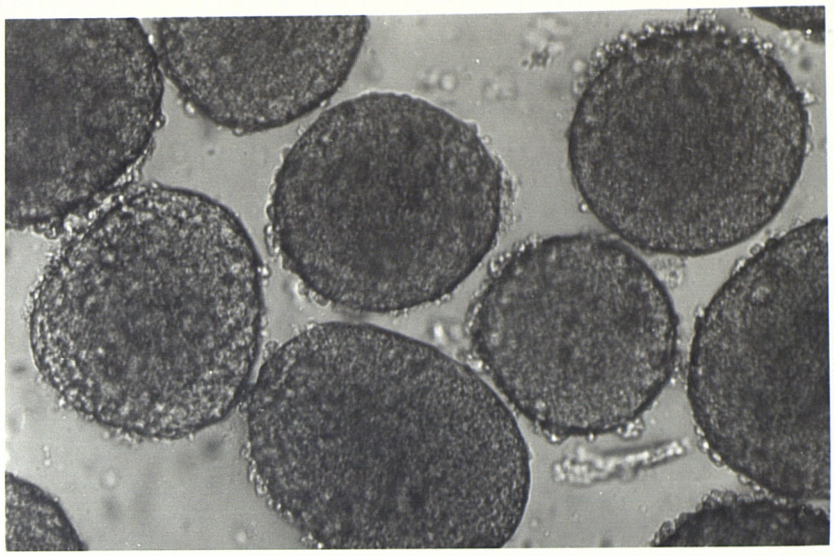
Ax-2 cells were shaken in inhibitor solutions of different strengths for a period of 24 hours. 500 units/ml of penicillin and streptomycin (Flow Laboratories, Scotland) were added to prevent infection. At the end of this period the suspensions were photographed (Fig. 6.8.) to show the degree of agglomeration at each inhibitor concentration, and the mean aggregate size. At 100% inhibitor (these values are expressed as the percentage of the concentration found in stationary phase medium). The cells were still almost completely single; a few existed in couplets or triplets but most were without contact with another cell. The number of cells cohering in small aggregates increases through the diminishing concentrations of inhibitor. At 0% (buffer alone) large round aggregates formed. Similar aggregates formed from cells which had been shaken in 100% inhibitor for 24 hours (i.e. contact completely prevented) and then in buffer for a further 24 hours. After each suspension was photographed it was centrifuged down, washed, and the cells dissociated by trituration. A drop of cell suspension was then placed on a slide and stained with FITC-labelled anti-spore serum (Forman and Garrod, 1977 a,b.). This serum causes the prespore vesicles within differentiating cells to fluoresce. Prestalk cells, however, remain unstained, under an ultraviolet light source. Each sample was then viewed on

(To face page 174)

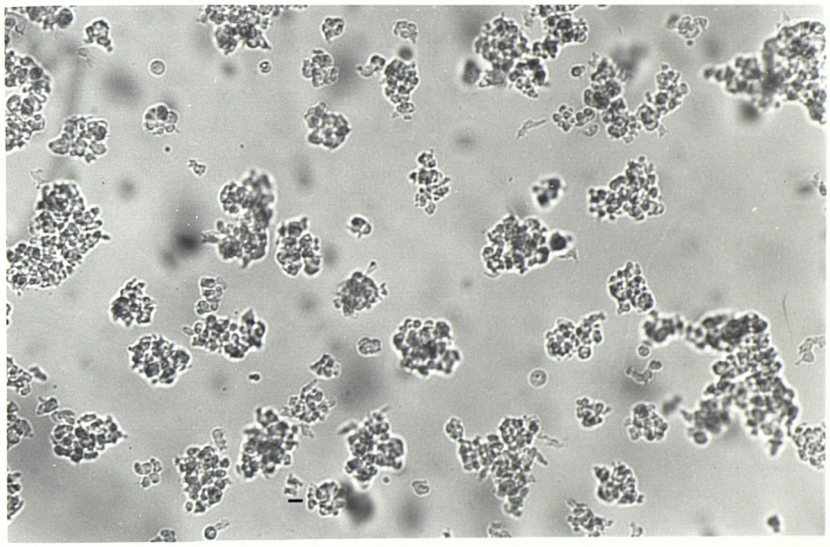
Fig. 6.8

The blocking of aggregation of Ax-2 cells in suspension by the inhibitor. Concentration of inhibitor is shown beside each photograph. In 100% inhibitor stable cell-cell contacts are prevented. Decreasing inhibitor concentrations allow greater degrees of aggregation. The last photomicrograph shows aggregates formed by cells which were kept in 100% inhibitor for 24 hours and transferred to buffer for a further 24 hours.

