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STUDIES ON THE COHESION MECHANISM OF AXENIC *DICTYOSTELIUM DISCOIDEUM* CELLS

BY

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To my parents and Marina,
To Klaus

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ABSTRACT

FACULTY OF MEDICINE
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STUDIES ON THE COHESION MECHANISM OF AXENIC *DICTYOSTELIUM DISCOIDEUM* CELLS

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The cohesive behaviour of slime mould cells during axenic growth has been studied. Axenically-grown log phase Ax-2 cells cohere rapidly when shaken in phosphate buffer. After 3.5 days in stationary phase, cells become completely non-cohesive. However, stationary phase cells form mutual contacts with both log phase and aggregation-competent cells. Mixed cohesions of these 2 cell types is inhibited by both EDTA and a low-molecular-weight inhibitory factor present in stationary phase medium. The effect of this factor seems to be specific against contact sites B-mediated cohesion.

Plasma membranes isolated from log phase Ax-2 cells inhibit cohesion of homologous cells completely, and partially inhibit cohesion of aggregation-competent cells. Evidence is presented which suggests that these inhibitory effects are directed against contact sites B, mediated by a glycoprotein and non-specific for other species. A soluble extract obtained from log cell membranes with lithium diiodosalicylate (LIS), contains an active component which inhibits cohesion of log phase cells completely, and that of aggregation-competent cells only partially. The active material is sensitive to periodate, stable against heat and binds to Con A-Agarose beads, suggesting it is a glycoprotein. Evidence is presented to suggest that the extractable inhibitory activity binds to the surface of intact log phase cells. A ligand(glycoprotein) -receptor (carbohydrate-binding protein) model is proposed for the cohesion mechanism of vegetative slime mould cells, and the evidence discussed in relation to known aspects of behaviour in these cells.

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1.1.1. The general problem

" The harmonious cooperation of all beings arose, not from the orders of a superior authority external to themselves, but from the fact that they were all parts in a hierarchy of wholes forming a cosmic pattern, and what they obeyed were the internal dictates of their own natures"

Chung Tzu

The existence, development and successful evolution of multicellular organisms depend on a series of phenomena among which is the ability of embryonic cells to differentiate into various cell types. The final organisation of these cell types into tissues, specialized in a particular function, involves a variety of cell-cell interactions, among which the capacity of cells to adhere to each other is the most apparent.

This important basic biological phenomenon has attracted the attention of many biologists, but as yet, experimental data defining the phenomenon in molecular terms are largely lacking. Interest in the mechanism of cell adhesion has been further inspired by the fact that malignancy may be the result of changes in the normal adhesive properties of the cells, culminating in a pathological condition.

In the following paragraphs, a few investigations that have provided concrete information on the nature of the molecular constituents that mediate cell adhesion in various systems will be reviewed.

1.1.2. How do cells stick together?

Most cells bear negative charges at their surfaces, due to the presence of charged groups of ionised carboxylic groups of sialic acid residues (Eylar et al., 1962; Cook et al., 1961), phosphate groups (Mehrishi, 1970) and the carboxylic groups of aspartic and glutamic acid residues (Cook et al., 1962; Cook & Eylar, 1965). Since these charged groups create an electrostatic force which would tend to repeal charges of the same sign, two negatively charged cells would therefore tend to repeal each other.

Since cells are in fact capable of forming stable contacts between themselves, the immediate question to this would be: does the surface charge control the adhesiveness among cells? Many theories have been developed to answer this question. Due to the fact that cells will tend to repeal each other, it has been necessary to postulate the existence of attractive forces in order to account for cell adhesion.

Pethica (1961) has given a list of possible forces of adhesion between cell surfaces. The DLVO theory (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948) for example, proposed that the physical interaction between a pair of colloidal particles can be controlled by the relative magnitude of two forces: one electrostatic repulsive force originating from the overlap of interacting electrical double layers between surround charged particles suspended in an electrolyte solution, and a Van der Waals attractive force. Van der Waals forces are weak forces originating by the attraction caused by the polarization of the charge distribution of one atom, so that it is opposite to the oscillating charge distribution in another atom. Since these forces act over considerable distances (100 Å), they could

explain the mutal attraction of cells, their cohesion, and the persistence of a force resisting the separation of the cells.

Curtis (1960, 1962) made extensive use of this theory, and postulated the existence of two types of adhesions: those with surfaces in molecular contact, and those adhesions with a gap between plasmalemmae. Fairly good agreement has been found between prediction and measurement . By studying electron micrographs of cell contacts (Curtis, 1967) he found two types of cell contacts. In one type, the cells appeared to be in molecular contact (zonula occludens), whereas in the other they appeared to be 10-20 nm gaps (zonula adherens). Van der Waals forces were then the appropiate candidates for the attractive forces which opposed electrostatic forces suff ciently to allow adhesion at distances of less than 40 and 75-200 Å.

Another approach to the same question has been taken by Garrod and Gingell (1970) who investigated the surface properties of pre-aggregating D. discoideum cells by cell electrophoresis. This study was stimulated by the finding that cells in aqueous suspensions could be made to adhere by adding solutions of sodium and calcium chlorides (Born & Garrod, 1968). They found that the surface charge density of slime mould pre-aggregation cells was spontaneously and progressively reduced as the cells approached the chemotactic aggregation stage. This observation, however, could not reveal the nature of the forces responsible for cell adhesion. The authors concluded that under certain conditions, "the electrostatic repulsive force between cells may be a limiting factor in determining adhesive stability".

Gingell (1975) also developed a method to investigate the adhesion of cells to flat oil/water interfaces by which the extent of adhesiveness to the interface

could be observed microscopically. He found that negatively charged red blood cells adhered completely to the uncharged octadecanol-hexadecane/saline interface, whereas cell adhesion to uncharged interfaces required an attractive force to overcome the electrostatic repulsion between the cells. Gingell et al. (1977) have also presented evidence that adhesion of red blood cells to oil/water interfaces is mediated by an attractive force acting at a distance exceeding 10 nm. Although the author's analysis strongly implicated a long-range attractive force, its role in cell adhesion, in physiological conditions, has not been defined.

Investigations on the molecular basis of the adhesive interactions among cells have provided information sometimes difficult to interpret and often in conflict. The next paragraphs will review the experimental evidence supporting a general mechanism of cell adhesion based on molecular complementarity.

1.1.3. Complementarity and cell adhesion

As first introduced by Fisher (1894), the term "molecular complementarity" accounts for the phenomenon which takes place when two reacting molecules approach very closely to each other, so that "the projecting constituent atoms or groups of atoms of one molecule can fit into complementary depressions or recesses in the other" by means of weak interactions such as hydrophobic and hydrophylic bonds, Van der Waals forces and ionic interactions.

Based on this theoretical statement, Tyler (1946) and Weiss (1947) independently attempted to correlate the cell adhesion phenomenon with the principle of molecular complementarity. They suggested that cell surfaces might contain complementary molecules which could interact with each other specifically in a lock-and-key fashion. Molecules

of this type, according to Weiss, would have at least two functions: first, they would be determinants in intercellular recognition if both molecules were bound to the external surfaces of particular cell types; second, cells might communicate by releasing soluble factors (activators or inhibitors) which would bind to their complementary receptors on the surface of other cells. Cell adhesion could then be imagined as the result of the complementation of active groups between two cell surfaces, or by ion pairing between adjacent cells.

Since this theory was postulated, many approaches have been attempted to test its feas bility. At present, experimental evidence is available in favour of a general mechanism of cell adhesion, involving complementary molecules, i.e., ligands and receptors, such as those proposed by Weiss and Tyler. Aggregation factors, that is, macromolecules isolated from the medium in which the cells are grown and originated either from the cell surface (ligands, receptors) or as a secretion product of the cells (effectors) have been isolated from many systems (Humphreys, 1963; Moscona, 1968; Lilien, 1968). Cohesion inhibitors (Bozzaro & Gerisch, 1978; Swan et al., 1977; Merrell et al., 1975), high molecular weight cell surface proteins (Hynes, 1973), surface lectins and their receptors (Barondes & Rosen, 1976) and surface "contact sites" (Müller & Gerisch, 1978), have all been designated as reasonable candidates for cell adhesion complementary molecules in various biological systems. The next paragraphs will review the accumulated evidence in favour of a complementary-like mechanism of cell adhesion, and the role of these molecules as mediators of this phenomenon.

(i) An aggregation factor from marine sponges

The first succesful demonstration of a specific macromolecule involved in cell adhesion was achieved with marine sponges (Humphreys, 1963). Everything started when Wilson (1907) observed that if marine sponges of the Microciona genus were mechanically dispersed into single cells by cutting the organism into small pieces, passed through a silk cloth in Ca^{2+} and Mg^{2+}-free sea water, and let the resulting cell suspension settle in a glass dish, the cells attached and eventually reconstituted a small functional sponge.

The readhesion of aggregating sponge cells was subsequently shown to be preferentially species-specific (Wilson, 1907; Wilson, 1910; Galtsoff (1925), i.e., in mixtures of cells from two different species, each species tended to adhere to homologous cells. Most recently (Turner, 1978) it has been demonstrated that only three bispecific mixtures exist in which species-specific recognition accompanies cell adhesion. These properties made sponge cells an ideal model for studying the molecular basis of selective adhesion.

Microciona prolifera and M. parthena cells, dissociated in Ca^{2+}-free sea water were shown by Humphreys (1963) to release a high molecular weight factor into the supernatant in which the cells were washed. In the absence of this factor, the cells were found to be non-cohesive, whereas the addition of the supernatant restored the ability of the cells to cohere to each other. In addition, material which promoted adhesion of homologous cells was also obtained from several species (Humphreys, 1963; Moscona, 1968).

More recently, McClay (1974) confirmed that crude supernatants obtained from dissociated cells of five different species were found to be species-specific in

their aggregation-promoting activity, and that labelled supernatants appeared to bind to homospecific cells only. However, although the species-specificity of sponge aggregation factor has been widely demonstrated, it does not seem to be absolute, as was first pointed out by MacLennan and Dodd (1967). Burger and co-workers have concluded that the apparent specificity of the aggregation factor-cell interaction is in some cases due to "quantitative rather than qualitative differences". They explained that for example, at high concentrations, the Haliclona occulata aggregation factor was capable of aggregating Microciona prolifera cells, whereas "its specific activity for homospecific versus heterospecific cell aggregation differed by an order of magnitude" (Turner & Burger, 1973). Moreover, with crude factors isolated from different families of sponges, far less specificity has been found (MacLennan & Dodd, 1967).

The Microciona aggregation factor has been biochemically characterized in detail, and shown to be a 21×10^6 dalton proteoglycan complex (Cauldwell et al., 1973; Henkart et al., 1973), containing similar proportions of protein and carbohydrate, including 10-20% of hexuronic acid.

Kuhns and Burger (1971) have shown that the factor binds plant lectins and concluded that carbohydrates might be exposed on the aggregation factor surface. Burger et al. (1971), and subsequently Turner and Burger (1973) demonstrated that the glucuronic acid disaccharide, cellobiuronic acid, inhibited the aggregation factor-mediated reaggregation of Microciona prolifera cells obtained by dissociating the sponge in Ca^{2+}-free sea water, but failed to inhibit Cliona celata cell aggregation. This inhibition was found to be unique to glucuronic and cellobiuronic acids and did not occur with other monosaccharides such as

galacturonic acid and D-glucose. Turner and Burger (1973) also demonstrated that a crude preparation of glycosidases destroyed the activity of the Microciona factor. However, in the presence of glucuronic acid, the glycosidase treatment had no effect on the activity of the factor. If glucose or galacturonic acid were present during the incubation of the aggregation factor with the crude enzyme preparation, the activity of the factor was abolished. The ability of crude glycosidase preparation to destroy the aggregation factor activity was associated with glucuronidase activity in the glycosidase preparation, and the inhibition of the factor-mediated reaggregation of Microciona cells by glucuronic acid was interpreted to indicate that glucuronic-like receptors on the cell surface might act as a complementary acceptor for the factor molecule (Turner & Burger, 1973; Turner et al., 1974).

The cell surface component involved in this recognition event has been found to be released from the cells by osmotic shock (Weinbaum & Burger, 1973). It was found that when the cells were treated with the appropriate low concentration of saline, they lost the ability to reaggregate with added aggregation factor. The incubation of these shocked cells with the low salt wash, restored their ability to reaggregate in the presence of the factor. The active component released by hypotonic treatment was named "baseplate" (Jumblatt et al., 1975). The baseplate appeared to be a peripheral protein since it could be removed from the cell surface by mild hypotonic shock, and since it could not be sedimented at 105,000 g for 90 minutes. Since the baseplate preparation failed to promote, but rather inhibited, the reaggregation of mechanically dispersed cells, and because it neither precipitated the aggregation factor nor promoted the aggregation of factor--coated beads (Weinbaum & Burger, 1973), it was concluded that this molecule was a functionally monovalent macro-

molecule.

In order to investigate at what level the specificity of the factor-cell interaction resided, and also the role of divalent cations in the reaggregation process, Burger and Jumblatt (1977) quantitated the binding of partially purified aggregation factor to glutaraldehyde--fixed cells. They showed that Ca^{2+} was essential for sponge cell reaggregation, and predicted that this cation would be required for the binding of the factor to the cells, since the aggregation factor could be released from mechanically dissociated cells by soaking them in Ca^{2+}-free sea water. Subsequent studies (Burger et al., 1978) have established that the treatment of the aggregation factor with EDTA, low Ca^{2+} and heat all failed to produce cell aggregation, but did not affect its binding to the cells. It was also found that a crude preparation of the baseplate, at low concentrations, was an effective inhibitor of the binding of the factor to the cells.

According to these studies, Burger and co (1978) have proposed a model for the factor-mediated cell recognition system in Microciona prolifera. They have suggested that aggregation factor molecules, held in stable association by Ca^{2+} ions, contain carbohydrate residues which interact with surface sites, namely the baseplate component. This implies that sponge cells reaggregate via a complementary interaction between their baseplate components and the aggregation factor. The model proposed by Burger et al. (1978) provides a clear example of molecular complementarity in cell-cell interactions.

(ii) Cell surface glycosyltransferases

The binding of an enzyme to its substrate is perhaps the clearest demonstration of complementarity

in biological systems. This type of association has also been related to the cell adhesion phenomenon by Roseman (1970) who suggested that the intercellular bonds responsible for cell adhesion were mediated by an enzyme-substrate interaction. He illustrated this theory with specific reference to cell surface glycosyltransferases, which are enzymes that catalyse the transfer of sugars from sugar nucleotides to appropriate acceptors. The idea was that if cells possess surface glycosyltransferases and their appropiate substrates, and these are prohibited to interact with each other on a single cell surface, then the enzyme-substrate binding will occur between these two cells when they make contact. This association would then account for intercellular adhesion.

Glycosyltransferase activities have been found to be associated with the outer surface of plasma membranes of various cell systems. In isolated chick embryonic neural retina cells, for example, it has been found that the cells are capable of catalysing the transfer of radioactively-labelled galactose from uridine diphosphate galactose, to either endogenous or exogenous acceptors (Roth et al., 1971). Roth and co-workers have also demonstrated that these enzymatic activities were present in the surface of the cells, and postulated that in neural retina these enzyme-substrate complexes might exist between adjacent cells, and might govern at least partially the adhesiveness demonstrated by these cells.

Glycosyltransferases have also been found in human blood platelets (Jamieson et al., 1971). In this case, the platelet surface glucosyltransferases are thought to bind galactosyl residues present in collagen fibers when these are exposed by endothelial wounding. According to Jamieson et al. (1971), haemostasis would be the final result of this platelet-collagen interaction.

Another system in which cell surface glycosyltransferases have been reported is that of cultured mouse cells (Roth & White, 1972). However, in this system the enzymes seem to be involved in the synthesis of cell surface oligosaccharides rather than in controlling cell adhesion.

The cell surface location of glycosyltransferases has been strongly questioned, and many criticisms have been made by various workers who considered that the assigned roles of these enzymes in intercellular adhesion has been premature (Keenan & Moore, 1975). One of the weak points has been the demonstrated ability of cell surface enzymes to catalise the hydrolysis of necleotide sugars (e.g., UDP-galactose) to free sugars (e.g., galactose), which could then be incorporated intracellularly into glycoproteins, thereby not allowing the accurate measurement of the glycosyltransferase (galactosyltransferase) activity. Most recently, Porzig (1978) has demonstrated that neural retinal cells do catalyse the hydrolysis of UDP-galactose and that the products of this hydrolysis can be minimized in order to examine accurately the galactosyltransferase which catalyses the transfer of galactose from UDP-galactose to an exogenous acceptor, i.e., N-acetyl-D-glucosamine, to form N-acetylgalactosamine.

At present, the role of surface glycosyltransferases in cell adhesion has not yet been unambiguously substantiated. These enzymes could just function in the synthesis of surface molecules (e.g., glycoproteins) and may play no direct role in either recognition or communication between cells.

(iii) An aggregation-enhancing factor from embryonic cell culture medium

The rather straightforward approach developed

by Burger and co-workers with sponge cells has not proved to be applicable to vertebrate embryonic cells. One of the limitations for studying the cell adhesion mechanism in embryonic cells has been the method used to prepare single cell suspensions. With embryonic cells, treatment with proteolytic enzymes, most often trypsin, is used for that purpuse. Although very little direct evidence is available suggesting that the adhesive capacity is significantly altered by this procedure, strong circumstantial evidence has been presented (Roth & Weston, 1967; Roth, 1968). Other techniques of single cell preparation, such as soaked the tissue in Ca^{2+} and Mg^{2+} free salt buffers, or treatment with EDTA, have generally been ineffective in obtaining large amounts of viable single cells, and also seem to remove significant amounts of associated material from the cell surface (Kemp et al., 1967).

A completely different approach to the problem of isolating cell surface adhesive components from embryonic cells was initiated by Moscona (1962), who thought that if embryonic cells could be maintained under physiological conditions but prevented to form aggregates, the cells might continue to synthesize adhesive molecules which would accumulate in the culture medium. According to this idea, serum-free medium from such cultures of embryonic neural retina cells was added to cells dissociated by trypsin treatment, and the size of the aggregates formed after 24 hours of rotation was compared with controls which did not have "conditioned medium". An increase in aggregate size was observed when conditioned medium was present. However , the aggregation of cells from other tissue types, was not affected to the same extent as were neural retina cells (Moscona, 1962).

Following these observations, Lilien and Moscona (1967) and Lilien (1968), subsequently reported that serum-free medium from monolayer cultures of embryonic

chick neural retina cells caused an increase in the aggregate size. It was also found that after dialysis, this medium was highly specific when tested with heart, liver and limb bud cells, i.e,, only the aggregation of neural retina cells was increased. The active factor(s) present in this medium had no effect when added to trypsin-dissociated cells 2 hours after the onset of aggregation, or if the cultures were maintained at 4°C , in the presence of cycloheximide. However, when freshly dispersed neural retina cells were incubated in the presence of monolayer conditioned medium at 4°C, complete loss of activity from the medium was observed. This was found to be different for the incubation with heterotypic cell types. In this case, the retinal factor activity could be completely recovered.

On the basis of these findings, Moscona (1962) proposed that "embryonic cell recognition and selective cell adhesion are mediated by molecular complexes located in the cell membranes, which function on one hand, as cell identity markers and recognition sites and, on the other hand, as specific cell-cell ligands". According to this hypothesis the cell adhesion phenomenon would be a function of the cell-ligand complementarity.

The biochemical characterization of the retina cell aggregation-enhancing factor was first undertaken by McClay and Moscona (1974). They related its activity to a glycoprotein-containing fraction which, following fractionation and electrophoresis on SDS polyacrylamide gels, was found to band in a region corresponding to 50,000 molecular weight. Further purification of this material by electrofocusing techniques showed that it was a glycosylated protein (Hausman & Moscona, 1975). The analysis of the amino acid and carbohydrate composition of the purified preparation demonstrated a relatively high proportion of acidic amino acid residues and a total polysaccharide content of less than 20%.

Garfield et al. (1974), using the purified glycoprotein, confirmed that its molecular weight was 50,000 by sedimentation analysis. They suggested that the molecule responsible for the retina cell aggregation activity might exist in the factor as a 50,000 dalton unit, but did not exclude the possibility that the cell-ligand activity required larger complexes of these molecules.

The biological activity of this preparation was found to be destroyed by trypsin, but not by neuraminidase or periodate oxidation, suggesting that the carbohydrate moity did not play an important role in its activity (Hausman & Moscona, 1975). The factor was also found to be lacking galactosyltransferase activity (Garfield et al. 1974). Hausman and Moscona (1975) reported that proflavine inhibited the aggregation of embryonic neural retina cells, possibly by inhibiting the synthesis of the aggregation-enhancing factor. This was supported by the fact that the factor restored the ability of the cells to aggregate in the presence of proflavin.

More recently, Hausman and Moscona (1976) reported that the aqueous phase from butanol extracts obtained from plasma membranes of neural retina cells contained a component with similar characteristics and activity to those present in the monolayer conditioned medium. This activity could not be extracted from embryos of more than 13 days, and cells older than 13 days did not respond to the factor either. These facts suggested some kind of temporal regulation of both the accumulation of the factor, and the response to it. The authors also found that the aggregation enhancement seemed to be a function of the progressive enhancement of the size of the cell clumps which started one hour after the onset of aggregation. They suggested that the neural retina factor did not affect the initial rate of aggregation, but needed a period of meta-

bolic activity. Moscona and coworkers have considered this factor to be the intracellular ligand in the chick embryonic system. However, from the data available, this factor could rather have a catalytic role or an "effector" activity in stimulating the synthesis of adhesive components.

(iv) A ligand-receptor model for cell adhesion of chick embryonic cells.

Based on the observations made by Moscona (1962) and the fact that homotypic cells were able to remove an active factor from cell aggregation-enhancing supernatant of chick embryonic tissues, Lilien and coworkers (Balsamo & Lilien, 1974a) attempted to establish the role of such a factor in intracellular adhesion by studying radioactively labelled factors, isolated from different organs, to the cells. They tested 10-day embryonic chick neural retina supernatants against 10-day embryonic cerebral, optic lobe, and liver cells, and found that in each case the binding of heterotypic cells was minimal whereas that to homotypic neural retina cells was maximal. Further characterization of the binding reaction using sugars as competitors and digestion of the factor with glycolytic enzymes indicated that the binding of the retinal factor was mediated by a terminal N-acetyl galactosamine residue, whereas that of the cerebral lobe factor was mediated by a terminal mannosamine-like residue (Balsamo & Lilien, 1975).

Lilien and co-workers purified the binding factor and demonstrated that it bound a single macromolecule containing both protein and polysaccharide. However, the binding studies did not established the role of this molecule in cell adhesion (see Lilien et al, 1978).

Balsamo and Lilien (1974b) approached the question of whether the bound material was involved in cell

adhesion by assaying the agglutinability of glutaraldehyde-fixed cells in suspension over a monolayer of monotypic or heterotypic cells. Freshly dispersed cells, cells with bound factor and cells with pronase-treated bound factor were tested. It was found that only those cells with intact bound factor were agglutinable.

Based on these findings, Balsamo and Lilien (1974b) proposed an adhesive mechanism for neural retina and cerebral lobe chick embryonic cells. They suggested that at least three distinct components mediated cell adhesion: (1) a cell surface receptor defined on the basis that following trypsinization, there were surface sites which acted as specific receptors for the binding factors; (2) the binding factor (ligand) itself and (3) a component (the "ligator") from monolayer cultures which was able to agglutinate fixed cells only if they had been previously exposed to intact binding factor. In addition, they suggested that the activity of this component might depend on the integrity of the polypeptide portion of the binding factor. This model, although it was proposed as the only possibility, predicted that free ligand would inhibit the agglutination of the cells by competing for the ligand binding sites on the ligator. More recently this inhibition has been shown to be tissue-type-specific, i.e., ligands from cerebral lobe cells did not compete in the retina system and vice versa (Lilien & Rutz, 1977).