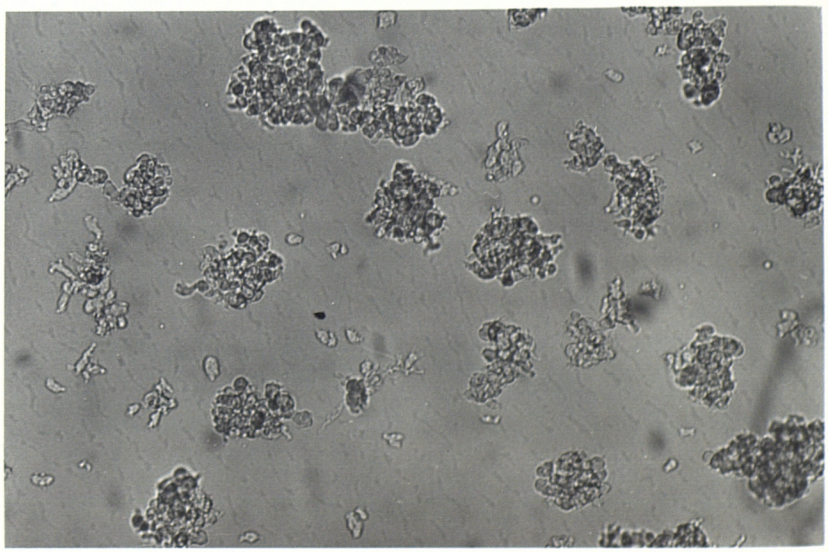
0%



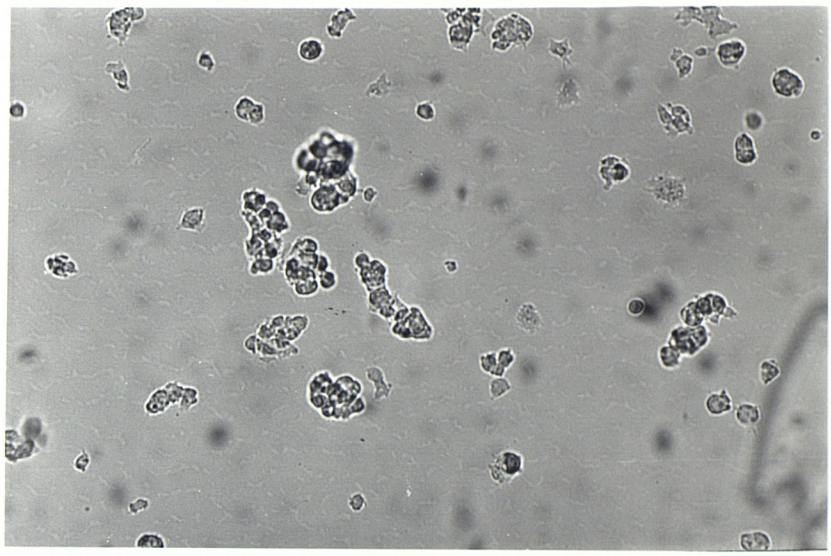
20%



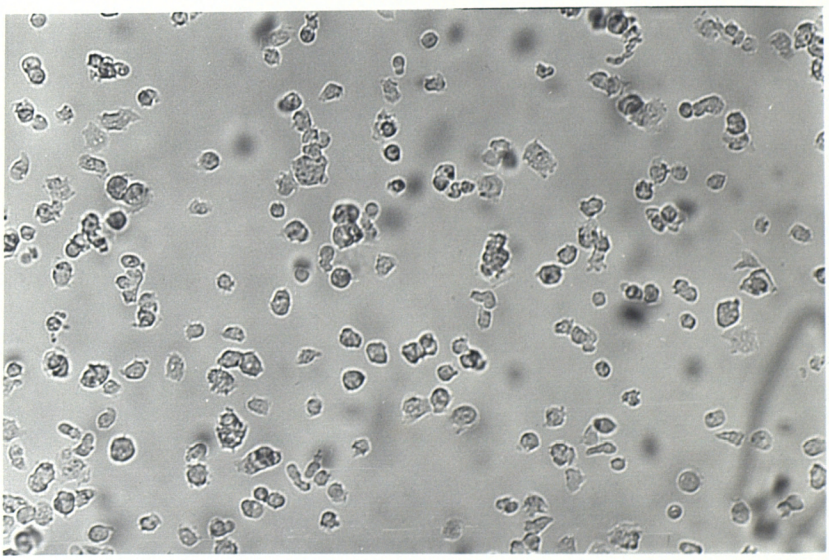
40%



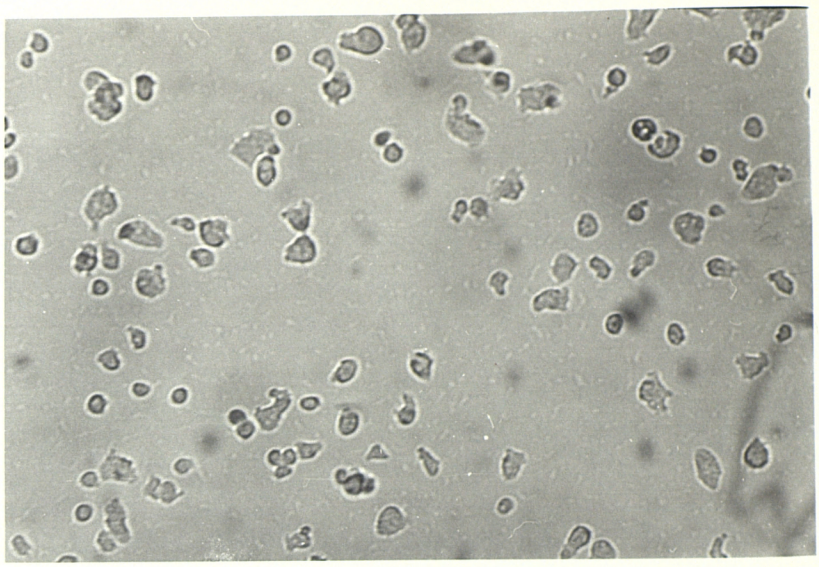
60%



80%



100%



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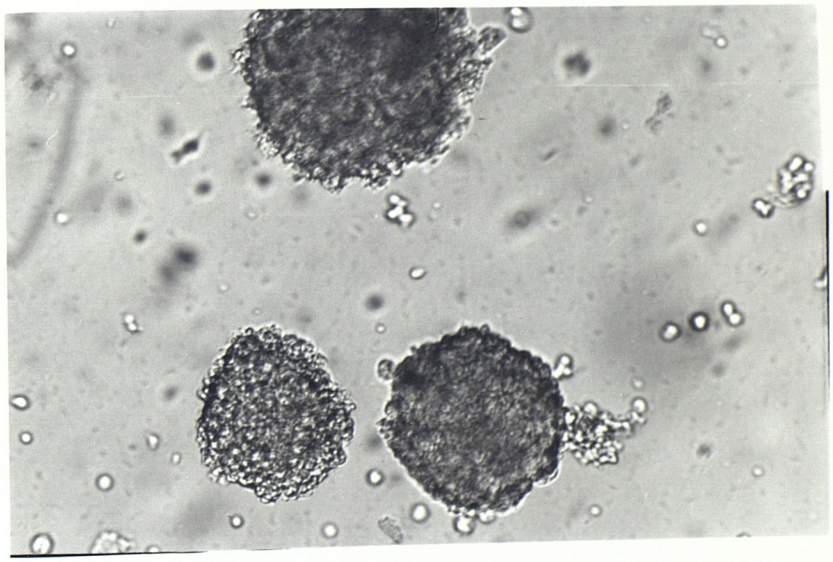

a Leitz Ortholux (Wetzlar, Germany) Ultraviolet Microscope. The total number of cells, and the number of fluorescing were counted in successive fields until at least 500 cells from each sample had been counted. At least three samples from each suspension were used and the experiment was performed twice. The results were pooled and are presented in Fig.6.9. Lack of time prevented further repeats of this experiment. Had these been done, these results would have been more acceptably representative. The consistency, however, in the two trials which were done warrants the inclusion of these results. They may be accepted as indicative of a trend but further data is needed before they can be taken as statistically valid.

Cells which had been maintained in a completely dissociated state (100% inhibitor) showed no evidence of prespore vesicles and hence of differentiation. Cells shaken in 80% inhibitor (which allowed only a minimal degree of cohesive contact) exhibited a correspondingly minimal amount of differentiation to prespore cells. Of 555 cells, only 10 showed any fluorescence. In 60% inhibitor small aggregates of up to 10 cells formed over 24 hours. When the cells were disaggregated and stained with FITC-labelled antispore serum the proportion of prespore cells had increased to almost 1 in 32 cells. At lower inhibitor concentrations the number of cells cohering in aggregates and the proportion of prespore cells rose correspondingly. In buffer alone, the ratio of prespore to prestalk cells was

% inhibitor	Stained cells (PSV'S)	Unstained cells (No PSV'S)	Ratio S/U
0%	2061	1356	1 : 0.66
20%	266	1227	1 : 4.61
40%	76	1663	1 :21.88
60%	22	1101	1 :50.05
80%	10	1045	1:104.50
100%	-	1024	1 : ∞
100% (buffer 24h)	364	1172	1 : 3.22

Fig.6.9. Effect of the inhibitor on differentiation in suspension of Ax-2 cells.

The blocking of differentiation of cells in suspension by the inhibitor. 'Stained' cells are those which contain fluorescing prespore vesicles (PSV's) Unstained cells do not possess these vesicles and are either prestalk cells, or undifferentiated cells of either type. The "normal" level of PSV formation is seen in the control population incubated in buffer alone.

approximately 2:1. This figure is in agreement with previous data both from Forman's shaking suspension work (Forman and Garrod, 1977 b) and measurements made on the final fruiting bodies (Garrod and Ashworth, 1972). It, therefore, appears from these results that cell contact is an essential requirement for cell differentiation in D.discoideum, or at least for the formation of prespore vesicles.

Cells which had been shaken for 24 hours in 100% inhibitor and subsequently for 24 hours in buffer gave an interesting result. The proportion of cells with prespore vesicles was high, about 1 in 5. This was higher than that obtained with 20% inhibitor, and approaching that of buffer alone. A sample of these cells taken after the 24 hours' shaking in buffer, showed no signs of prespore vesicle formation. Whilst shaking in buffer for a further 24 hours, the cells cohered into large round aggregates. During this time the prespore vesicle formation (i.e. differentiation) occurred, whilst the cells maintained prolonged contact. Under these conditions differentiation proceeded at a rate almost comparable to that of the control populations. This result also provides the additional reassurance that after 24 hours in 100% inhibitor, the cells are still viable and functioning normally.

6.3. DISCUSSION.

Several interesting points have emerged in this section concerning first the inhibitor and its action and second the role of cell contact in slime mould development.

The Ax-2 cells would not aggregate on Millipore filters in the presence of inhibitor. Once removed from this inhibitory influence, however, they underwent aggregation in a much shorter time than is usual. Moreover, the longer they were exposed to inhibitor the shorter their aggregation time. This suggests that although the cells are prevented in some way from aggregating, they still undergo the biochemical changes necessary for aggregation (i.e. they become aggregation-competent but are unable to aggregate). If this suggestion is true, the formation of contact sites A would probably occur during this period of inhibition. Once the inhibitory block is released, the process of aggregation can then proceed as normal. Since all the differentiative changes have already been made all that remains is the actual cell locomotory component of aggregation, which is then completed in a short period of time. If the cells have received a relatively short exposure to inhibitor (viz.2-3 hours) then the aggregation period is necessarily longer, since the differentiation process has not been completed. Once the cells have formed firm aggregates they remain unaffected by the inhibitor's action.

The reason why cells are unable to stream in the presence of the inhibitor has not been fully resolved.

Two possible explanations seem likely. First, cells may be prevented from adhering sufficiently strongly to the Millipore substratum by the inhibitor and, therefore, could not physically move over the filter surface. Second, the presence of inhibitor may prevent the appearance on the cell surface of contact sites A. To investigate the former possibility more fully the S.E.M. study was undertaken. It was concluded from this study that the cells were able to adhere to the substratum.

Whether this adhesion was sufficient to allow free movement over the surface is not clear from the photomicrographs, but since the degree of flattening of inhibited cells was similar to that of the aggregating cells, they certainly appear to have no impediments in this area.

The second possibility, that the inhibitor may prevent the appearance of contact sites A, was also investigated. Shaking the cells in inhibitor for 8 hours did not block the acquisition of aggregation-competence. The cells were able to form end-to-end contacts when washed and placed in buffer. In addition, cells 'inhibited' on filters for 8 or 10 hours were seen to begin streaming almost immediately when released from the block. Therefore, it seems unlikely that CSA are actually formed in this short time. It is more likely that these sites are not exposed on the cell surface in the presence of inhibitor, but on removing the inhibitory influence they may be exteriorised. Whilst this is only a hypothesis there is further evidence to support it: the cells which <u>become</u> aggregation-competent

when shaken in the inhibitor do not cohere to the same degree as normal aggregation-competent cells that have been placed in inhibitor. They will, however, form the characteristic chains within 30 minutes when washed and placed in buffer. Second, these cells show a 10-15 minute delay over control cells in forming these chains by end-to-end contacts. This period may possibly constitute the time taken for the CSA to become exteriorised.

If this hypothesis is correct, the disaggregation of streams of cells on Millipore filters by inhibitor up to the firm aggregate stage must be due to the disruption of the contact site B-mediated side-to-side cohesions. This is possible because cohering cells are readily disaggregated by the inhibitor even while shaking in suspension. Once cells are cohering by means of the CSA system, however, disaggregation is not complete. This is supported in the photographs of Fig.6.6. where the end-to-end (CSA-mediated) contacts held firm when inhibitor was added, although the cell-substrate adhesions were broken.

The likelihood that EDTA-insensitive (CSA-mediated) and inhibitor-insensitive cohesions occur via the same set of surface sites is also supported by data shown in Fig.6.5. These two types of cohesion develop in parallel, strongly suggesting that they are mediated by one and the same system.

Not surprisingly, in view of the results using shaking suspensions (5.2.2.(i)), D.mucoroides and P.violaceum cells on Millipore filters are also prevented from developing by the inhibitor. D.mucoroides cells, however, appear less susceptible to the inhibitory effect than are

P.violaceum cells. This is not reflected in the cohesion of these cells in suspension in the presence of inhibitor (Fig.5.2.) and once again it is possible that the D.mucoroides cells had already begun to acquire aggregation-competence before they were harvested. In culture on E.coli, cells of this species clear the bacteria in a shorter and less predictable length of time than cells of other species. It is quite possible, therefore, that the 'vegetative' cells used in this set of experiments were, in fact, verging on aggregation-competence, which may explain this result.

The use of the inhibitor to investigate the role of cell contact in slime mould development supported the findings of Forman and Garrod (1977 a,b; Garrod and Forman,1977). These authors were able to achieve cell differentiation when cells were shaken in suspension under conditions which allowed the formation of large cell aggregates. No signs of differentiation were observed, however, if the cell suspensions were shaken so fast as to prevent the formation of cell cohesions. If the shaking speed was then reduced, aggregate formation occurred and differentiation ensued.

The use of inhibitor mimics the effects of fast-shaking but avoids the possibility of adverse effects from a high shear force on the cells. Under conditions of complete inhibition there was no evidence of differentiation after 24 hours' shaking. Decreasing inhibitor levels allowed an increase in the formation of stable cohesions. This resulted in a commensurate increase in the degree of differentiation. Cells shaken in 100% inhibitor for

24 hours differentiated only when allowed to agglomerate subsequently in buffer over 24 hours.

The necessity for cell-cell contact in development and differentiation in the slime moulds is, therefore, confirmed by this preliminary investigation using the cohesion inhibitor. The ease with which the degree of cell contact can be controlled using this system merits further work which may reveal more information on the degree and type of contact crucial for differentiation.

SECTION SEVEN:

DISCUSSION.

The most fundamental and pertinent question raised by this work concerns the biological importance of the cohesion inhibitor. Does this inhibitor fulfil a useful function in the axenic medium in which it appears? It seems unlikely that it is produced by bacterially-grown cells, for if it were present in cultures grown in association with E.coli then the cells would be unable to aggregate. This suggests that it is either destroyed at some point before aggregation begins, or is produced in quantities that are sufficiently small to allow normal development to take place. If the inhibitor were present, moreover, it would be difficult to visualise a useful role for it in these cultures. It is possible that the substance itself could have another purpose and its action on cell cohesion may be a side-effect. Since this 'side-effect' is so detrimental to aggregation, however, it does not seem plausible for this type of substance to have been evolutionarily useful. It is far more likely that the cohesion inhibitor is produced only by axenically-grown cells — cells which are well known to differ biochemically in many ways from their wild-type parent strain (Ashworth, 1971; Firtel and Bonner, 1972.). The biological significance of this inhibitor in axenic cultures is nevertheless difficult to understand, especially since it is produced only in stationary phase. Stationary phase cells in liquid culture do not proceed to aggregation-competence as do log phase cells starved of nutrients.

Several authors now believe that the attainment of stationary phase may not necessarily be directly due to a depletion of nutrients, but is the result of a response to other environmental factors, such as the accumulation with increasing cell density, of a macromolecular growth inhibitor (Hanish, 1975).

Stationary phase cells will, however, given the correct conditions, undergo morphological and behavioural alterations and revert to either log phase cells when in axenic culture, or aggregation-competent cells when on a solid surface. Both these phenotypic changes take place only after a lag period which is not observed when normal dividing cells are subjected to these procedures.

Stationary cells may, therefore, be regarded as a new phenotype differing in many ways from log phase axenic cells, or developing cells in their morphology and biochemistry (Weeks, 1973; Rossomando et al, 1974, Malkinson and Ashworth, 1973; Cocucci and Sussman, 1970; Mirick et al, 1974; Yarger et al 1974, Soll and Sussman, 1973) and in their cohesive behaviour (Swan and Garrod, 1975). At present, the real biological significance of the production of inhibitors of cohesion, division and transcription in this phase remains obscure. All these observable activities may simply be the effects of the end-products of metabolism which are accumulating in the medium.

Although the cohesion inhibitor cannot be assigned a functional role at present, a study of its actions was

still important for what it could reveal about the mechanism of cohesion in slime moulds. It has been possible to answer some of the major questions relating to the biological effects of the cohesion inhibitor. These answers in turn have helped to formulate a tentative hypothesis regarding the mode of action of this inhibitor. It is desirable at this point to summarise the work presented in this thesis. The most effective way to do this is in question and answer form. The possible modes of action of the inhibitor are then discussed in relation to these answers, followed by a schematic representation of the most probable method by which the inhibitor exerts its effects.

Which cells produce the cohesion inhibitor?

It appears that log phase cells do not excrete inhibitor or, if they do, only in quantities insufficient both to allow detection and to inhibit the cohesion of log phase cells in their growth medium. It is most likely that the inhibitor is produced only by cells which have ceased to divide.

Which cells are sensitive to the cohesion inhibitor?

Of axenically-grown (Ax-2) cells, only log phase cells are sensitive to the inhibitor. Stationary phase cells are intrinsically non-cohesive anyway. Aggregation-competent Ax-2 cells are partially prevented from cohering by the inhibitor. Grex cells show

the same cohesive pattern as aggregation-competent cells.

Is this sensitivity to the inhibitor specific to D.discoideum?

Preaggregation (vegetative) cells of D.mucoroides, D.purpureum and P.violaceum are susceptible to this inhibitor's effects. In addition, vegetative cells of the wild-type strain (NC-4) of D.discoideum, grown in association with E.coli, are also inhibited by this substance.

Does the inhibitor have any effect on the development of cells on a surface?

Cells exposed to the inhibitor until the formation of firm aggregates will dissociate, and further development will not occur while inhibitor is present. When removed from inhibited to buffered conditions, the cells proceed with development as usual, but after prolonged exposure to inhibitor the timing may be altered. In such cases, development is completed far more quickly than normal. The development of cells of D.mucoroides, D.purpureum, P.violaceum and D.discoideum (NC-4) is also blocked by this inhibitor.

Given that aggregation-competent cells can cohere to some degree in inhibitor, but are blocked on a surface, does the inhibitor prevent the acquisition of aggregation-competence?

Cells shaken in the presence of inhibitor exhibit

the cohesive behaviour characteristic of aggregation-competence on a glass surface after the normal time-span of 8 hours. They do not, however, cohere whilst still in suspension to the same degree as cells which have become aggregation-competent in buffer and have been subsequently transferred to inhibitor.

Does the inhibitor bind to the cells?

Preliminary evidence, based on the loss of the inhibitor from the medium when incubated with successive batches of fixed log phase cells, suggests that the inhibitor was being absorbed from the medium by these cells. The simplest explanation, since the cells were fixed, is that the inhibitor binds to the cell surface.

Does the inhibitor affect the contact sites A which appear at aggregation-competence?

The cohesion inhibitor does not disrupt contact sites A which are already maintaining cohesive contact between aggregating cells, although it does cause detachment of the cell streams from their substratum. Cells exposed to inhibitor before the onset of aggregation-competence, however, do not form these cohesions either under shaking conditions or on a surface. The development of EDTA-insensitive (i.e. contact-sites-A-mediated) and inhibitor-insensitive cohesion occurs in parallel.

Since the inhibitor may be binding to the surface, or at least to some component(s) of fixed cells, it is possible that it acts by blocking some type of adhesive or cohesive site on the surface membrane. Work reported from other sources suggests several possible alternative explanations. The relative merits of these are now discussed:

1. The inhibitor functions as a chelating agent.

The most important divalent cations in the development of D.discoideum are calcium (Ca^{2+}) and manganese (Mn^{2+}) (Sussman and Boschwitz, 1975,b). Cohesion of preaggregation cells is EDTA-sensitive, which demonstrates the necessity for divalent cations in some component of the cohesive process. As the cells become aggregation-competent, the binding sites for these cations increase and the cations become more tightly bound (Sussman and Boschwitz, 1975 a,b). Should the inhibitor be acting as a chelator, it may be expected to have a reduced effect on cell cohesion at the aggregation-competent stage, which it has. To this extent, the hypothesis fits the evidence. However, when log cells are shaken in inhibitor they remain non-cohesive for at least up to 24 hours. They will exhibit the cohesive behaviour characteristic of aggregation-competence when placed, in buffer, on a surface. Cells shaken in EDTA-buffer for 8 hours, however, will not only show this cohesion on a surface, but also begin to cohere in the EDTA-buffer from about

4 hours shaking. If the inhibitor were functioning in the same way as EDTA, then, this gradual development of cohesion in suspension would be expected. Moreover, EDTA considerably alters cell morphology by causing the cells to round up. The inhibitor does not seem to have this effect.

2. The inhibitor affects the cAMP system.

Work by Brachet and Klein (1975), in particular, pinpoints the blocking of aggregation and/or growth of D.discoideum cells by certain hormones to interference with the cAMP chemotactic system. For example, progesterone (at 4×10^{-5} M) and to a reduced extent, dehydroepiandrosterone, oestradiol and testosterone inhibit growth and aggregation of D.discoideum amoebae. Other hormones, such as pregnenolone, are active only on growth. Progesterone, however, is active throughout the aggregation phase, preventing starved cells from becoming aggregation-competent, and inhibiting the aggregation of already-competent cells. After a brief rounding-up, the cells reattach to solid supports and are able to form pseudopods. In many of these ways, progesterone mimics the effects of the inhibitor. (The acquisition of aggregation-competence was tested in the presence of hormone, whereas with inhibitor the cells were first resuspended in buffer. Had this step not been taken, the cells would appear to have failed to develop aggregation-competence). Could the inhibitor, therefore, be

having an adverse effect on the cAMP system? Two points may be argued against this. First, cells which are already oriented and in the process of aggregating are dissociated when exposed to inhibitor. Any factor affecting the chemotactic system could have this effect, since it could disorientate the cells sufficiently for their cohesions to be broken and for them to move apart. After aggregate formation, however, the inhibitor has no effect on the cells. At these later developmental stages, though, the cAMP system is still essential to the ordered process of successful development (Town and Gross, 1978). It follows that the inhibitor, if disrupting the cAMP system, would adversely affect these stages of development, which it does not do.

Second, there is no evidence to suggest that cAMP has any importance in the cohesive processes of pre-aggregation or log phase cells. It is at these growth stages that the inhibitor is at its most effective. It is possible that the inhibitor may have side-effects which interfere with the cAMP system, but it seems unlikely that it exerts its primary effect in this manner.

3. <u>The inhibitor affects the topography of membrane components</u>.

Recently, much information on the importance of surface receptor topography and membrane fluidity has been reported in connection with cell adhesion studies.

For example, fixation of the cell membrane prevents clustering of conA receptors on lymphoma cells and inhibits cell agglutination by the lectin although the binding of this lectin is not affected (Inbar et al, 1973). The clustering of these receptors into patches is not a metabolically-driven process, but the more complete process of capping is metabolically-dependent (Shields, 1975). Microtubules and microfilaments have been implicated to be important in these processes (Shields, 1975; Yahara and Edelman, 1972, 1975; Ukena et al.1974; Tahara and Edelman, 1975).

Agglutinating factors, which can mediate specific cell-cell interations, can control the surface topography of their receptors, and it has been suggested that they are important in the recognition, adhesion and histotypic rearrangement of specific cell types with regard to cells of other types (McDonough and Lilien, 1975). These agglutination factors may hold the surface in the appropriate configuration for stable contacts to be formed. Membrane fluidity and lectin agglutinability, however, have been negatively correlated in mouse ascites tumour cells (Bales et al, 1977).

It seems unlikely that the drastic and potent effects of the inhibitor are due to alterations in membrane fluidity or the receptor arrangements. These two factors do not seem to be significantly altered during slime mould development, and as such would not seem to be of great importance per se. Molday et al(1976)

showed that the arrangement of ConA and WGA receptors on the cell surface remained unchanged between the vegetative and aggregation stages, although the ConA agglutinability does alter (Weeks and Weeks, 1975). The change in membrane lipid composition during development (Long and Coe, 1974) is not paralleled by a change in the fluidity of the lipids within the membrane (Von Dreele and Williams, 1977).