Subsequent to the binding and agglutination assays, Lilien and colleagues reported that the organ culture -conditioned media inhibited the induced capping of cell surface receptors by three different plant lectins (McDonough & Lilien, 1975). This activity was shown to be tissue-specific and, like the binding, as labile to N-acetyl hexosaminidase or alpha-mannosidase for neural retina or cerebral lobe cells respectively. The dependence of the binding and capping assays on the same terminal sugar indicated that a similar or

identical ligand was responsible. The identity of these two activities was confirmed by Lilien and Rutz (1977) who demonstrated that neural retina cells became agglutinable by monolayer-conditioned medium with the same kinetic patterns as they lost the ability of capping by lectins. The release of the capping inhibitor from the cell surface was also found to correlate with the loss of agglutinability (McDonough et al., 1977).

Lilien and colleagues (1978) have integrated all these ideas and proposed that "a surface adhesive ligand exists in a terminally unglycosylated form in association with a glycosyltransferase, presumabily a N-acetyl galactosaminyltransferase for retina cells, within an intracellular pool". According to the authors the pool of ligands and transferase is mobilized to the cell surface and the polypeptide portion of the ligand becomes exposed and is free to interact with the ligator. The release of the ligand would then be accompanied by its terminal glycosylation followed by a conversion of the transferase, which would now recognize the glycosylated ligand, and this ligand might interact with the cells. It is interesting to note that since this model implies glycosyltransferases in the turnover of the ligand, it could be related with the hypothesis proposed by Roseman (1970), although the view of the adhesion mechanism is different from the original theory. On the other hand, a cell adhesion model involving a "ligator" or bridging agent, has also many similarities to the proposed model for sponge cell recognition and adhesion (Weinbaum & Burger, 1973). The sponge "baseplate" component may be analogous to the retina binding factor, and the surface receptors for the retinal factor may be analogous to the surface receptors to which the baseplate binds. In any case, both models represent good examples of molecular complementarity in intercellular interactions.

(v) A cohesion inhibitory activity from plasma membranes of chick embryonic cells

Merrell and Glaser (1973) have taken an entirely different approach than that used by Lilien in the identification of surface macromolecules which may be involved in intercellular adhesion of chick embryonic cells. They prepared a plasma membrane-enriched fraction from 8-day embryonic chick neural retina and cerebellum cells. These membranes were assayed for their effect on both homotypic and heterotypic cell adhesion by counting the number of single cells remaining in suspension during the first hour of aggregation by rotation. It was found that the membrane fraction was capable of inhibiting homotypic but not heterotypic cell aggregation. Moreover, they showed that radioactivelly-labelled membranes bound only to homotypic cells. This binding was found to be temperature-dependent, being reduced at 4°C. Following these observations, Merrell and Glaser (1973) suggested that the cells might repair or replace components removed during the dispersion of the tissue for binding to occur.

On the basis of studies of membrane inhibition of cell aggregation as a function of development in various regions of the embryonal nervous system, Gottlieb et al. (1974) demonstrated that plasma membranes isolated from 7, 8 and 9-day neural retina cells showed their highest inhibitory activity on neural retina cells of the corresponding age, whereas with cells of different ages their activities was found to be very low. This fact suggested that a membrane factor or the component to which the membrane bound, might undergo an age dependent change.

Merrell et al. (1975) achieved a 20-fold purification of the active component from neural retina and optic tectum chick embryonic cells, by lipid extraction of

the membranes with acetone, extraction with lithium diiodosalicylate (LIS) and filtration through an Amicon PM-10 membrane. The purified materials showed the same biological and temporal activities observed in the membrane themselves, and also were trypsin-labile. They showed that the interaction of freshly prepared homologous single cells with the extract resulted in a decrease of inhibitory activity in the supernatant. This indicated that the active component was being adsorbed by the cells. The active fraction was found to be a glycoprotein in nature.

A similar approach has been taken by Vicker (1976) who trypsinized baby hamster kidney (BHK) cells and digested the soluble fraction with pronase. He was able to obtain a glycopeptide fraction which was fractionated by high voltage electrophoresis and tested for its effect on the rate of cell adhesion. It was found that two fractions had inhibitory activities on cell adhesion, and that this activity could be abolished by treatment with periodate or galactose oxidase. However, the activity of one of these two fractions was found to be enhanced by neuraminidase treatment. This fact was interpreted to indicate a masking effect of terminal sialic acid residues on the fractionated activity, and was found by Vicker to be consistent with the enhanced adhesion of BHK cells following treatment with neuraminidase (Vicker & Edwards, 1972).

The inhibition of cell adhesion by isolated plasma membranes or glycopeptides could be explained in terms of a direct surface interaction of two complementary molecules. The active fractions, i.e., inhibitory fractions, would compete with the cells for their complementary groups thus blocking cell-cell adhesions.

(vi) High molecular weight cell surface proteins as mediators of cell adhesion in chick embryonic cells

Based on studies of the specific interaction between molecules released from the cell surface, and the possible relationship between the properties of these molecules and their role in cell adhesion, Rutishauser and colleagues have proposed a model for cell adhesion in retinal and brain cells of chick embryos (Rutishauser *et al.*, 1976) which also involves complementary molecules.

Proteins on the surface of chick embryonic neural cells were compared with proteins released from the cells and collected in the culture medium. One component released, denominated F1, was found to be a protein of 140,000 molecular weight derived by proteolytic cleavage of a cell surface precursor (240,000 dalton). Another component, F2, obtained from the culture medium as a dimer of molecular weight 110,000 seemed to be a product of limited proteolysis of F1. On the other hand, it was found that neural retina cells had a surface protein of molecular weight 150,000 which also appeared to be derived from the cell surface precursor by proteolytic cleavage.

Antibodies to F2 were raised, and showed that they interacted with determinants on the cell surface protein of molecular weight 150,000 and specifically inhibited the homologous and heterologous aggregation between dissociated neural retina and brain cells. By contrast, antibodies raised against F1 failed to inhibit cell adhesion and did not show cross reaction with F2.

From these observations Rutishauser *et al.* (1976) suggested that the cell surface protein of molecular weight 150,000 was involved in cell adhesion of both neural retina and brain cells, and that the molecular mechanism of cell adhesion was the same in these two tissues.

According to Rutishauser et al. (1976), a cell adhesion molecule (CAM) would contain the antigenic determinants demonstrated on F2, and CAM would contain F2 as an integral portion of its polypeptide chain. Another molecule, called ProCAM, would be the cell surface precursor from which CAM was generated by proteolytic cleavage and which was found on the surface of neural retina cells grown in monolayers. This precursor, according to Rutishauser, could be cleaved upon activation by adjacent surface proteases, to yield CAM molecules still attached to the cell surface.

Assuming that CAM participates directly in cell adhesion, the authors proposed three possibilities for the nature of cellular interactions: (1) CAM molecules on different cells could bind directly to each other; (2) CAM molecules on different cells could be bridged by an unidentified molecule, i.e., an aggregation factor, and (3) CAM molecules on one cell could bind to an unidentified receptor on the surface of an adjacent cell.

Subsequent studies by Rutishauser et al. (1978 a) have shown that the CAM molecule exists on the plasma membranes of neural retina chick embryonic cells. Furthermore, two cell populations which differed in their rates of aggregation seemed to have a different relative density or accessibility of CAM molecules on their surfaces, as measured by antibody absorption techniques.

Rutishauser et al. (1978 b) have also determined the distribution of CAM molecules on plasma membranes of nerve processes of the chick embryo. Their results indicated that the cell adhesion molecule was distributed over the whole surface of nerve processes and a major function was postulated for CAM: to mediate side-to-side adhesion between neurites to form fascicles. However, these studies did not provide any information about the direct participation of CAM in cell adhesion of embryonic cells.

High molecular weight surface macromolecules have also been described in other systems. The transformation-sensitive protein" (LETS) described by Hynes (1974) may in fact resemble ProCAM, the precursor of CAM molecules reported by Rutishauser et al. (1976). It has been shown that LETS is a glycoprotein with a molecular weight of 250,000 which might be involved in some way with growth control and agglutination reactions (Yamada et al., 1975).

Although the structure, location and probable general function of these related surface proteins are known, many important questions still remain open. For example, if CAM is an adhesive protein, how does it function in vivo and what is the mechanism by which these proteins mediate cell adhesion?

It is important to note that the comparison between the studies reviewed on the embryonic cell recognition system is limited, mainly due to differences in the assay procedures. However, in all cases a ligand-receptor interaction is proposed to explain the adhesive behaviour of these cells and the properties demonstrated by aggregation factors, ligands, receptors, precursors. The role of carbohydrates on such interactions has also been strongly suggested, not only in embryonic cells but also in other systems (Turner & Burger, 1973; Roseman, 1970; Barondes & Rosen, 1976; Swan et al., 1977).

(vii) Complementary molecules as mediators of cell cohesion in cellular slime moulds

Complementary molecules have also been suggested as attractive candidates involved in intercellular adhesion of slime mould cells. These include surface lectins and their receptors (Barondes & Rosen, 1976) and glycoproteins

and their receptors (Müller & Gerisch, 1978; Swan *et al.*, 1977). Cohesion inhibitory activities have been isolated and also related to the cell adhesion phenomenon (Swan *et al.*, 1977; Bozzaro & Gerisch, 1979). The next section will review studies on the biochemical and immunological characterization of these molecules, in relation to their role in a general cell adhesion mechanism based on cell complementarity.

1.2. The cellular slime moulds

1.2.1. Why slime moulds?

Dictyostelium discoideum has been attracting the attention of many workers who have realized its unique advantages as a model eukaryotic system in which to study the phenomenon of cell adhesion. The main reason for this is that this organism presents a clear developmental transition, readily controlled by the availability of nutrients between a non-social and a social stage. Over the several hour period in which the cells differentiate to an aggregation-competent state, it is possible to study accompanying cell surface changes by morphological, immunological and biochemical techniques. These studies are aided by the fact that slime mould cells are easily cultured, and large amounts of cells at any stage of development or during growth can be obtained.

In addition, single cell suspensions can be prepared by simply pipetting the cell pellet in an appropiate buffer and no trypsinization or other treatments which could damage the cells, are involved. The analysis of cell adhesion mechanisms can also be assisted by aggregateless mutants which are readily generated.

All these advantages have been considered, and the slime mould cell system chosen as a suitable system in which to study the cell adhesion mechanism. This was encouraged by the hope that a better understanding of the molecular basis of cell adhesion in this lower eukaryote might be relevant for the understanding of related phenomena in other systems. The next paragraphs will give a general description of the organism and will review the biophysical, biochemical and immunological studies which relate to the mechanism of cell adhesion.

1.2.2. The organism: a general description

The growth or vegetative stage of *Dictyostelium discoideum* consists of small solitary amoebae which feed on bacteria by phagocytosis and divide by fission every 3 to 4 hours. Essentially, each amoeba behaves independently showing no consistent orientation with respect to its neighbours, and forming no stable associations. When the food supply is depleted, a unique and characteristic series of developmental events occurs which distinguishes *D. discoideum* from less social organisms.

Guided by a chemotactic system, the amoebae align and move over large distances towards discrete aggregation centres, forming streaming patterns (Loomis, 1975). Within the streams, the cells are closely adherent with their long axes predominantely aligned in the direction of movement. At each centre, the cells assemble into a peg-like multicellular aggregate surrounded by a slime sheath. These multicellular units topple over onto the surface and migrate horizontally still surrounded by the sheath, and as such are referred to as pseudoplasmodium, grex or slug.

After a variable period of migration, the pseudoplasmodium stops migrating and the anterior tip moves vertically in preparation for the construction of the fruiting body. The fuiting body, which marks the endpoint of differentiation, consists of two distinct differentiated cell types: the vacuolated structural cells of the stalk, or stalk cells and the spore cells responsible for the continuation of the cell line. In general, stalk cells arise from the anterior portion of the pseudoplasmodium, whereas the spore cells derive from the posterior section (Loomis, 1975).