Finally, Cappuccinelli and Ashworth (1976) have presented complementary evidence implying that microtubular or microfilamentous structures may modulate the action of the cell surface molecules involved in aggregation in the slime moulds. Inhibitors of microtubule and microfilament function (e.g. colchicine, vinblastine, griseofulvin and isopropyl-N-phenylcarbamate) all inhibit or delay aggregation of slime mould amoebae. Since the effect of the inhibitor alters considerably during the developmental progression of slime mould cells, it would appear that its action cannot be correlated with membrane fluidity and receptor arrangement properties.

4. <u>The inhibitor interacts in some way with the slime mould lectin system</u>.

Small molecules such as the inhibitor can drastically alter the activities of lectins. The most effective agents for blocking these activities are simple mono- or disaccharides, which act by binding competitively to the lectin binding site, thus

preventing attachment of the lectin to the cell surface. ConA, for example, binds to ∝-glucopyranosyl and ∝-mannopyranosyl residues, while the agglutinin RCA-1 from Ricinus communis is blocked by D-galactopyranosyl residues. Rosen et al (1975) have shown that although it is possible to discriminate between lectins from different slime mould species on the basis of inhibition by a series of simple sugars, some species are very similar in their sugar specificities and are often only easily discriminable after consideration of the effects of two or more sugars. The appearance of native lectins on the cells of several slime mould species (including strain NC-4) occurs only after about 4 - 6 hours' starvation. Cell cohesion in the vegetative stage, therefore, is mediated by other means. Ax-2 cells, however, possess high levels of discoidin during the log phase of growth. A maximum level is attained at a cell density in the culture of 7×10^6/ml and declines thereafter (Gerisch, 1977).

If the inhibitor were acting by blocking the lectin systems (being sufficiently non-specific to block the lectins of all four species shown to be affected) it might be expected to block Ax-2 log cell cohesion. It should, however, have no effect on the feeding stage (preaggregation) cells of NC-4, D.mucoroides, D.purpureum and P.violaceum. Since these cells are susceptible to the inhibitor but do

not possess lectin on their surfaces at this stage (at least at currently undetectable levels) it appears highly unlikely that the inhibitor functions as a lectin-blocker. It remains possible that the inhibitor may adversely affect the lectins by some other means.

5. <u>The inhibitor affects the contact sites on the cell surface</u>.

The arguments against the involvement of the cohesion inhibitor with contact sites A has already been put forward. Aggregation-competent cells in shaking suspension show a reduced susceptibility to the inhibitor's effects. Contact site A-mediated cohesion, once formed, persists in the presence of inhibitor. The development of the ability to cohere in inhibitor as the cells progress towards full aggregation-competence parallels the development of EDTA-insensitive cohesion (CSA-mediated). The most likely, and most attractive, theory, therefore, is that inhibitor-insensitive cohesion occurs via the contact sites A.

Anti-cell-homogenate Fab fragments prevent the aggregation of NC-4 cells and also render these cells non-adhesive to glass (Gerisch,1974). The inhibitor also has these two effects. As well as containing Fab fragments against contact sites A and B, anti-homogenate Fab also contains Fab fragments directed against antigens 'I' and 'II', i.e. the carbohydrate-

containing antigens. This anti-carbohydrate Fab alone, however, does not impede aggregation. It seems unlikely, therefore, that the inhibitor blocks this process by acting at the surface sites of antigens I or II.

Contact sites B are present on both vegetative and aggregation-competent cells (Beug et al, 1970). Cohesion of vegetative cells is mediated through these sites, and they also function in the side-to-side cohesion of aggregating cell streams.

The blocking of these contact sites at the vegetative stage would, therefore, result in a complete inhibition of cohesion whereas aggregation-competent cells could cohere partially via the CSA. Blocking at aggregation would be expected to have a disruptive effect at the stage where the cells need to cohere by side-to-side means. In addition, contact sites B may be the mediators of adhesion to glass, which is blocked by anti-homogenate Fab. These predictions compare favourably with the actual observations using the inhibitor, and with the findings of Gerisch (1977) using EDTA to block sites B. It is, therefore, tentatively suggested that the inhibitor acts by blocking contact sites B in some way. Aggregation-competent cells are able to cohere via contact sites A, but these sites apparently do not begin to function in the presence of inhibitor. It may be, therefore, that the inhibitor prevents their initial expression on the cell surface. This hypothesis is presented in a diagrammatic scheme in Fig.7.1.

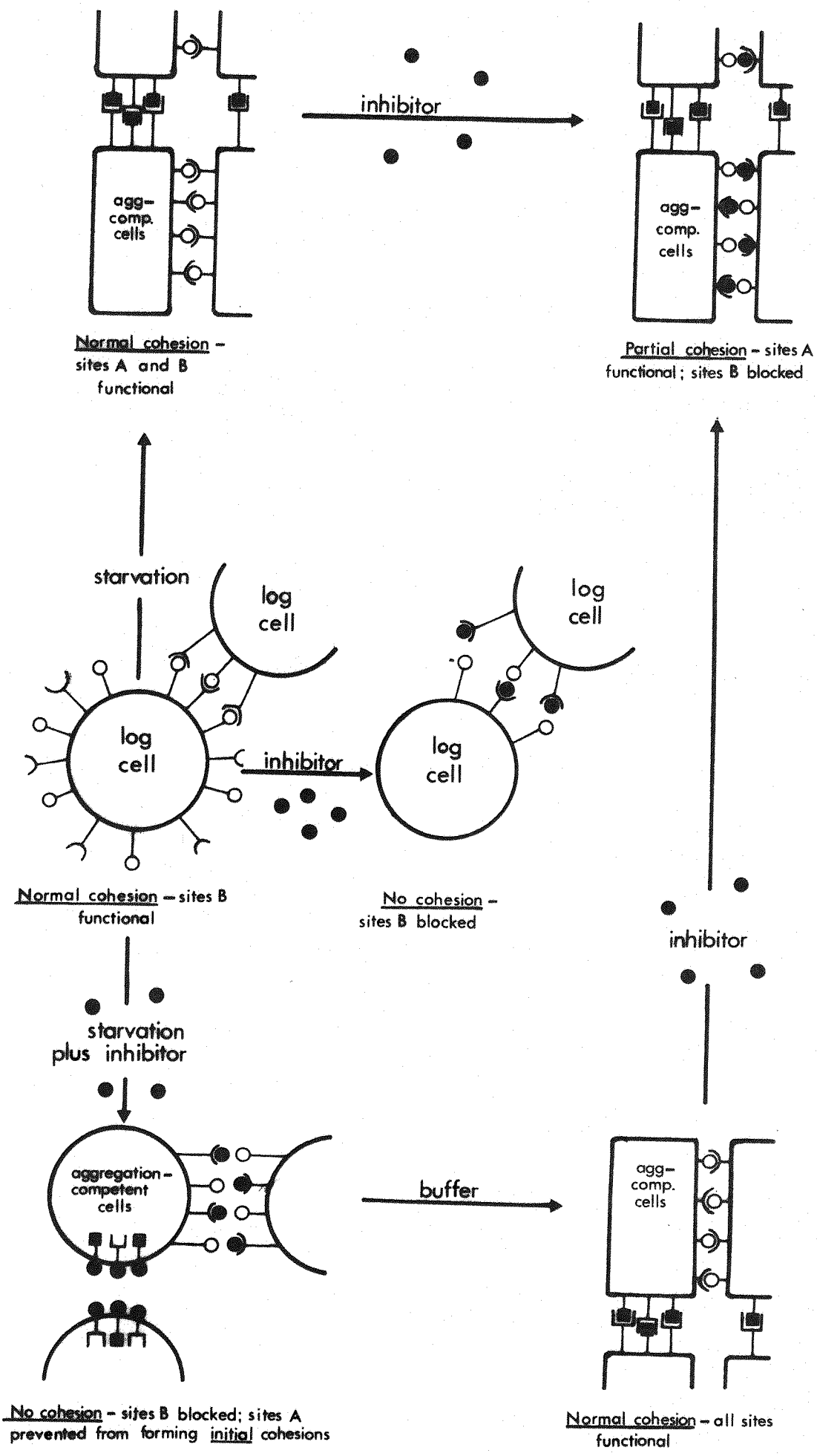

Fig. 7.1. Suggested action of the inhibitor

This scheme is supported by all the available evidence, providing one accepts contact sites B to be at least partially responsible for the adhesion of vegetative cells to glass.

The scheme raises several questions. Is this proposed mechanism biologically feasible? If so, what sort of molecular identity could the inhibitor have? What could it tell us about the cohesive mechanisms of slime moulds? How might stationary phase cells fit into this scheme?

Certainly the possibility of a small molecule blocking cell surface contact sites is biologically acceptable. The case of monosaccharide-blocking of lectin systems has already been cited, and specific Fab fractions will effectively inhibit immunoglobulin molecules from attaching to their particular surface antigens. The only requirement for such a mechanism is that the blocking agent must possess a complementary molecular configuration to the cell surface sites to which it binds. A precise fit is not entirely necessary, for a molecule possessing a sufficiently similar configuration to the 'proper' binding molecule can effectively block a surface site.

In this thesis it is suggested that the inhibitor binds to contact sites B. The most likely identity for this inhibitor is a carbohydrate since Fab-binding to contact sites is periodate-sensitive, suggesting that a carbohydrate moiety is involved in the process (Gerisch, 1974). The contact sites are assumed to be

bi-partite, thus a carbohydrate which binds to one portion of the site effectively prevents coupling between this and the complementary portion.

It is possible that the inhibitor is of a different molecular species, although at present it is difficult to predict what this could be. It could possess certain atomic groupings which would disrupt, for example, certain proteins from functioning effectively. Nevertheless, in view of recent evidence on the molecular aspects of cell adhesion (cf.section 1), the most likely identity of the inhibitor at this stage remains that of a carbohydrate.

The inhibitor has important implications concerning the mechanism of adhesion and cohesion in slime moulds. First, if it <u>does</u> act on contact sites B then the extent of the role played by these CSB in aggregation can be assessed. Similarly, the participation of each type of contact site in the adhesion of slime mould cells to glass, or to Millipore filters can be investigated. Most importantly, it may enable the relative importance of the lectin and contact site systems to be evaluated. Evidence presented here suggests that the lectins, unaffected by the inhibitor, must therefore play a minor role in the aggregation and development of slime moulds.

The position of stationary phase cells in this scheme remains difficult to determine. These cells are non-cohesive until they alter their properties to become either vegetative dividing cells, or aggregation-

competent cells. Even after washing, the stationary cells' cohesiveness in buffer only develops after a lag period. It would not appear, then, that their non-cohesiveness in stationary phase is due to a high level of inhibitor blocking their surface sites. It is more likely that, amongst other things, the increasing pH of the medium in stationary phase may affect the cohesive mechanisms of these cells. Additional factors may also be partly responsible, but at this stage there is little indication, or profit in speculation, of what these might be.

Two lines of research look promising when considering further work with the cohesion inhibitor.

First is to fully characterise this molecule. This would facilitate the production of a pure radiolabelled preparation which could prove invaluable in determining the precise site of action, the amount bound per cell, the location on aggregation-competent cells and the rate of removal of this molecule from the cell surface after binding.

The second approach would be an investigation into the role of cell contact during differentiation. The inhibitor provides a simple and effective way either of keeping cells separated, or of maintaining a <u>controlled degree</u> of agglomeration. This could be extremely useful in elucidating the critical factors necessary for successful differentiation in the slime moulds; for example, how many cells are necessary for the initiation of differentiation, and what is the

minimum duration of contact needed? It has already been shown that contact *per se* is necessary for the production of certain enzymes involved in differentiation (Gross *et al* 1977) although production can be initiated in the case of at least one of these developmentally-regulated enzymes, alkaline phosphatase, by pulsing separated cells with cyclic AMP (Rickenberg *et al*, 1977). Even continuous high levels of cAMP have now been found to be effective in some instances (Gross *et al* 1977). The addition of cAMP to cell suspensions maintained at various degrees of contact by the inhibitor may, therefore, help in evaluating the extent of cAMP-dependent and contact-dependent processes.

REFERENCES.

Aldrich, H.C., and Gregg, J.H.(1973) Unit membrane structural changes following cell association in Dictyostelium. Exp.Cell Res. 81, 407-412.

Alexander, S, Brackenbury, R., and Sussman, M.(1975). Tryptic destruction of aggregative competence in Dictyostelium discoideum and subsequent recovery. Nature, 254, 698-99.

Aminoff D.(1961) Methods for the quantitative estimation of N-acetylneuraminic acid and application to hydrolysates of sialomucoids. Biochem J., 81, 384-92.

Andrews P. (1964) Molecular sieve chromatography applied to molecular size and molecular weight estimation. Lab. Pract. 16, 851-56.

Armstrong P.B.(1966) On the role of metal cations on cellular adhesion: effect on cell surface charge. J.Exp.Zool. 163, 99-109.

Armstrong, P.B., and Jones, D.P. (1968). On the role of metal cations in cellular adhesion: cation specificity. J.Exp.Zool. 167, 275-82.

Ashworth J.M.(1971). Cell development in the cellular slime mould. Dictyostelium discoideum. Symp.Soc.Exp. Biol. 25, 27-49.

Bales,B.L., Lesin,E.S., and Oppenheimer, S.B.(1977) On cell membrane lipid fluidity and plant lectin agglutinability. A spin label study of mouse ascites tumour cells. Biochim. Biophys. Acta. 465, 400-407.

Barlow, D.I. (1977). Rapid-freeze technique for preparation of cells for scanning electron microscopy. (Pers. comm.)

Beug.H, Gerisch G, Kempff S., Riedel V, and Cremer G., (1970). Specific inhibition of cell contact formation in Dictyostelium by univalent antibodies. Exp.Cell Res. 63, 147-158.

Beug., H. Gerisch,G., and Muller, E.(1971), Cell dissociation: univalent antibodies as a possible alternative to proteolytic enzymes. Science 173, 742-43.

Beug, H., Katz, F.E., and Gerisch, G.(1973)(a) Dynamics of antigenic membrane sites relating to cell aggregation in Dictyostelium discoideum. J. Cell Biol. 56, 647-58.

Beug, H., Katz, F.E., Stein A., and Gerisch, G.(1973) (b) Quantitation of membrane sites in aggregating Dictyostelium cells by use of tritiated univalent antibody. Proc.Nat. Acad. Sci. 70, 3150-4.

Bonner, J.T., (1947). Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mould, Dictyostelium discoideum. J.Exp.Zool. 106, 1-26

Bonner, J.T., (1957). A theory of the control of differentiation in the cellular slime moulds. Quart. Rev.Biol. 32, 232-46.

Bonner, J.T., and Adams, M.S.(1958). Cell mixtures of different species and strains of cellular slime moulds. J.Emb.Exp.Morph. 6, 346-56.

Bonner, J.T., Barkley, D.S., Hall, E.M., Konijn, T.M. Mason, J.W., O'Keefe, G., and Wolfe, P.B.(1969). Acrasin, acrasinase, and the sensitivity to acrasin in Dictyostelium discoideum. Dev. Biol., 20, 72-87

Bonner, J.T., Sieja, T.W., and Hall, E.M.(1971). Further evidence for the sorting out of cells in the differentiation of the cellular slime mould, Dictyostelium discoideum. J.Emb.Exp.Morph. 25, 457-65.

Bosmann, H.B. (1971). Platelet adhesiveness and aggregation: the collagen: glycosyl, polypeptide: N-acetylgalactosaminyl, and glycoprotein: galactosyl transferases of human platelets. Biochem. Biophys.Res.Comm. 43, 1118-1124.

Bosmann, H.B. (1972a) Cell surface glycosyl transferases and acceptors in normal and RNA- and DNA-virus transformed fibroblasts. Biochem. Biophys.Res.Comm. 48, 523-529.

Bosmann, H.B. (1972b) Platelet adhesiveness and aggregation II. Surface sialic acid, glycoprotein: N-acetylneuraminic acid transferase, and neuraminidase of human blood platelets. Biochim.Biophys.Acta. 279, 456-74.

Brachet, P. and Klein, C.(1975). Inhibition of growth and cellular aggregation of Dictyostelium discoideum by steroid compounds. Exp. Cell Res. 93, 159-65.

Burdick, M.L. (1974a). Cell sorting out according to species in aggregates containing mouse and chick embryonic limb mesoblast cells. J. Exp. Zool. 175, 357-368.

Burdick, M.L. (1974b). Differences in the morphogenetic properties of mouse and chick embryonic liver cells. J. Exp. Zool. 180, 117-126.

Burdick, M.L., and Steinberg, M.S.(1969). Embryonic cell adhesiveness: Do species differences exist among warm-blooded vertebrates? Proc.Nat.Acad.Sci., 63, 1169-1173.

Burger, M.M.(1974). The isolation of surface components involved in specific cell-cell adhesion and cellular recognition. Proc.Mexican Soc.Biochem.Symp., Oaxaca, Mexico (Ed: Estrada-O and Gitler) Acad. Press, N.Y., and London. pp.509-521.

Burger. M.M., and Turner, R.S. (1975), Kuhns, W.J. and Weinbaum G. A possible model for cell-cell recognition via surface macromolecules. Phil.Trans.Roy.Soc.London B 271, 379-393.

Cappuccinelli P., and Ashworth J.M. (1976). The effect of inhibitors of microtubule and microfilament function on the cellular slime mould, Dictyostelium discoideum. Exp. Cell Res. 103, 387-93.

Chang, C.M., Reitherman, R.W., Rosen, S.D., and Barondes, S.H.(1975). Cell surface location of discoidin, a developmentally regulated carbohydrate-binding protein from Dictyostelium discoideum. Exp.Cell Res. 95, 136-42.

Chang, C.M. Rosen, S.D., and Barondes, S.H.(1977). Cell surface location of an endogenous lectin and its receptor in Polysphondylium pallidum. Exp.Cell Res. 104, 101-9.

Coccucci, S., and Sussman, M.(1970). RNA in cytoplasmic and nuclear fractions of cellular slime mould amoebas. J. Cell Biol. 45, 399-407.

Cohen, M.H., and Robertson, A.(1971). Wave propagation in the early stages of aggregation of cellular slime moulds. J. Theor.Biol. 31, 101-18.

Coman, D.R.(1944). Decreased mutual adhesiveness, a property of cells from squamous cell carcinomas. Cancer Res. 4, 625-629.

Cook, G.M.W., and Stoddart, R.W.(1973). Surface carbohydrates of the eukaryotic cell. Academic Press, New York.

Curtis, A.S.G.(1961). Timing mechanisms in the specific adhesion of cells. Exp.Cell Res. Suppl.8, 107-122.

Curtis, A.S.G.(1973). Cell Adhesion. In: Prog.Biophys and Molec.Biol. (Ed: Butler, A.J.V., and Noble, D.) 27, 315-86. Pergamon Press, Oxford.

Curtis, A.S.G. and Greaves, M.F.(1965). The inhibition of cell aggregation by a pure serum protein. J. Emb. Exp. Morph. 13, 309-326.

Curtis, A.S.G., and Van de Vyver, G. (1971). The control of cell adhesion in a morphogenetic system. J. Emb. Exp. Morph. 26, 295-312.

Curtis, A.S.G. and de Sousa, M.(1975). Lymphocyte interactions and positioning. I. Adhesive interactions. Cell. Immunol. 19, 282-97.

Darmon, M., Brachet, P., and Pereira Da Silva, LH (1975) Chemotactic signals induce cell differentiation in Dictyostelium discoideum. Proc.Nat.Acad.Sci.72, 3163-3166.

Darmon, M., and Klein, C.(1976). Binding of Concanavalin A and its effect on the differentiation of Dictyostelium discoideum. Biochem. J. 154, 743-50.

De Long, G.R., and Sidman, R.L.(1970). Alignment effect of reaggregating cells in cultures of developing brains of reeler mutant mice. Dev.Biol. 22, 584-99.

Deman, J.J., and Bruyneel, E.A.(1974). Evidence for long-range electrostatic repulsion between Hela cells. Exp. Cell Res. 89, 206-16.

Edelman, G.M. Rutishauser, U., and Millette, C.F.(1971). Cell fractionation and arrangement on fibres, beads and surfaces. Proc. Nat. Acad.Sci. 68, 2153-2157.

Edwards, J.G., Campbell, J.A., Robson,R.T. and Vicker, M.G.(1975). Trypsinised BHK21 cells aggregate in the presence of metabolic inhibitors and in the absence of divalent cations. J. Cell Sci.19, 653-67.

Ellingson, J.S, (1974). Changes in the phospholipid composition in the differentiating cellular slime mould, Dictyostelium discoideum. Biochim.Biophys.Acta.337, 60-67.