Depending on species, the fruiting body consists of one or more spore caps borne on an unbranched or

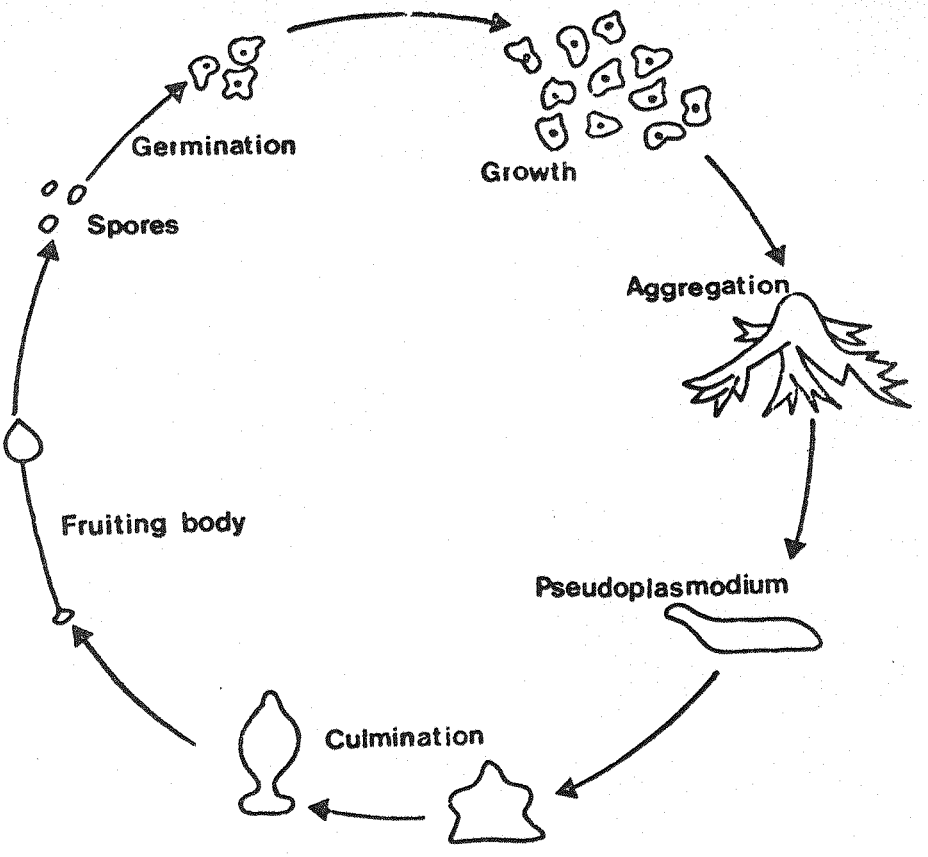

Figure 1-1: The life-cycle of Dictyostelium discoideum. The diagram is not drawn to scale. Spores are 8 μm long, vegetative cells 10 μm in diameter, and the multicellular stages are usually composed of 10^4 to 10^5 cells.

branched stalk. In some instances, two species are distinguished mainly by the pigmentation of their spore caps.

When the spores are dispersed, they germinate by splitting the spore case and escaping as single amoabae. The sequence of developmental stages for Dictyostelium discoideum is shown schematically in figure 1-1.

1.2.3. Mechanism of cell adhesion

Various attempts have been made to study the cohesion mechanism of Dictyostelium discoideum. These can classified into these categories:

(1) Biophysical studies on the plasma membrane fluidity, and the relationship between this parameter and definite changes that could be associated with intercellular adhesion (Von Dreele & Williams, 1977). tudies on the electrophoretic properties of cells at different stages of development (Garrod & Gingell, 1970; Yabuno, 1970; Lee, 1972). Although it has been speculated that a reduced surface charge might contribute to the strength of cell-cell cohesion (reviewed in section 1.1.2) and also that plasma membrane fluidity could be a critical determining factor in aggregation (Ellingson, 1974), no direct evidence has been reported yet.

(2) Biochemical studies, in which cell surface proteins, in particular those related the development of cohesiveness, have been attempted to be identified and isolated. These include the work by Smart and Hynes (1974) and Siu et al. (1975) who used the lactoperoxidase-mediated radioiodination of external membrane proteins to study the pattern of labelled proteins associated with development of Dictyostelium discoideum. Both groups did agree that a polypeptide of molecular weight 130,000 became accessible to labelling

after 12 hours of differentiation.

Another approach used to correlate surface protein changes with differentiation was to determine the polypeptide composition of isolated plasma membranes from D. discoideum cells at different stages of development. Hoffman and McMahon (1977), using high resolution gradient polyacrylamide SDS gel electrophoresis, resolved 55 proteins corresponding to vegetative cells. They observed that during the first 12 hours of development, 8 bands diminished and 5 bands either increased or appeared for the first time. Three of these 5 bands appeared to be external, however, there was no apparent correlation between these three and th external proteins labelled by Smart and Hynes (1974).

Hoffman and McMahon (1977) also analysed the glycoprotein components of Dictyostelium discoideum plasma membranes using the periodic acid Schiff (PAS) staining of SDS polyacrylamide gradient gels. They found that membranes isolated from vegetative cells contained 15 glycoproteins and by 12 hours of development, one of these had disappeared, two had diminished and three new bands had appeared. However, non of these proteins were isolated or directly proposed as cell cohesion mediators.

Most recently, Gilkes et al. (1979) have detected qualitative and quantitative changes in the protein and glycoprotein components of plasma membranes isolated from D. discoideum cells. They also analysed the neutral and amino sugar composition of the membranes and found that it remained essentially unchanged during the first 12 hours of differentiation. The major sugars were found to be mannose, fucose, and glucosamine. These studies suggested that aggregation might involve a number of surface components; however, they did not define the function of each of these components in cell adhesion.

The changes in cell surface components during

growth and development has also been approached by studying the susceptibility of vegetative and aggregating cells to plant lectins (carbohydrate-binding proteins). Weeks (1973) and Kawai and Takeuchi (1976) for example, found that Concanavalin A, a plant lectin which binds predominantly to alpha-glucopyranosyl or alpha-mannopyranosyl residues, agglutinated vegetative D. discoideum cells. The susceptibility to ConA agglutination was found to decrease gradually over the first 10 hours of development (Weeks & Weeks, 1975). Furthermore, Weeks and Weeks (1975) demonstrated a gradual decrease in the susceptibility of slime mould cells to agglutination by ConA as the cells went from the exponential growth phase to stationary phase in axenic medium.

Direct studies of the cell surface "receptors" for lectins have also been carried out by Weeks (1975), Geltosky et al. (1976) and Darmon and Klein (1976), who have found a very small increase in the number of cell surface binding sites in vegetative and aggregation-competent cells. However, in terms of lectin sites per unit surface area, there was a marked increase of lectin sites with differentiation (Weeks, 1975; Geltosky et al., 1976). They indicated that the decreasing agglutination observed with differentiation was therefore not due to a diminished density of ConA receptors. The question of whether lectin receptors on the surface of D. discoideum cells, in particular those which are developmentally regulated, function in cell-cell adhesion, still remains unsolved.

(3) Immunological studies in which the role of surface antigens in cell cohesion have been investigated. Gregg (1956) provided early evidence that many antigens appeared on the surface of D. discoideum, D. purpureum and Polysphondilium violaceum cells between the vegetative and aggregation stages. Other studies (Gregg & Trygstad, 1958) indicated that some aggregateless mutants of D. discoideum

had altered surface antigens. Bonneborn et al. (1964) first tried to study the role of new surface antigens in the aggregation process. They raised an aggregation-specific antibody against D. discoideum aggregates, and absorbed this with vegetative cells. The new absorbed antibody was found to be capable of blocking the aggregation of cells on an agar surface without affecting their viability.

However, the most comprehensive immunological approach to identify the relevant antigens in cell cohesion of D. discoideum cells has been taken by Gerisch and co-workers. Gerisch's work will be reviewed in detail in the next paragraphs.

At present, two main lines of evidence exist in favour of a complementary-like mechanism of slime mould cell-cell adhesion. These are originated from immunological work by Gerisch and co-workers who suggest that cohesion in D. discoideum is mediated by specific surface antigens, i.e., "contact sites", and the works by Barondes and Rosen, and Lerner and co-workers who postulate that cell-cell interactions in D. discoideum is mediated by developmentally-regulated carbohydrate-binding proteins, i.e., lectins. These two cohesive models are reviewed in detail as follows.

(i) Contact sites A and B

Gerisch has made use of quantitative cohesiveness assays to investigate the mechanism of cell cohesion in D. discoideum. Early work (1961) demonstrated that both vegetative and aggregation-competent cells cohered in gyrated suspension. This was observed by counting the proportion of aggregates of various sizes under the microscope. However, the cohesiveness of vegetative cells was inhibited almost completely by EDTA, whereas that of aggregation-competent cells persisted in the presence of the chelating agent.

Following these observations, Beug et al. (1970) attempted to block selectively the formation of cell contacts between aggregating cells, by antibodies attached to their surfaces. The main questions to be answered were: Does the binding of antibodies to any cell surface component prevent cell cohesion?, must the antibody be attached to specific sites in order to inhibit? Antibodies raised against crude extracts of different D. discoideum cells were used to answer these questions. These antibodies were degraded to univalent Fab fragments in order to avoid immune agglutination of the cells. The low molecular weight of Fab fragments was also considered as an advantage since the target region which was going to be covered by attached Fab would be smaller than that covered by intact bivalent antibodies.

It was found that Fab against homogenized aggregation-competent cells, were able to block adhesion of these cells to each other and to a substratum (Beug et al., 1970). This inhibition suggested the binding of Fab molecules to certain cell surface antigens. However, the following questions remained open: (1) What is the function of these target sites?, (2) How many different inhibiting Fab-species exist?, (3) Does the cell surface possess complementary "contact sites" which specifically interact with each other according to a ligand-receptor model?. These questions were partially answered by Beug et al. (1973 a). They prepared univalent antibodies from antisera obtained against different cells, and absorbed them with vegetative cells. This new Fab fragment was able to block the EDTA-resistent cohesion of aggregation-competent cells (Gerisch, 1961) but failed to affect the EDTA-sensitive cohesion of vegetative cells. The appearance of an EDTA-resistent cohesion in differentiated cells was also correlated with the ability of these cells to form firm end-to-end contacts on a substratum (Beug et al., 1973 a). The target antigens to which aggregation-specific Fab bound were defined as "contact sites A" (CS A).

On the other hand, Fab prepared against vegetative cells were capable of blocking EDTA-sensitive cohesion of those cells, but did not affect the EDTA-resistent cohesion of aggregation-competent cells. In this case, the target antigens were called "contact sites B" (CS B) (Beug et al., 1973 a).

It was also possible to quantify CS A and CS B at various stages of development, by determining the loss of inhibitory activity of appropriate Fab after the absorption with particulate fractions derived from the cells. CS A were undetectable before 4 hours of development, but between 4 and 9 hours there was a steep increase in the amount of CS A which closely paralleled the development of aggregation-competence, as measured by the EDTA-resistent cohesion at this stage. By contrast, the amount of CS B, did not vary substantially over the first 9 hours of development (Beug et al., 1973 a).

The binding of ^{3}H-labelled aggregation-specific Fab molecules to aggregation-competent cells was studied by Beug et al. (1973 b). They found that 3×10^{5} Fab molecules per cell were sufficient to completely block contact sites A. Similarly, contact sites B were completely inhibited by binding of 2×10^{6} Fab (non-absorbed) molecules per cell.

The electron microscope localization of antigens has also been performed with ferritin-labelled antibodies directed against plasma membranes of aggregating cells, from which the antibodies against growth phase cells had been removed (Gerisch et al., 1974). No accumulation of antigens at the ends of single aggregation-competent cells was found. The presence of contact sites A all over the cell surface was interpreted to indicate that every part of the surface was a "potential tip of the cell " (Gerisch et al., (1975).