Every, D., and Ashworth, J.M.(1973). The purification and properties of extracellular glycosidases of the cellular slime mould, Dictyostelium discoideum. Biochem. J.133(1973) 133-37.

Every, D., and Ashworth, J.M.(1975). Rates of degradation and synthesis of glycosidases de novo during growth and differentiation of Dictyostelium discoideum. Biochem. J. 148, 169-77.

Firtel, R.A., and Bonner, J.T.(1972). Developmental control of alpha 1-4-glucan phosphorylase in the cellular slime mould, Dictyostelium discoideum. Dev. Biol.29,85-103.

Forman, D.,and Garrod, D.R.(1977a) Pattern formation in Dictyostelium discoideum. I. Development of prespore cells and its relationship to the pattern of the fruiting body. J. Emb. Exp. Morph.40, 215-228.

Forman, D.,and Garrod, D.R.(1977b) Pattern formation in Dictyostelium discoideum II. Differentiation and pattern formation in non-polar aggregates. J.Emb.Exp.Morph. 40, 229-243.

Frazier, W.A., Rosen, S.D., Reitherman, R.W., and Barondes, S.H.(1975). Purification and comparison of two developmentally regulated lectins from Dictyostelium discoideum. J. Biol. Chem. 250, 7714-21.

Garber, B.B., and Moscona, A.A.(1972). Reconstruction of brain tissue from cell suspensions II. Specific enhancement of aggregation of embryonic cerebral cells by supernatant from homologous cell cultures. Dev.Biol.27, 235-271.

Garrod, D.R.,(1974b). The cellular basis of movement of the migrating grex of the slime mould, Dictyostelium discoideum: chemotactic and reaggregation behaviour of grex cells. J. Emb. Exp. Morph. 32, 57-68.

Garrod, D.R.,(1974a). Cellular recognition and cell adhesion in cellular slime mould development. Arch. Biol. (Bruxelles) 85 , 7-31.

Garrod, D.R., and Gingell, D.(1970). A progressive change in the electrophoretic mobility of preaggregation cells of the slime mould, Dictyostelium discoideum. J.Cell.Sci. 6, 277-284.

Garrod, D.R., and Ashworth, J.M.(1972). Effect of growth conditions on development of the cellular slime mould, Dictyostelium discoideum. J.Emb.Exp.Morph.28 463-479.

Garrod, D.R., and Ashworth, J.M.(1973). Development of the cellular slime mould, Dictyostelium discoideum. Symp. Soc. Gen. Microbiol. XXIII, 407-435.

Garrod, D.R., and Forman, D.(1977). Pattern formation in the absence of polarity in Dictyostelium discoideum. Nature, Lond.265, 144-146.

Geltowsky, J.E., Siu, C.H., and Lerner, R.A.(1976). Glycoproteins of the plasma membrane of Dictyostelium discoideum during development. Cell 8, 391-6.

Gerisch, G.(1961). Zellfunktionen und zell-funktionwechsel in der entwicklung von Dictyostelium discoideum. V.Stadienspezifische zellkontaktbildung und ihre quantitative erfassung. Exp.Cell Res. 25, 535-54.

Gerisch, G.(1976). Extracellular cAMP phosphodiesterase regulation in agar plate cultures of Dictyostelium discoideum. Cell Differ. 5, 21-25.

Gerisch. G.(1977). Membrane sites implicated in cell adhesion: their developmental control in Dictyostelium discoideum. In: International Cell Biology 1976-1977. (Eds: B.R.Brinkley and K.R.Porter). Rockefeller Univ. Press, N.Y., pp 36-42.

Gerisch, G., Malchow, D., Riedel V., Muller, E., and Every, M.(1972). Cyclic AMP phosphodiesterase and its inhibitor in slime mould development. Nature,New Biol. 235, 90-92.

Gerisch, G., Beug, H., Malchow, D., Schwarz, H., Stein,A.V., (1974). Receptors for intercellular signals in aggregating cells of the slime mould, Dictyostelium discoideum. In 'Biology and chemistry of eucaryotic cell surfaces' Miami Winter Symp. Vol.7, 49-66.

Gerisch, G., Frowin, H., Huesgen, A., and Wick, U., (1975). Control of cell-contact sites by cAMP pulses in differentiating Dictyostelium cells. Nature 255, 547-9.

Gillette, M.U., and Filosa, M.F.(1973). Effect of Concanavalin A on cellular slime mould development: premature appearance of membrane-bound cyclic AMP, phosphodiesterase. Biochem. Biophys.Res.Comm. 53,1159-1166.

Gottlieb, D.I., and Glaser, L.(1975). A novel assay of neuronal cell adhesion. Biochem.Biophys.Res.Comm. 63, 815-821.

Gregg, J.H.(1956). Serological investigations of cell adhesion in the slime moulds, Dictyostelium discoideum, and Polysphondylium violaceum. J. Gen.Physiol. 39,813-20.

Gregg, J.H.(1971). Developmental potential of isolated Dictyostelium myxamoebae. Dev.Biol. 26, 478-85.

Gregg and Badman (1970). Morphogenesis and ultrastructure in Dictyostelium. Dev.Biol. 22, 96-111.

Gross, J., Kay R., Lax A, Peacey, M., Town, C., and Trevan, D.(1977). Cell contact, signalling and gene expression in Dictyostelium discoideum. In "Development and Differentiation in the Cellular Slime Moulds" Developments in Cell Biology Vol.1 (Eds: Cappucinelli, P, and Ashworth, J.M.) Elsevier/North-Holland, Amsterdam, New York. pp 135-172.

Gregg, J.H., and Trygstad, C.W.(1958). Surface antigen defects contributing to developmental failure in aggregateless variants of the slime mould Dictyostelium discoideum. Exp. Cell Res. 15, 358-69.

Gustafson, T. and Wolpert, L.(1967). Cellular movement and contact in sea urchin morphogenesis. Biol.Rev.42, 442-98.

Hanish, M.D.(1975). A possible cause of termination of cell growth in the cellular slime mould, Dictyostelium discoideum. Dev. Biol. 45, 340-48.

Harrison, R., and Lunt, G.G.(1975). Membrane carbohydrate and cell surface specificity. In: "Biological Membranes", Blackie and Son, Glasgow and London. pp 140-146.

Hausmann, R.E., and Moscona, A.A.(1975). Purification and characterisation of the retina-specific cell-aggregating factor. Proc.Nat.Acad.Sci. 72, 916-920.

Hayashi, M., and Takouchi, E.(1976). Quantitative studies on cell differentiation during morphogenesis of the cellular slime mould. Dev.Biol. 50, 302-309.

Henkart, P. Humphreys, S., and Humphreys, R.T.(1973). Characterisation of sponge aggregation factor. A unique proteoglycan complex. Biochemistry 12, 3045-3050.

Hoffman, S., and McMahon, D.(1977). The role of the plasma membrane in the development of Dictyostelium discoideum II. Devtl. and topographic analysis of polypeptide and glycoprotein composition. Biochim.Biophys. Acta. 465, 242-59

Huesgen, A., and Gerisch, G.(1975). Solubilised contact sites A from cell membranes of Dictyostelium discoideum. FEBS Letters 56, 46-49.

Inbar, M., Huet, C., Oseroff, A., Benbassat, H., and Sachs, L. (1973). Inhibition of lectin agglutinability by fixation of the cell surface membrane. Biochim. Biophys. Acta 311, 594-9.

Jamieson, G.A., Urban, C.L., Barber, A.J.(1971). Enzymatic basis for platelet: collagen adhesion as the primary step in haemostasis. Nature N.B. 234, 5-7.

Johnson, G.S., and Pastan, I.(1972). Cyclic AMP increases the adhesion of fibroblasts to the substratum. Nature, N.B., 236, 247-8.

Jones, M.E., and Robertson, A.(1976). Cyclic adenosine monophosphate and the development of Polysphondylium J.Cell.Sci. 22, 41-47.

Kemp, R.B.(1968). Effect of the removal of cell surface sialic acids on cell aggregation in vitro. Nature 218, 1255-6.

Kemp. R.B.(1970). The effect of neuraminidase (3:2:1:18) on the aggregation of cells dissociated from ambryonic chick muscle tissue. J.Cell Sci. 6, 751-66.

Kemp. R.B., and Jones, B.M.(1970). Aggregation and electrophoretic mobility studies of cells.I. The effects of p-benzoquinone and tannic acid. Exp.Cell.Res.63, 293-300.

Kondo K.(1974). Demonstration of a reaggregation inhibitor in sea urchin embryos. Exp.Cell Res. 86, 178-81.

Konijn, T.M. Barkley, D.S., Chang, Y.Y., and Bonner,J.T. (1968). Cyclic AMP: a naturally occurring acrasin in the cellular slime moulds. Amer.Nat. 102, 225-33.

Leach, C.K., Ashworth, J.M. and Garrod, D.R. (1973). Cell sorting out during the differentiation of mixtures of metabolically distinct populations of Dictyostelium discoideum. J.Emb.Exp.Morph. 29, 647-661.

Lee, K.C.(1972). Cell electrophoresis of the cellular slime mould, Dictyostelium discoideum I. Characterisation of some of the cell surface ionogenic groups. J.Cell Sci. 10, 229-48.

Lilien, J.E.(1969). Toward a molecular explanation for specific cell adhesion. Curr.Topics in Dev.Biol. 4,169-95.

Lloyd, C.W., and Cook, G.M.W.(1974). On the mechanism of the increased aggregation by neuraminidase of 16C malignant rat dermal fibroblasts in vitro. J.Cell Sci. 15 575-590.

Lloyd, C.W., and Kemp, R.B.(1971). A biochemical investigation into the inhibitory effects of glucosamine and N-acetylglucovannine on the aggregation in vitro of embryonic chick muscle cells. J.Cell Sci. 9, 85-102.

Lloyd, C.W., Rees, D.A., Smith, C.G., and Judge, F.J.(1976) Mechanisms of cell adhesion: early-forming junctions between aggregating fibroblasts. J. Cell Sci. 22, 671-84.

Lodish, H., and Alton, T.(1977). Translational and transcriptional control of protein synthesis during differentiation of Dictyostelium discoideum. In: Development and differentiation in the cellular slime moulds (Eds: P. Cappuccinelli and J.M.Ashworth). Elsevier/ North Holland, Amsterdam. pp.253-272.

Lond, B.H., and Coe., E.L.(1974). Changes in neutral lipid constituents during differentiation of the cellular slime mould, Dictyostelium discoideum. J.Biol.Chem. 249, 521-29.