Aggregateless mutants have also been used to evaluate the significance of contact sites A. In one class of mutants, both CS A and the ability to form EDTA-resistent contacts did not develop with starvation (Beug et al., 1973 a; Gerisch et al., 1974). Another group showed no loss of CS A absorbing activity, indicating that either CS A were functionally inactive or that the mutant lacked a membrane component involved in cell cohesion for which there was inadequate inhibition by Fab. However, the role of contact sites in cell cohesion has been greatly dependent on the purification and characterization of the antigens involved.

Contact sites A have been solubilized by sodium deoxycholate and the Fab-binding assay (Beug et al., 1973 a) has been used for their purification (Huesgen & Gerisch, 1975). By DEAE-cellulose chromatography and gel filtration, Huesgen and Gerisch (1975) separated CS A from the major membrane proteins and from membrane-bound cyclic AMP phosphodiesterase. Both pronase and sodium periodate treatments destroyed the Fab-binding activity of solubilized contact sites A (Gerisch et al., 1975). This result indicated that carbohydrate residues might be required for the structural integrity of these sites.

More recently, Müller and Gerisch (1978) purified CS A and identified them as a Concanavalin A-binding glycoprotein of molecular weight 80,000, which appeared as a minor constituent (2×10^5 copies per cell) on the surface of aggregation-competent cells. From these results the authors have proposed some possibilities on how CS A might function in cell cohesion. According to them, cell adhesion in D. discoideum might be due to the interaction of complementary molecules such as (1) a transmembrane dimer, formed by protein-protein interaction between the sites on the surface of the cells, (2) a dimer formed by mutual protein-

carbohydrate interaction, or (3) mediated by a third component, i.e., a ligand, which would interact with high affinity to one of the cell surface sites, activating the complementary association between two adjacent cells.

However, the cohesive apparatus of vegetative Dictyostelium discoideum cells, i.e., contact sites B, has not been described in detail.

(ii) Cell surface carbohydrate-binding proteins

Early work by Rosen (1972) demonstrated that when tannic acid-treated formalin-fixed erythrocytes were exposed to a crude extract of D. discoideum slugs, the red blood cells spontaneously agglutinated. The most striking observation was that the level of agglutination activity in the slug extracts was significatively higher than in extracts obtained from growth phase cells. Furthermore, over the first 12 hours of development, the specific haemagglutinatinin activity in the cell extracts increased over 400-fold, showing a steep rise between 6 and 9 hours after starvation. It was also found that the agglutination activity was heat-labile, trypsin-sensitive and non-dialysable, indicating a protein involved.

Further investigations (Rosen et al., 1973) established that the agglutination activity found in slime mould extracts prepared from cells harvested between 3 and 9 hours after starvation, could be inhibited by specific saccharides. Rosen et al. (1973) found that N-acetyl-D-galactosamine was a strong inhibitor, although other sugars such as glucose also blocked the agglutination activity but were less effective. These observations clearly suggested that the haemagglutination activities found in slime mould extracts were carbohydrate-binding proteins, i.e., lectins. Since these lectins appeared to be present on the surface

of aggregating but not vegetative cells, the authors suggested the possibility that a developmentally-regulated carbohydrate-binding protein could function in cell cohesion, however, did not present any direct evidence.

The appearance of a lectin-mediated agglutination activity in soluble extracts of D. discoideum as well as in P. pallidum cells, was also found to correlate the development of cellular cohesiveness (Rosen et al., 1973; 1974). In D. discoideum, both the lectin activity and the cell cohesiveness (quantified by measuring the EDTA-resistent cohesion in gyrated suspension as described by Beug et al., 1973 a) were undetectable in the first 2 hours after depletion of nutrients, but increased markedly between 6 and 9 hours of development. By contrast, P. pallidum growth phase cells showed a significant amount of lectin activity which increased after 4 hours of development in parallel with the acquisition of cell cohesiveness.

Because of its affinity for galactosyl residues, the lectin from D. discoideum soluble extracts could be purified by affinity chromatography on Sepharose 4B (a linear polymer of galactose residues) (Simpson et al., 1974). After resolving the purified agglutinin fraction in DEAE-cellulose, 2 distinct species were found: discoidin I and discoidin II (Frazier et al., 1975). It was found that they were tetramers with molecular weights of about 100,000 (Simpson et al., 1974; Reitherman et al., 1975) each made of 4 identical subunits of 26,000 (discoidin I) and 24,000 (discoidin II) (Frazier et al., 1975). The carbohydrate specificities of discoidin I and II were found to be different, as measured by the ability of different sugars to inhibit their haemagglutinin activities (Frazier et al., 1975). They also possessed different relative activities in agglutinating sheep, rabbit and human erythrocytes, indicating that their binding sites might be different

(Frazier et al., 1975). Discoidin I and II were also found to be different in their developmental regulation. This was demonstrated by the amounts extracted from cells at different stages of development. In vegetative cells, both lectins were undetectable, whereas between 6 and 12 hours of development, discoidin I increased 20-fold and discoidin II increased 3-fold to a level that was only 10% of that achieved by discoidin I (Frazier et al., 1975).

Pallidin, the purified lectin from P. pallidum was isolated by Simpson et al. (1975). This protein showed an approximate molecular weight of 250,000. Its aminoacid composition, peptide map, isoelectric point and carbohydrate -binding specificities were clearly different from those determined for discoidin I and II (Frazier et al., 1975; Rosen et al., 1974). Four other species of slime moulds have shown to contain lectin activities (Rosen et al., 1975). The major lectins from D. mucoroides (mucoroidin) and D. purpureum (purpurin) were very similar to discoidin I and II, but their carbohydrate-binding specificities were clearly different.

The location of the agglutination activities found in slime mould cell extracts has been widely studied. It has been found that the lectins of P. pallidum and D. discoideum are located on the cell surface. This was first demonstrated by the finding that intact cohesive cells formed rosettes with erythrocytes, which could be inhibited by lectin-specific sugars but not by non-specific sugars (Rosen et al., 1973; 1974). The formation of rosettes was interpreted to indicate the interaction between cell surface lectins and oligosaccharides on the surface of red blood cells.

Using antibodies against purified discoidin, Chang et al. (1975) showed its presence on the surface of

cohesive D. discoideum cells by immunofluorescence and immuno-ferritin labelling techniques. Siu et al. (1976) confirmed the surface location of discoidin and its developmental regulation. They radioiodinated cells at different stages of development, and prepared extracts of the labelled cells. These were treated with antidiscoidin and the immune precipitates analysed by SDS-gel electrophoresis to measure the extent of iodination of discoidin. Similar studies have demonstrated the presence of pallidin in P. pallidum cells, however, in contrast to D. discoideum cells, Chang et al. (1977) found detectable amounts of lectin in growth phase cells.

Discoidin has also been detected in purified plasma membranes, by SDS-gel electrophoresis (Hoffman & McMahon, 1977). Membranes isolated from vegetative Ax-3 cells and 12-hour differentiated cells, both appeared to contain discoidin. In both cases, discoidin was eluted from the gels and found to contain galactose-binding activity (West & McMahon, 1977).

All these observations led to investigate the nature of the association between surface lectins and the plasma membrane. The search for lectin receptors on the surface of slime mould cells was initiated by Rosen et al. (1974) who first provided evidence that they existed, by inhibiting the pallidin activity of aggregation-competent cells with D-galactose. They heat-treated aggregation competent P. pallidum cells to reduce the endogenous cohesiveness, and then agglutinated the cells by adding pallidin in a gyrated suspension. D-galactose was found to inhibit cohesion of the cells, whereas D-glucose, a non-specific sugar, had no effect. Similar studies were performed by Reitherman et al. (1975) who demonstrated changes in the discoidin-induced agglutinability of D. discoideum cells at different stages of development. This study suggested the

possibility of a "receptor modification in structure or topography during development".

The interaction between isolated slime mould lectins and cell surface receptors, has been approached by studying the direct binding of those molecules to the cells (Reitherman et al., 1975). These studies have shown that 9-hour D. discoideum cells bound a maximum of 4-5 x 10^5 discoidin I or discoidin II molecules per cell, whereas vegetative cells bound slightly less of these lectins, with 25-fold lower affinities. These results suggested the possibility that specific complementarity between lectins and receptors might play an important role in intercellular adhesion.

The chemical nature of lectin receptors on the surface of slime mould cells has not been determined yet. However, in early experiments with P. pallidum cells, an inhibitor of pallidin was solubilized from crude membranes (Rosen et al., 1976). This inhibitor was presumed to contain complex carbohydrates.

On the other hand, Lerner and co-workers have also postulated carbohydrate-binding proteins (CBP) as mediators of cell adhesion in slime mould cells. Most recently they attempted to isolate a cross-reacting material mutant which seemed to have a defect in the structural gene for the 26,000 dalton subunit of the carbohydrate-binding protein described by Frazier et al. (1975). This mutant was shown to be non-cohesive, lack haemagglutination activity, and failed to develop (Ray et al., 1979). The fact that wild-type cells (NC-4) were able to complement the mutant for development indicated that except for the defect in CBP-26 (the 26,000 dalton subunit), the mutant cells were completely competent to adhere, aggregate and proceed through the life cycle.

This was interpreted to indicate that during development, CBP bound to a carbohydrate receptor, and although the mutant lacked CBP, it might have the receptor and thus could be complemented by wild-type cells which contained CBP. According to this, the authors proposed various models for the molecular basis of cell cohesion. They included (a) the trans-membrane dimer formation between CBP-26 of adjacent cell surfaces, (b) a dimer formation by interaction between complementary protein-carbohydrate molecules on opposing cell surfaces and (c) the CBP-26, acting as a cell ligand between the carbohydrate receptors on adjacent cell surfaces.

Since Müller and Gerisch (1978) have shown that purified contact sites A from D. discoideum aggregation-competent cells, is distinct from both CBP and its receptor (Huesgen & Gerisch, 1975), Ray et al. (1979) suggested that more than one molecular system is necessary for cell cohesion and development. However, no direct evidence has been presented to explain the role of cell surface carbohydrate-binding proteins in cell cohesion.

1.2.4. The cohesion inhibitor: A potential tool for the study of the cohesion mechanism of vegetative cells

Recent investigations on the cohesive properties of axenically grown slime mould cells (Swan & Garrod, 1975) have demonstrated that stationary phase medium from cultures of D. discoideum Ax-2 cells contained a low molecular weight (approx. 500) factor which (1) completely inhibited the cohesion of log phase Ax-2 cells, (2), partially inhibited the cohesion of aggregation-competent Ax-2 cells, (3) partially inhibited the cohesion of cells of other slime mould species, (4) reduced the adhesion of D. discoideum cells and other species to glass, and (5) reversebly inhibited

the development of D. discoideum cells prior the aggregation stage (Swan et al., 1977; Garrod et al., 1978).

Gas-liquid chromatography analysis of this factor has indicated that it is a carbohydrate (Swan, 1978), however, its chemical characterization is still under investigation. The molecule has been found to be trypsin and heat-stable, as well as dialysable. A series of sugars and cyclic nucleotides have been tried in order to study whether the effects of the cohesion inhibitor could be mimiced. cohesion of log phase Ax-2 cells was found to be unaffected by a range of sugars, including maltose, lactose, sucrose, fructose and ribose. This indicated that these simple sugars were not responsible for the effects described for the factor (Swan, 1978). Moreover, this low molecular weight inhibitory factor has been suggested to bind the surface of log phase cells (Swan et al., 1977; Swan, 1978). All these observations however, could not descriminate the possibility of some kind of relationship between the low molecular weight factor and a transcription inhibitor reported by Yarger on stationary phase cultures (Yarger et al., 1974; Yarger & Soll, 1975). On the other hand, the fact that the cohesion inhibitor was effective against cohesion and adhesion of other species of slime moulds, was suggested to be due to lack of specificity (Nicol & Garrod, 1977; Garrod et al., 1978).

Although there is considerable amount of evidence implicating cell contacts in various aspects of slime mould development (Newell et al., 1971; Newell et al., 1972; Gregg & Badman, 1970; Gregg, 1971; Aldrich & Gregg, 1973; Yu & Gregg, 1975; Sakai & Takeuchi, 1971; Garrod & Forman, 1977; Forman & Garrod, 1977), there has been no direct evidence that the inhibitory effect of the factor accumulated in stationary phase cultures, on development, is just due to the inhibition of cell cohesion.