Loomis, W.F.(1975). In:"Dictyostelium discoideum: a developmental system". Acad.Press, New York.

Maeda, Y., and Takeuchi, I (1969). Cell differentiation and fine structures in the development of the cellular slime moulds. Develop., Growth and Differ. 11, 232-45.

Maeda, Y., and Maeda, M.(1974). Heterogeneity of the cell population of the cellular slime mould, Dictyostelium discoideum. Exp.Cell Res. 84, 88-94.

Malchow, D., Nagele, B., Schwarz, H., and Gerisch, G.(1972). Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of Dictyostelium discoideum. Eur.J.Biochem. 28,136-42.

Malchow, D., and Gerisch, G.(1973). Cyclic AMP binding to living cells of Dictyostelium discoideum in presence of excess cyclic GMP. Biochem.Biophys.Res.Comm. 55, 200-204.

Malchow, D., and Gerisch, G.(1974). Short-term binding and hydrolysis of cyclic 3′:5′- adenosine monophosphate by aggregating Dictyostelium cells. Proc.Nat.Acad.Sci. 71, 2423-7.

Malkinson, A.M. and Ashworth, J.M.(1972). Extracellular concentrations of adenosine 3′: 5′ - cyclic monophosphate during axenic growth of myxamoebae of the c.s.m, Dictyostelium discoideum. Biochem J. 127, 611-2.

Malkinson, A. and Ashworth, J. (1973). Adenosine 3′:5′ - cyclic monophosphate concentrations and phosphodiesterase activities during axenic growth and differentiation of cells of the cellular slime mould Dictyostelium discoideum Biochem. J. 134, 311-19.

McClay, D.R., and Moscona, A.A.(1974). Purification of the specific cell-aggregating factor from embryonic neural retina cells. Exp.Cell Res. 87, 438-43.

McDonough J. and Lilien, J.(1975). Inhibition of mobility of cell-surface receptors by factors which mediate specific cell-cell interactions. Nature, Lond.256, 416-7.

McGuire, E.J.(1972). A possible role for carbohydrates in cell-cell adhesion. In "Membrane Research" Proc.ICN-UCLA Symp. on Molec.Biol. (Squaw Valley, Calif.) (Ed: C.Fred Fox) Acad.Press N.Y., and London, pp 347-368.

McGuire, E.J.(1976) Intercellular adhesive selectivity. II. Properties of embryonic chick liver cell-cell adhesion. J.Cell Biol 68, 90-100.

McMahon. D. (1973). A cell contact model for cellular position determination in development. Proc.Nat.Acad. Sci. 70, 2396-2400.

McQuiddy, P., and Lilien, J.(1971). Sialic acid and cell aggregation. J.Cell Sci. 9, 823-833.

Merrell, R. Pulliam, M.W., Randono, L. Boyd, L.F., Bradshaw, R.A., and Glaser L. (1975). Temporal changes in tectal cell surface specificity induced by nerve growth factor. Proc.Nat.Acad.Sci. 72, 4270-74.

Molday, R., Jaffe, R., and McMahon, D.(1976). Concanavalin A and wheat germ agglutinin receptors on Dictyostelium discoideum. Their visualisation by S.E.M. with microspheres. J.Cell Biol. 71, 314-22.

Moscona, A.A.(1957). The development in vitro of chimaeric aggregates of dissociated embryonic chick and mouse cells. Proc.Nat.Acad.Sci. 43, 184-194.

Moscona, A.A.(1969). Development of heterotypic combinations of dissociated embryonic chick cells. Proc.Soc. Exp.Biol. Med. 92, 410-16.

Moscona, A.A.(1974). In: "The cell surface in Development" (Ed.A.A.Moscona) pp.67-99. John Wiley and Sons, New York.

Nanjundiah V., and Malchow, D.(1976). A theoretical study of the effects of cAMP phosphodiesterases during aggregation in Dictyostelium. J.Cell Sci. 22, 49-58.

Newell, P.C., Longlands, M. and Sussman, M., (1971). Control of enzyme synthesis by cellular interaction during development of the cellular slime mould, Dictyostelium discoideum. J. Mol. Biol. 58, 641-54.

Newell, P.C., Franke, J. and Sussman, M.(1972). Regulation of four functionally related enzymes during shifts in the developmental programme of Dictyostelium discoideum. J. Mol. Biol. 63, 373-82.

Nicol, A., and Garrod, D.R.(1977). Mutual adhesion and cell sorting out among different species of cellular slime mould (In preparation).

Oppenheimer, S.B.(1975). Functional involvement of specific carbohydrate in teratoma cell adhesion factor. Exp.Cell.Res. 92, 122-26.

Oppenheimer, S.B., Edidin, M. Orr,C.W., and Roseman, S. (1969). An L-glutamine requirement for intercellular adhesion. Proc.Nat.Acad.Sci. 63, 1395-1402.

Overton, J.(1977). Formation of junctions and cell sorting in aggregates of chick and mouse cells. Dev.Biol.55,103-116.

Pan, P. Bonner, J.T., Wedner, H.J., and Parker, C.W.(1974). Immunofluorescence evidence for the distribution of cyclic-AMP in cells and cell masses of the cellular slime moulds. Proc.Nat.Acad.Sci. 71, 1623-25.

Raper, K.B., and Thom, C.(1941). Interspecific mixtures in the Dictyosteliaceae. Amer.J.Bot. 28, 69-78.

Reitherman, R.W. Rosen, S.D., Frazier, W.A., and Barendes, S.H. (1975). Cell surface species - specific high affinity receptors for discoidin: Developmental regulation in Dictyostelium discoideum. Proc.Nat.Acad. Sci. 72, 3541-5.

Rickenberg, H.V., Rahmsdorf, H.J., Campbell, A., North, M.J., Kwasniak, J. and Ashworth, J.M.(1975). Inhibition of development in Dictyostelium discoideum by sugars. J.Bacteriol. 124, 212-219.

Rickenberg, H.V., Tihon, C., and Guzel, O. (1977) The effect of pulses of 3′:5′ cyclic adenosine monophosphate on enzyme formation in non-aggregated amoebae of Dictyostelium discoideum. In:"Development and differentiation in the cellular slime moulds" EMBO Sardinia Symposium 1977. (Eds: P.Cappuccinelli and J.M.Ashworth) pp. 173-188.

Robertson, A., Drage, D.J., and Cohen, M.H.,(1972). Control of aggregation in Dictyostelium discoideum by an external periodic pulse of cyclic adenosine monophosphate. Science, 175, 333-335.

Roseman, S.(1970). The synthesis of complex carbohydrates by multiglycosyl transferase systems and their potential function in intercellular adhesion. Chem.Physics.Lipids 5, 270-297.

Rosen, S.D., Kafka, J.A., Simpson, D.L. and Barondes, S.H., (1973). Developmentally regulated, carbohydrate-binding protein in Dictyostelium discoideum. Proc.Nat.Acad.Sci. 70 2554-57.

Rosen, S.D., Simpson, D.L., Rose. J.E., and Barondes,S.H. (1974). Carbohydrate-binding protein from Polysphondylium pallidum implicated in intercellular adhesion. Nature, 252, 128 and 149-151.

Rosen, S.D. Reitherman, R.W., and Barondes, S.H.(1975). Distinct lectin activities from six species of cellular slime moulds. Exp.Cell Res. 95, 159-66.

Rosen, S.D., Haywood, P.L., and Barondes, S.H.(1976). Inhibition of intercellular adhesion in a cellular slime mould by univalent antibody against a cell-surface lectin. Nature 263, 425-7.

Rosen, S.D., Chang, C.M., Barondes, S.H.(1977). Intercellular adhesion in the cellular slime mould, Polysphondylium pallidum inhibited by interaction of asialofetuin or specific univalent antibody with endogenous cell surface lectin. Dev.Biol. 61, 202-213.

Rossomando, E.F., Steffek, A.I., Mujwid, D.K., and Alexander, S. Scanning EM observations on cell surface changes during aggregation of Dictyostelium discoideum. Exp.Cell Res. 85 (1974), 73-78.

Roth, S.A.(1968). Studies on intercellular adhesive selectivity. Dev.Biol. 18, 602-631.

Roth, S.A., (1973). A molecular model for cell interactions. Quart. Rev. Biol. 48, 541-63.

Roth, S.A., and Weston, J.A.(1967). The measurement of intercellular adhesion. Proc.Nat.Acad.Sci. 58, 974-980.

Roth, S., McGuire, E.J., and Roseman, S.(1971a). Evidence for cell surface glycosyl transferases. Their potential role in cellular recognition. J.Cell Biol. 51, 536-47.

Roth, S., McGuire, E.J. and Roseman S.(1971b). An assay for intercellular adhesive specificity. J.Cell Biol. 51, 525-35.

Rubin, J. and Robertson, A.(1975). The tip of Dictyostelium discoideum pseudoplasmodium as an organiser. J.Emb.Exp.Morph. 33, 227-41.

Runyon, E.H. (1942). Aggregation of separate cells of Dictyostelium discoideum to form a multi-cellular body. Collecting Net. 17, 88.

Rutishauser, U., Thiery, J.P., Brackenbury, R., Seta,B.A., and Edelman, G.M.(1976). Mechanisms of adhesion among cells from neural tissues of the chick embryo. Proc.Nat. Acad.Sci. 73, 577-81.

Sakai, Y. and Takeuchi, I.(1971). Changes of the prespore specific structure during dedifferentiation and cell type conversion of a slime mould cell. Dev.Growth and Differ. 13, 231-240.

Shaffer, B.M.(1953). Aggregation in cellular slime moulds: in vitro isolation of acrasin. Nature, 171, 975.

Shaffer, B.M.(1957a) Aspects of aggregation in cellular slime moulds. 1. Orientation and chemotaxis. Amer.Nat. 91, 19-35.

Shaffer, B.M.(1957b). Variability of behaviour of aggregating cellular slime moulds. Quart.J.Microsc. Sci. 98, 393-405.

Shaffer, B.M.(1964). In: "Primitive Motile Systems in Cell Biology" pp 387-405. (Ed. Allen, R.D., and Kamiya,N.) Acad. Press, New York.

Shaffer, B.M.(1972). Secretion of cyclic AMP induced by cyclic AMP in the cellular slime mould, Dictyostelium discoideum. Nature. 255, 549-52.

Sharon, N. and Lish (1972). Lectins: Cell-agglutinating and sugar-specific proteins. Science 177, 949-959.

Shields, R.(1975). Microtubules and membrane topography. Nature. 256, 257-8.

Shur. B.D., and Roth. S.(1975). Cell surface glycosyl-transferases. Biochim.Biophys.Acta. 415, 473-512.

Simpson, D.L., Rosen, S.D., and Barondes, S.H.(1974). Discoidin, a developmentally-regulated carbohydrate-binding protein from Dictyostelium discoideum. Purification and characterisation. Biochemistry 13, 3487-93.

Singer, S.J. and Nicolson, G.L.(1972). The fluid mosaic model of the structure of cell membranes. Science 175, 720.

Siu, C.-H., Lerner, R.A., Ma, G., Firtell, R.A., and Loomis, W.F.(1976). Developmentally regulated proteins of the plasma membrane of Dictyostelium discoideum. The carbohydrate-binding protein. J.Mol.Biol.100, 157-78.

Smart, J.E., and Hynes, R.O.(1974). Developmentally regulated cell surface alterations in Dictyostelium discoideum. Nature 251, 319-321.

Soll, D.R., and Sussman, M.(1973). Transcription in isolated nuclei of the slime mould, Dictyostelium discoideum. Biochim.Biophys.Acta. 319, 312-22.

Soll. D.R., Yarger, J., and Mirick.M.(1976). Stationary phase and the cell cycle of Dictystelium discoideum in liquid nutrient medium. J.Cell Sci. 20, 513-23.

Sonneborn, D.R., Sussman, M., and Levine, L.(1964). Serological analyses of cellular slime mould development. 1. Changes in antigenic activity during cell aggregation. J.Bacteriol. 87, 1321-1329

Steinberg. M.S.(1964). The problem of adhesive selectivity in cellular interactions. Cellular membranes in development (ed:Locke) 22nd symp. of Soc. for Study of Devt. and Growth. P.321-66.

Steinberg, M.S.(1970). Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a heirarchy among populations of embryonic cells. J.Exp.Zool.173, 395-434.

Steinberg, M.S.(1975). Adhesion-guided multicellular assembly: a commentary upon the postulates, real and imagined, of a differential adhesion hypothesis, with special attention to computer simulations of cell sorting. J.Theor.Biol. 55, 431-43.

Steinberg, M.S.(1975). Adhesion-guided multicellular assembly: a commentary upon the postulates, real and imagined, of a differential adhesion hypothesis, with special attention to computer simulations of cell sorting. J. Theor Biol. 55, 431-443.

Steinberg. M.S., Armstrong P.B., and Granger, R.E.(1973). On the recovery of adhesiveness by trypsin-dissociated cells. J.Membrane Biol. 13, 97-128.

Sternfeld, J. and Bonner, J.T.(1977). Cell differentiation in Dictyostelium under submerged conditions. Proc.Nat.Acad.Sci. 74, 268-271.

Sussman, M.(1966). Biochemical and genetic methods in the study of cellular slime mould development. In: "Methods in Cell Physiology" Vol.2. (Ed:D.Prescott) pp 397-410. Acad.Press, New York.

Sussman, M. and Boschwitz, C.(1975a). Adhesive properties of cell ghosts derived from Dictyostelium discoideum. Dev. Biol. 44, 362-68.

Sussman, M. and Boschwitz, C.(1975b). An increase of calcium/manganese binding sites in cell ghosts associated with the acquisition of aggregative competence in Dictyostelium discoideum. Exp.Cell Res. 95, 63-6.

Swan, A.P. and Garrod, D.R.(1975). Cohesive properties of axenically grown cells of the slime mould, Dictyostelium discoideum. Exp.Cell Res. 93, 479-484.

Tahara, I and Edelman, G.M.(1975). Electron microscopic analysis of the modulation of lymphocyte receptor mobility. Exp.Cell Res. 91, 125-42.

Takeuchi, I.(1969). Establishment of polar organisation during slime mould development. In: Nucleic Acid Metabolism, Cell Differentiation and Cancer Growth, (Ed: E.V. Cowdry and S. Seno) pp.297-304. Pergamon Press, Oxford.

Tekeuchi, I.(1977). Cell differentiation and pattern formation in Dictyostelium. In:"Development and differentiation in the cellular slime moulds". (Eds: P.Cappuccinelli and J.M.Ashworth). EMBO Sardinia Workshop, 1977. Elsevier/North-Holland, Amsterdam, New York. pp 1-16.

Takeuchi, I. and Yabuno, K.(1970). Disaggregation of slime mould pseudoplasmodia using EDTA and various proteolytic enzymes. Exp.Cell Res.61, 183-90.

Tilney. L.G.(1968). Studies on the microtubules in Heliocoa: IV. The effect of colchicine on the formation and maintenance of the axopodia and the redevelopment of pattern in Actinosphaerium nucleofilum. J.Cell Sci.3, 549-62.

Town, C., and Gross J.(1978). The role of cyclic nucleotides and cell agglomeration in post-aggregative enzyme synthesis in D.discoideum. (In press) Dev Biol.

Townes, P.L., and Holtfreter, J.(1955). Directed movements and selective adhesion of embryonic amphibian cells. J.Exp.Zod.128, 53-118.

Trinkaus, J.P.(1963). The cellular basis of Fundulus epiboly. Adhesivity of blastula and gastrula cells in culture. Dev. Biol. 7, 513-32.

Tyler, A. (1946). An auto-antibody concept of cell structure, growth and differentiation. Growth 10, (Symp.6), 7-19.

Ukena, T.E., Borysenki, J.Z., Karnovsky, M.J., and Berlin, R.D.(1974). Effects of colchicine, cytochalasin B and 2-deoxyglucose on the topographical organisation of surface-bound Concanavalin A in normal and transformed fibroblasts. J.Cell Biol. 61, 70-82.

Vicker, M.G., and Edwards, J.G.(1972). The effect of neuraminidase on the aggregation of BHK 21 cells and BHK 21 cells transformed by polyoma virus. J.Cell Sci. 10, 759-68.

Von Dreele, P.H., and Williams, K.L.(1977). Electron spin resonance studies of the membranes of the cellular slime mould, Dictyostelium discoideum. Biochim.Biophys. Acta. 464, 378-88.

Vosbeck K. and Roth, S.(1976). Assay of intercellular adhesiveness using cell-coated Sephodex beads as collecting particles. J.Cell Sci. 22, 657-60.

Walther, B.T., Ohman R and Roseman, S.(1973). A quantitative assay for intercellular adhesion. Proc.Nat.Acad. Sci. 70, 1569-73.

Watts. D.J., and Ashworth, J.M.(1970). Growth of myxamaebae of the cellular slime mould, Dictyostelium discoideum in axenic culture. Biochem.J. 119, 171-4.

Weeks, G.(1973). Agglutination of growing and differentiating cells of Dictyostelium discoideum by concanavalin A. Exp.Cell Res. 76, 467-70.

Weeks, G.(1975) Studies of the cell surface of Dictyostelium discoideum during differentiation. The binding of ^{125}I-Concanavalin A to the cell surface. J.Biol.Chem. 250, 6706-6710.

Weeks, C. and Weeks, G.(1975). Cell surface changes during the differentiation of Dictyostelium discoideum interaction of cells with ConA. Exp.Cell Res. 92, 372-82.

Weiss, P.(1947). The problem of specificity in growth and development. Yale J.Biol.Med. 19, 235-278.

Weiss, L.(1961). The measurement of cell adhesion. Exp.Cell Res. Suppl.8, 141-153.

Wessels, N.K., Spooner, B.S., Ash, J.F., Bradly,M,O., Luduena, M.A., Taylor, E.L., Wrenn,J., and Yamada, K.M. (1971). Microfilaments in cellular and developmental processes. Science 171, 135.

West, C., and McMahon, D.(1977). Multiple ConA receptors and discoidin in plasma membranes from Dictyostelium discoideum. J.Cell Biol. 70, 411a.(Abstract.)

Willingham, M.C., and Pastan, I.(1975). Cyclic AMP modulates microvillus formation and agglutinability in transformed and normal mouse fibroblasts. Proc.Nat. Acad.Sci. 72, 1263-7.

Wilson, H.V.(1907). On some phenomena of coalescence and regeneration in sponges. J.Exp.Zool. 5, 245-58.

Wolpert, L.(1969). Positional information and the spatial pattern of cellular differentiation. J.Theor. Biol. 25, 1-47.

Wolpert, L.(1971). Positional information and pattern formation. Curr.Topics. Dev.Biol.6, 183-222.

Yabuno, K.(1970). Changes in electronegativity of the cell surface during the development of the cellular slime mould, Dictyostelium discoideum. Develop., Growth and Differ. 12, 229-39.

Yahara. I., and Edelman, G.M.(1972). Restriction of the mobility of lymphocyte immunoglobulin receptors by Concanavalin A. Proc.Nat.Acad.Sci. 69, 608-612.

Yahara, I., and Edelman, G.M.(1975). Electron microscopic analysis of the modulation of glyphocyte receptor mobility. Exp.Cell Res. 91, 125-142.

Yanagida, M. and Noda H. Cell contact and cell surface properties in the cellular slime mould, Dictyostelium discoideum (1967). Exp.Cell Res.45, 399

Yarger, J., Stults, K., and Soll, D.R.(1974). Observations on the growth of Dictyostelium discoideum in axenic medium. Evidence for an extracellular growth inhibitor synthesised by stationary phase cells. J. Cell. Sci. 14, 681-90.

Yarger, J., and Soll, D.R.(1975). Transcription and division inhibitors in the medium of stationary phase cultures of the slime mould, Dictyostelium discoideum. Biochim. Biophys. Acta. 390, 46-55.

Yu, N.Y., and Gregg, J.H.(1975). Cell contact mediated differentiation in Dictyostelium. Dev.Biol. 47,310-318.

Zahn, R.K., Muller, W.E.G., Geisert, M. Reinmuller, J., Michadis, M. Pondeljak, V., and Beyer, R.(1976). Species-specific aggregation factor in sponges. 1. Characterisation of the large circular proteid particle. Cell Differ. 5, 129-37.

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Alma P. Swan and D.R. Garrod 1975. AN INHIBITOR OF CELL COHESION FROM AXENICALLY GROWN CELLS OF THE SLIME MOULD, DICTYOSTELIUM DISCOIDEUM. Journal of Experimental Cell Research. Vol. 93, No. 2, July 1975, pp. 479-484