On the other hand, Bozzaro and Gerisch (1978) have isolated an inihibitory activity from soluble fractions of vegetative D. discoideum cells. This activity was shown to inhibit cell differentiation and also cell aggregation. It appeared to be a low molecular weight (1,000-1,300) molecule, stable against heat, alkali, periodate and pronase treatment. This inhibitor was shown to block the increase of adenyl cyclase activity, the formation of EDTA-stable cell contacts (contact sites A, as defined by Beug et al., 1973 a) and the release of an inhibitor of cyclic-AMP phosphodiesterase into the extracellular medium. The authors suggested that if there is a relationship between their factor and the extracellular inhibitory factor described by Swan et al. (1977), the former might be a precursor of the extracellular factor. However, the mechanism of action of these molecules and the role that they might play in cell cohesion and cell development of D. discoideum ha not been clearly defined.

As it has just been reviewed, specific molecules have been proposed as possible mediators of intercellular adhesion in slime moulds. The candidated molecules are usually said to mediate cell cohesion because either the isolated molecules induce cell cohesion or antibodies against them are able to inhibit cell cohesion. Most of these studies however have been concerned with the mechanism of cohesion of aggregating cells, and very little is known about the cohesive apparatus of vegetative cells. The low molecular weight inhibitory factor found in stationary phase cultures appears as a potential tool for the study of the molecular basis of cohesion between growth phase cells. The present work provides evidence for a ligand-receptor model of vegetative cell cohesion, in which the cohesion inhibitor plays an important role.

Chapter two: Materials and Methods

2.1. Growth and development conditions

2.1.1. Axenically grown cells

Most of the experiments reported in the present work make use of *Dictyostelium discoideum* strain Ax-2 (Watts & Ashworth, 1970). Axenic medium was made up as follows:

Oxoid Yeast Extract	7.15gr
Oxoid Bacteriological Peptone	14.3gr
$Na_2HPO_4.12\ H_2O$	1.28gr
KH_2PO_4	0.49gr
H_2O	to 1 litre, pH 6.7

The medium was aliquoted into 60 or 700 ml quantities, placed in 250 or 2000 ml flasks respectively and autoclaved. Cells were then inoculated into the medium under sterile conditions, and supplemented with 86 mM glucose (final concentration). The cultures were then incubated in a rotary shaker with a radius of rotation of 2.75 cm at 22°C and 140 r.p.m. Under these conditions, the cells attained a final concentration of 1-2 x 10^7 per ml, with a generation time of 8-9 hours. "Log phase" cells were harvested from the growth medium when they had reached a density of 1-5 x 10^6 cells/ml; "stationary phase" cells were harvested when the cell count had remained constant at approximately 1-2 x 10^7 cells/ml for 3.5 days (at or after point E in Fig. 2a) except where otherwise stated.

Harvesting was performed by centrifugation at 350 g for 5 minutes at 4°C (or 2000 g for 15 mins in the case of 700 ml volumes). The cells were then washed with cold distilled water and 17 mM phosphate buffer, pH 6.0 before resuspending in the required medium.

2.1.2. Bacterially grown cells

Dictyostelium discoideum (strain NC-4), Dictyostelium mucoroides (Dm), Dictyostelium purpureum (Dp) and Polysphondylium violaceum (Pv) were grown with a bacterial nutrient source. For such growth, 3-4 spore heads of the relevant species were inoculated with 0.2 ml of 1-2 -day old bacterial broth (Escherichia coli B/r) onto sterile nutrient agar plates prepared as follows (Sussman, 1966):

Oxoid gar	20gr/1t
Glucose	10gr/1t
Oxoid Bacteriological Peptone	10gr/1t
$MgSO_4.7\ H_2O$	2.06gr/1t
K_2HPO_4	1.0gr/1t
KH_2PO_4	1.5gr/1t

After spreading the spores over the entire surface, the agar plates were incubated in the dark at 22°C under humid conditions. The feeding cells cleared the bacteria in about 40 hours (Dm was quicker) and reached the aggregation stage 48 hours after the inoculation.

Vegetative cells were harvested from the plates after 40 hours of growth by flooding the agar surface with cold distilled water, and removing the cell layer. The cells were centrifuged at 350 g for 5 minutes at 4°C, and washed with cold distilled water until contaminating bacteria had been removed.

2.1.3. Development of cells

Ax-2 cells harvested at log phase of growth were allowed to develop by placing 0.5 ml of the cell suspension (1×10^8 cells/ml of distilled water) over the surface of washed 47 mm Millipore filters (AABP0-4700), resting on absorbent pads saturated with 1.6 ml of a solu-

tion containing:

50 mM Na_2HPO_4/ KH_2PO_4 buffer,
1.5 gr/lt KCl,
0.5 gr/lt $MgCl_2.6H_2O$
0.5 gr/lt Streptomycin sulphate, at pH 6.5.

The filters and adsorbent pads were maintained within 50 mm petri dishes, which were incubated in the dark under humid conditions at 22°C. Under these conditions, cells developed with high degree of synchrony (Sussman, 1966). To test the effect of a particular substance on development, the substance was included into the Millipore solution at the required concentration and the cells allowed to develop as described.

2.1.4. Acquisition of Aggregation-Competence in shaken suspension

Log phase Ax-2 cells were harvested, washed and suspended in 17 mM phosphate buffer, pH 6.0 at a final concentration of 1 x 10^7 cells/ml. Cells were shaken in a New Brunswick G-86 rotary shaker with a radius of rotation of 0.7 cm, at 22°C and 140 r.p.m. After 8 hours, the cells had achieved aggregation-competence. Cells were then dissociated by washing in cold distilled water, and repeated pippeting through a fine-tipped Pasteur pipette before use in cohesion assays or membrane isolation.

2.1.5. Test for Aggregation-Competence

After 8 hours in shaking suspension, the cells were washed, dissociated in cold distilled water, and resuspended in 17 mM phosphate buffer, pH 6.0 at a final concentration of 5 x 10^5 cells/ml. The single cell suspension was assayed for chemotactic aggregation-competence by

allowing the cells settle onto a glass cover-slip for 30 minutes. The cells on the cover-slip were then examined for the characteristic chaining patterns formed by end-to-end cell contacts (Gerisch, 1968).

2.2. Cohesion assays

The method used to assess the cohesiveness of cells in suspension consisted of counting the number of particles (single cells and cell aggregates) for a given initial cell number over a defined period of time. In all cases, the degree of cohesion was expressed as the percentage of the total initial count; thus, in a single-cell suspension, this value was 100%, and the percentage decreased as the cells cohered.

2.2.1. In phosphate buffer

Washed cells were suspended in 17 mM phosphate buffer, pH 6.0 at a final concentration of 1×10^6 cells/ml. This concentration gave a convenient haemocytometer count of 100. The cells were shaken at 140 r.p.m. in a New Brunswick rotary shaker maintained at 22°C. Cohesion was assessed by sampling the shaking suspension at intervals and counting the number of particles per ml.

2.2.2. In EDTA

To test the effect of EDTA on cell cohesion, the cells were shaken in 17 mM phosphate buffer, pH 6.0, containing 10 mM EDTA. Cohesion was assessed as described in the above section.

2.3. Cohesion inhibitory factor

The inhibitory factor accumulated in stationary phase cultures was isolated and purified as described by Swan, Garrod and Morris (1977).

2.3.1 Isolation and purification

For routine preparations of partially purified activity, 700 ml of stationary phase medium were dialysed overnight at 4°C against 2000 ml of distilled water. The inhibitory activity was concentrated from the dialysate into approximately 5 ml by evaporation under vacuum using a Searle rotary evaporator. The purification of the concentrated dialysed material was performed by:

a) Gel filtration chromatography in Sephadex G-50 (Fine),
b) Anion-exchange chromatography on QAE-Sephadex A-25,
c) Cation-exchange chromatography on SP-Sephadex C-25, and
d) Desalting in Sephadex G-10.

After each chromatographic step, the fractions containing inhibitory activity on cohesion of log phase cells were pooled and reduced to about 5 ml by rotary evaporation. The concentrated final product was kept frozen and used within 15 days; there was no loss of activity during this time.

2.3.2. Biological activity assay

To test the effect of the cohesion inhibitory factor on cohesiveness of various cell populations, a dose--response curve was first established in order to select the minimum concentration of product giving complete inhibition of cohesion. One unit of activity was defined as that amount required to give 100% inhibition of cohesion of log phase cells at 1×10^6 cells/ml in 17 mM phosphate buffer, pH 6.0

under the standard conditions of shaking.

After harvesting, the cells were washed and suspended in 17 mM phosphate buffer, pH 6.0 at a final concentration of 1 x 10^6 per ml. The inhibitory factor was added at the desired concentration, the cell suspension was shaken and cohesion assessed as described in 2.2.1.

2.4. Cell treatment

Throughout the course of this work, it was nessesary to treat the cells with cycloheximide in order to study its effects on cell cohesion. The technique used for such treatment is described as follows:

2.4.1. Cycloheximide treatment

Cells were harvested at log phase of growth, washed in cold distilled water and suspended at 1 x 10^6 per ml in 17 mM phosphate buffer, pH 6.0 containing 500 ugr/ml of cycloheximide. The cell suspension was shaken in a New Brunswick rotary shaker at 140 r.p.m. and 22°C. The cells were washed with cold distilled water before use in cohesion assays or plasma membranes isolation. When cycloheximide--treated cells (CH-cells) are refered, these correspond to log phase Ax-2 cells treated with CH for 8 hours in shaken suspension, unless otherwise stated.

2.5. Plasma membranes

2.5.1. Cell lysis

Obviously, the choice of a suitable cell lysis procedure is the key step in all fractionation work. This holds both for the isolation of soluble substances such as proteins and nucleic acids and for suspended particles, such

as cell organelles and plasma membranes which may aggregate, dissociate or generally change their state with time.

Riedel and Gerisch (1968) have proved that plasma membranes from Dictyostelium discoideum cells can be stabilized as ghosts when they are lysed with digitonin, a detergent which seems to block the formation of lysolecitin by inhibition of the phospholipase A which is present at high activity in cell homogenates. This method was chosen as the most suitable one, and is described as follows:

After harvesting, the cells were washed twice in cold phosphate buffer and once in 30 mM Tricine buffer, pH 7.0. The cells were then resuspended in Tricine buffer at a final concentration of 1 x 10^8 per ml. An equal volume of a solution containing Tricine-buffered digitonin (1 mg digitonin/ml in Tricine buffer, pH 7.0) was added to the cell suspension and the mixture stirred for one minute at room temperature (Riedel & Gerish, 1968). 60% sucrose (another stabilizing agent) was added to a final concentration of 6% and the suspension stirred for another 15 minutes. This suspension was examined in the light microscope to ensure that the majority of cells were lysed and cell ghosts were present. After 30 minutes centrifugation at 13,000 g and 4°C, the pellet was washed once in 20 mM Tris buffer, pH 7.5 and centrifuged again at 13,000 g for 30 minutes at 4°C. The pellet was then resuspended in 50 ml of 20 mM Tris buffer containing 0.5 mg DNase (Sigma Chem Co.) at pH 7.5, and centrifuged at 13,000 g for 30 minutes at 4°C. The supernatant was discarded and the pellet ("crude particulate fraction") was used for plasma membrane isolation by the two-phase dextran-polyethyleneglycol method.

2.5.2. The two-phase polymer system

A number of methods for the isolation of

mammalian cell membranes have been reported (Warren, Glick & Nass, 1967; Korn, 1969). Most of them are based on differences in density or sedimentation characteristics to achieve separation of the surface membranes from other cell organelles. However, these methods do not always result in satisfactory yield or purity of the isolated material (Wallach, 1967).

In the present work, the aqueous two-phase polymer system developed by Albertson (1971) has been used for the isolation of plasma membranes of Ax-2 cells as described by Brunette and Till (1971) for L-cell surface membranes. This method has proved suitable for the rapid isolation of plasma membranes from various sources with a good yield and high purity.

The basis for separation by a two-phase system is the selective distribution of substances between two phases. For soluble substances, distribution takes place mainly between two bulk phases, and the partition is characterized by the partition coefficient, K:

$$K = \frac{Ct}{Cb}$$

where Ct and Cb are the concentrations of the partitioned substance (in moles/lt) of top and bottom phase respectively. The interface between the phases should, however, also be considered because it has a certain capacity for adsorption of the partitioned substance. Therefore, in the separation of cell particles, there are in fact three phases to consider: the upper, the inner and the lower phase. It is the selective distribution between these phases which forms the basis for separation of particles by a two-phase system.

Aqueous-aqueous systems have been proved to be the most suitable systems for partition of biological particles. They consist essentially of two immiscible aqueous solutions of different polymers. A mixture such as that results in phase separation.

When partitioning macromolecules or particles, equilibrium is obtained by adding them to a phase system and then allowing them to distribute by their own thermal motion. In practice, this would take too long since the diffusion of the particles is very slow. To acieve equilibrium quickly, one centrifuges the suspension, supplying kinetic energy to the particles in addition to that of the thermal motion.

The two-phase system used for the isolation of plasma membranes of Dictyostelium discoideum Ax-2 cells was prepared as follows:

200 grms of 20% (w/w) dextran-500 in distilled water,
103 grms of 30% polyethylene glycol (PEG) in distilled water,
99 ml of distilled water,
333 ml of 0.22M phosphate buffer (sodium salts) at pH 6.5,
and 80 ml of 10 mM $ZnCl_2$ were mixed in a separatory funnel and allowed to settle overnight at 4°C. The two phases (Top and Bottom) were collected; some precipitate formed, which was collected along with the Bottom phase and allowed to settle out. Both phases were stores at 4°C.

2.5.3. Plasma membrane partition

The pellet obtained after the last centrifugation at 13,000 g for 30 mins (see section 2.5.1) was resuspended in 10 ml of Top phase and then 10 ml of Bottom phase were added. The two phases were mixed, placed in a 3 x 25 ml MSE swing rotor and spun at 8,500 r.p.m. for 10 minutes. On completion of this centrifugation, the membranes were found at the interface of the two-phase system. The interface material, together with the Top and Bottom phases were carefully transfered to another tube, remixed and centrifuged at 8,500 r.p.m. This was repeated until no pellet was observed at the bottom of the tube. This was done in order to wash the interface material. The final supernatant (i.e., the membranes

and the two phases) was similarly remixed and spun at 8,500 r.p.m. for 10 minutes at 4°C. The material at the interface was collected and diluted with approximately 5 times its volume of cold distilled water. The membranes were then sedimented out of the suspension by centrifugation at 17,000 r.p.m. for 15 minutes at 4°C. The pellet was washed twice in distilled water and centrifuged as described. The isolated plasma membranes were immediately used or otherwise stored in 30% (v/v) glycerol at -20°C (see fig. 2-1).

2.5.4. Protein determination

The concentration of protein was determined by the method of Lowry et al (1951) with bovine serum albumine as standard. Stock solutions were made up as follows:

Reagent A: 2% Na_2CO_3 in 0.1N NaOH
Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% sodium-potassium tartrate
Reagent C: 50 ml of reagent A mixed with 1 ml of reagent B
Reagent D: 1N Folin-Ciocalteu reagent (Sigma Chem. Co.)
Albumin stock: 1 mg/ml in sample buffer or distilled water.

To a protein sample containing 25-250 µgr of protein in 1 ml (standards were prepared by adding 25, 50, 100, 150, 200 and 250 ults of albumin stock to 975, 950, 900, 850, 800 and 750 µlts of buffer or distilled water respectively), 5 ml of reagent C were added. The tubes were well mixed and allowed to stand for 10 minutes at room temperature. 0.5 ml of reagent D were added with immediate mixing. The tubes were allowed to stand for 30 minutes and the optical density at 600 nm was read in a spectrophotometer. The calibration curve was made by plotting absorbance against corresponding albumin concentrations in µgrms of protein, and was used to calculate the test results.

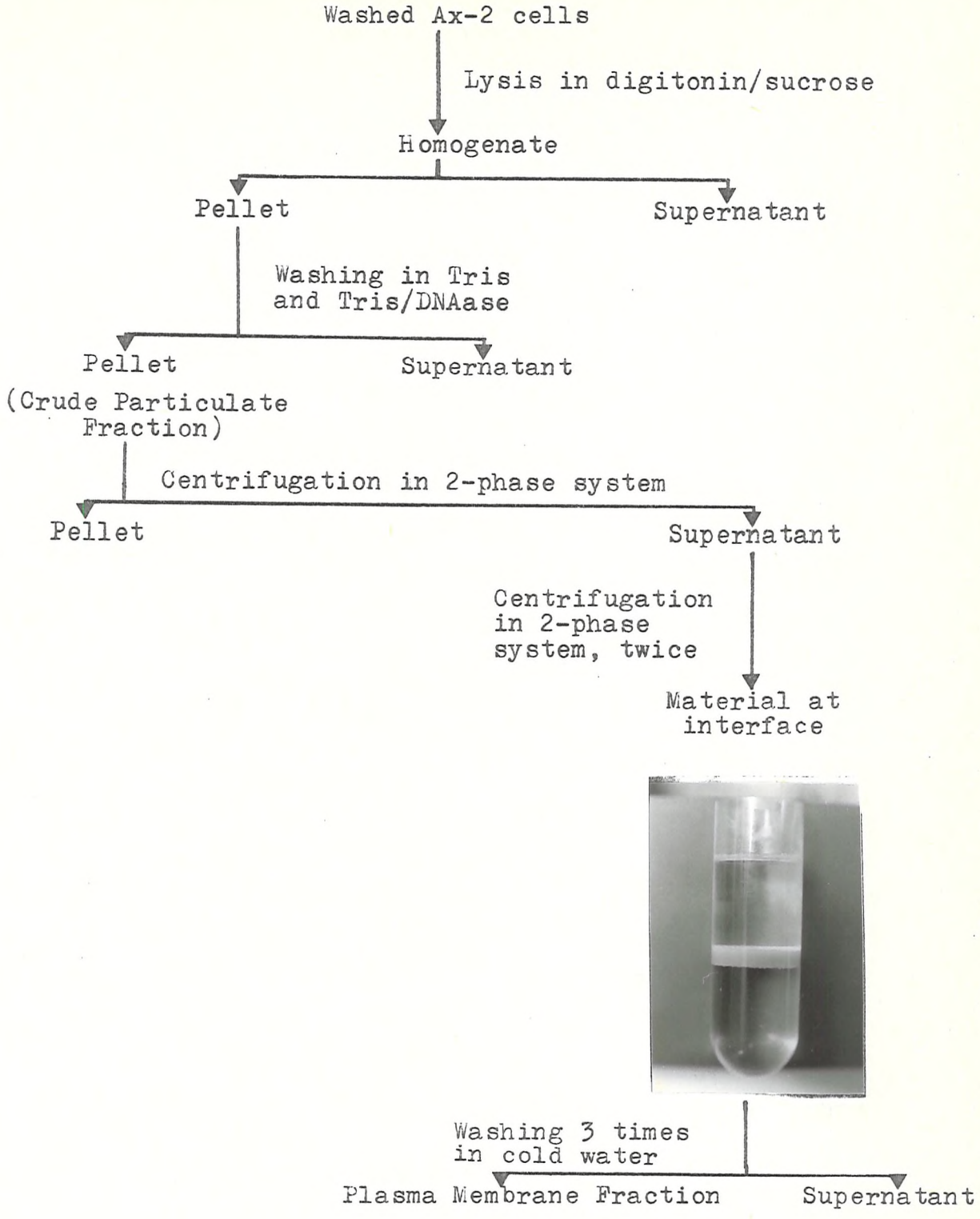

Figure 2-1: Summary of the procedure used for isolation of plasma membranes from Ax-2 *Dictyostelium discoideum* cells.

2.5.5. Biological activity assay

To test the effect of isolated plasma membranes on cohesiveness of various cell populations, a dose-response curve was established. After harvesting, the cells were washed and suspended in 17 mM phosphate buffer, pH 6.0 at a final concentration of 1 x 10^6 per ml. The plasma membranes were suspended in a small amount of the same buffer, and added to the cell suspension at the desired protein concentration. The cell suspension was shaken and cohesion assessed as described in section 2.2.1.

2.5.6. Stability against periodate

In order to investigate the possible role of carbohydrates on the inhibitory effect of cell membranes on cell cohesion, plasma membranes isolated from log phase Ax-2 cells were treated with periodate as described by Bozzaro and Gerisch (1978) and suggested by G. Weeks (personal communication).

2 mg of plasma membrane protein were incubated with 3 ml of 10mM or 50mM sodium periodate in phosphate buffer, pH 7.2, overnight at 4°C in the dark. The reaction was stopped by centrifuging the treated membranes and washing them three times with cold distilled water (20,000 r.p.m., 30 mins at 4°C). Periodate-treated membranes were tested for biological activity on cohesiveness of log phase cells as described in section 2.5.5.

2.6. Enzyme markers assays and electron microscopy

The marker enzymes alkaline phosphatase (EC 3.1.3.1), succinate dehydrogenase (EC 1.3.99.1) and glucose 6-phosphatase (EC 3.1.3.9) were used as indicators or monitors to assess the purity of the plasma membrane fractions, as

described by Green and Newell (1974).

2.6.1. Alkaline phosphatase

This enzyme was used as a plasma membrane marker; the activity was determined as a Mg^{2+}-dependent alkaline p-nitrophenyl phosphatase activity, as indicated in the reaction:

p-nitrophenyl phosphate $\rightleftharpoons$ p-nitrophenol + inorganic phosphorus

(colourless in alkali) (yellow in alkali)

The assay system contained in a test cuvette:

40 mM 2-amino 2-methyl 1-propanol, pH 10.3
5 mM $MgSO_4$
2 mM KF
and the enzyme sample.

$MgSO_4$ was replaced by 10 mM EDTA in the control cuvette. After 5 minutes of incubation, the reaction was initiated by addition of p-nitrophenyl phosphate to 10 mM final concentration. The inclusion of an EDTA control allowed correction for hydrolysis by other phosphatases, such as glucose 6-phosphatase, which does not require Mg^{2+} ions (Hübscher & West, 1965).

The rate at which p-nitrophenol was liberated at pH 10.3 and 22°C was measured as the change in absorbance (ΔA) per minute at 404 nm. The specific activity was expressed as umol of product liberated per min per mg of protein. The molar extinction coefficient for p-nitrophenol at 404 nm was 1.73×10^4 lt/mol/cm as reported by Bessey and Love (1952).

2.6.2. Succinate dehydrogenase

Mitochondria were located by assaying succinate dehydrogenase, an enzyme generally considered to be in

the inner mitochondrial membrane (Schneider, 1946). Succinate is oxidized to fumarate by the flavoprotein succinate dehydrogenase, which contains covalently bound flavin adenine dinucleotide (FAD). Its reducible coenzyme FAD functions as an hydrogen acceptor in the reaction:

$$\text{succinate} + \text{E-FAD} \rightleftharpoons \text{fumarate} + \text{E-FADH}_2$$

The reduced flavin dehydrogenase can be now reoxidized by certain artificial electron acceptors, such as phenazine methosulphate and 2,3-dichlorophenolindophenol (DPIP). These dyes undergo changes in their absorption spectra when they are reduced by the enzyme, as:

$$\text{E-FADH}_2 + \text{2,6-DPIP}_{ox} \rightleftharpoons \text{E-FAD} + \text{2,6-DPIP}_{red}$$

where 2,6-DPIP_{ox} and 2,6-DPIP_{red} represent the oxidized and reduced form of 2,6-dichlorophenolindophenol respectively. The rate of reduction of this dye measured at 600 nm, is proportional to the amount of enzyme present.

The activity of succinate dehydrogenase in the plasma membrane fraction was determined as described by Ells (1959). The incubation mixture contained:

50 mM phosphate buffer, pH 7.2
20 mM sodium succinate
10 mM KCN
0.018 mg/ml 2,6-DPIP
0,4 mg/ml phenazine methosulphate,
and the enzyme sample, in a total volume of 3 ml. Sodium succinate was replaced by phosphate buffer in the control cuvette. The rate of reduction of 2,6-DPIP was measured as the change of absorbance at 600 nm per min. The specific activity was reported as umol of product/min/mg of protein. The molar extinction coefficient used for 2,6--DPIP was 2.1×10^4 lt/mol/cm (King, 1967).

2.6.3. Glucose 6-phosphatase

This enzyme, considered to be a microsomal marker (Goldfischer, Essner & Novikoff, 1964) was used to locate endoplasmic reticulum. The activity was measured by the hydrolysis of glucose 6-phosphate, as indicated in the reaction:

$$\text{glucose 6-phosphate} \rightleftharpoons \text{glucose} + \text{inorganic phosphate}$$

and liberated inorganic phosphate was determined by the method of Fiske and Subbarow (1925). The incubation mixture contained:

43 mM maleate buffer, pH 6.0
28.5 mM glucose 6-phosphate
5 mM EDTA
2 mM KF

plus the enzyme sample. 5 mM EDTA and 2 mM KF were included in order to inhibit alkaline and acid phosphatase respectively. Glucose 6-phosphate was replaced by maleate buffer in the control tube. The mixtures were incubated at 37°C for 15 minutes, and the reaction stopped by addition of 1.3 ml of 10% trichloroacetic acid and 2 ml of 5% trichloroacetic-acid containing 12 gr/100 ml of Norit A (activated charcoal). The samples were centrifuged at 1,000 r.p.m. for 10 minutes, and inorganic phosphate determined in the supernatant.

2.6.4. Inorganic phosphorus determination

When a phosphorus-containing sample is reacted with ammonium molybdate in an acid medium, phosphomolybdate is formed. A mixture of sodium bisulphite, sodium sulphite and 1-amino 2-naphtol 4-sulphonic acid (Fiske & Subbarow reducer), reduces the phosphomolybdate to form phosphomolybdenum blue complex. The intensity of the colour is pro-

portional to the phosphate concentration and is measured at 660 nm in a spetrophotometer.

Stock solutions: The Fiske-Subbarow solution was prepared by dissolving 1 gr of Fiske-Subbarow reducer (Sigma Chem.Co.) in 6.3 ml of distilled water. The solution was filtered and stored in the dark at room temperature. The acid molybdate solution contained ammonium molybdate (1.25 gr/100 ml) in 2.5 N sulphuric acid. The phosphorus standard solution contained potassium phosphate, monobasic, equivalent to 20 ugr inorganic phosphorus per ml in diluted hydrochloric acid.

Calibration standards of inorganic phosphorus were prepared by adding 0.5, 0.75, 1.0, 1.25, 1.50 and 1.75 ml of the stock solution to distilled water to a final volume of 5 ml. The experimental samples contained 2 ml of the supernatant obtained as described in the previous section, and 3 ml of distilled water, whereas the experimental "blank" contained 2 ml of 10% (w/v) trichloroacetic acid and 3 ml of distilled water. 1 ml of the acid molybdate solution was added to each tube and the content mixed by swirling. 0.25 ml of the Fiske-Subbarow solution was then added, mixing each tube by inversion. They were allowed to stand 10 minutes at room temperature for colour development. The contents were transfered to cuvettes in order to read the absorbance at 660 nm. The calibration curve was constructed by plotting absorbance versus inorganic phosphorus concentration in nmol/lt, and was used to calculate the test results. The specific activity of glucose 6-phosphatase was reported as nmol of inorganic phosphorus liberated/min/mg of protein.

2.6.5. Electron microscopy

The plasma membrane fraction was pelleted by centrifugation at 60,000 g for 15 minutes; the pellet was treated for electron microscopy as described by Green and Newell (1974).

2.7. Plasma membrane solubilization

Solubilization of plasma membrane proteins usually requires treatment with reagents that destroy the structural integrity of the membrane. Numerous detergents (amphipathic substances with polar and non-polar moieties) are available for this purpose. They include negatively charged, positively charged and neutral species.

Recent studies (Razin, 1972) have shed some light on the mechanism by which detergents release integral proteins from the non-polar phospholipid matrix of plasma membranes. The hydrophobic portion of a non-ionic detergent seems to interact specifically with the hydrophobic portion of the protein, substituting for the fatty acid chains of the membrane phospholipids. The protein bound to the detergent is thereby released in an apparently molecularly dispersed form.

On the other hand, an ionic detergent will interact with and alter the conformation of the hydrophilic as well as the hydrophobic moiety of the protein. Little interaction is observed between typical hydrophilic proteins and non-ionic detergents.

The second group of agents used to solubilize biologically active plasma membranes are the aqueous denaturants or chaotropes, which includes urea, guanidinium chloride and lithium diiodosalicylate. They function by introducing "chaos" into the biological membranes, in part by disrupting the ordered arrangement of water molecules that surround these structures.

Organic solvents have also been used both separately and in conjunction with detergents and chaotropes (Razin, 1972).

Throughout the course of the present work, the

isolated plasma membrane proteins were exposed to different extraction treatments, in order to obtain a soluble fraction which showed complete retention of biological activity (see section 2.5.5). As the chemical nature of the active protein involved was not known, a wide variety of extracting agents were tried:

2.7.1. Extraction in chloroform:methanol

Plasma membranes isolated from log phase Ax-2 cells (see section 2.5.3) were extracted with cloroform :methanol according to Folch et al. (1961). 7 mg of membrane protein were suspended in 0.5 ml of distilled water. 10 vol. of cold chloroform:methanol (2:1 v/v) were added, the suspension mixed and kept at 4°C overnight. The precipitated protein and the methanol:water (top phase) soluble proteins were collected and centrifuged at 20,000 r.p.m. at 4°C for 30 minutes. The pellet was washed twice with 2:1 (v/v) cloroform:methanol, and collected by centrifugation at 20,000 r.p.m. at 4°C for 30 minutes. This pellet was suspended in phosphate buffer in order to test its biological activity as described in 2.5.5. The chloroform-soluble material was obtained by drying the chloroform phase (lower) under vacuum at 4°C. The dried material was suspended in phosphate buffer for the biological activity assay (see section 2.7.8).

2.7.2. EDTA extraction

EDTA has been generally used to extract peripheral proteins from plasma membranes by removal of Ca^{2+} and Mg^{2+} ions (Saier & Stiles, 1975). In the present work it was decided to use EDTA not just because of its divalent cation chelating property, but also because of its inhibitory effect on cohesion of log phase cells (Gerisch,

1961). It was thought that if 10 mM EDTA was able to impart complete inhibition on cohesion of vegetative slime mould cells, probably due to some kind of interaction between the chelating agent and the cohesive molecules involved in the cell surface (Beug et al., 1970), the treatment of plasma membranes isolated from log phase cells with 10 mM EDTA would give rise to the selective fractionation of those molecules, which would also be obtained in a soluble form.

Plasma membranes isolated from log phase Ax-2 cells were suspended in 50 mM Tris buffer pH 7.5 containing 10 mM EDTA and 2 mM phenyl-methyl-sulfonyl fluoride (PMSF, an inhibitor of proteases, Sigma Chem. Co.), at a final concentration of 5.6 mg protein/ml. The suspension was stirred for 1 hour at 4°C and then centrifuged at 30,000 g for 30 minutes. The supernatant was collected and dialysed overnight against 50 mM Tris buffer pH 7.5. The "control" buffer used in the cohesion assay (see 2.7.8) consisted of 50 mM Tris buffer pH 7.5 containing 10 mM EDTA and 2 mM PMSF, dialysed against Tris buffer in the same way.

When the extraction was carried out from the crude particulate fraction (see section 2.5.3) in order to improve the yield, the same procedure was followed except that the starting concentration of material was 12 mg of protein/ml.

2.7.3. Sodium deoxycholate extraction

The method used for the solubilization of membrane proteins with sodium deoxycholate (NaDOC) was that described by Pearlstein (1977). 4 mg of plasma membrane protein were suspended in 3 ml of 1% NaDOC in 50 mM Tris buffer pH 7.5 containing 2 mM PMSF. The suspension was stirred for 30 minutes at 4°C and centrifuged at 30,000 g for 30 minutes at 4°C. The supernatant was extensively

dialysed against several changes of Tris buffer, and then tested for biological activity (see section 2.7.8). The "control" buffer consisted of 1% NaDOC in 50 mM Tris buffer pH 7.5 containing 2 mM PMSF, dialysed against Tris buffer in the same way as the experimental samples.

2.7.4. Sodium dodecyl sulphate extraction

Plasma membranes isolated from log phase Ax-2 cells were suspended in 1% of sodium dodecyl sulphate (SDS) made up in 50mM Tris buffer pH 7.5, and containing 2 mM PMSF, at a final concentration of 1.3 mg of protein/ml. The suspension was stirred for 1 hour at room temperature and centrifuged at 30,000 g for 30 minutes at 4°C. The supernatant was collected and dialysed against several changes of 50 mM Tris buffer. The biological activity of the extract was tested as described in section 2.7.8. The "control" buffer consisted of 1% SDS in 50 mM Tris buffer pH 7.5 containing 2 mM PMSF and dialysed against Tris buffer in a similar way.

2.7.5. Triton X-100 extraction

Plasma membranes isolated from log phase Ax-2 cells were suspended in 0.1% Triton X-100 (v/v) containing 17 mM phosphate buffer pH 7.2 and 2 mM PMSF, at a final concentration of 1.3 mg/ml. The suspension was stirred at room temperature for 4.5 hours and then centrifuged at 30,000 g for 40 minutes. The detergent was removed from the supernatant by adsorption onto Bio-Beads SM-2 (Bio Rad Labs.), a neutral porous styrene divinyl benzene copolymer, as described by Holloway (1973).

66 ml of methanol were added to 10 grms of Bio-Beads SM-2. The mixture was stirred on a glass funnel and washed with a further 200 ml of methanol, The beads

were not allowed to dry but were immediately washed with 500 ml of distilled water. 1 gr of moist copolymer beads was added to a 2 ml protein sample (supernatant from the Triton X-100 extraction). The sample was gently agitated at 4°C for 2 hours, and aliquots were removed at various time intervals for determination of absorbance at 275 nm due to Trion X-100. After 2 hours, the beads were removed by centrifugation at low speed, and the protein sample stored in fresh beads overnight at 4°C. The biological activity of the Triton X-100-free soluble extract was tested as described in section 2.7.8. The "control" buffer for such assay consisted of 0.1% Triton X-100 in 17 mM phosphate buffer pH 7.2 and 2 mM PMSF, which had been treated with Bio-Beads as described above.

2.7.6. Nonidet P-40 extraction

Plasma membranes from log phase Ax-2 cells were suspended in 0.5% Nonidet P-40 (NP-40) in 17 mM phosphate buffer pH 7.2 containing 2 mM PMSF, at a final concentration of 2 mg of protein/ml, and stirred for 2 hours at 4°C as described in section 2.7.5. The extraction was performed in the same way as with Triton X-100, and the detergent removed with Bio-Beads as described in the previous section. The biological activity of the NP-40-extracted fraction was tested as described in section 2.7.8. The "control" buffer in this case consisted of 0.5% NP-40 in 17 mM phosphate buffer pH 7.2 containing 2 mM PMSF, treated with Bio-Beads as described with Triton X-100.

2.7.7. Lithium diiodosalicylate extraction

Lithium diiodosalicylate (LIS) has been used to extract plasma membrane proteins in a water-soluble form as indicated by Marchesi and Andrews (1971), and

Merrell et al. (1975).

Plasma membranes isolated from log phase Ax-2 cells were treated as follows:

a) suspended in 30 mM LIS in 50 mM Tris buffer pH 7.5 at 9.3 mg protein/ml and stirred at room temperature for 15 minutes;

b) suspended in 30 mM LIS in 50 mM Tris buffer pH 7.5, at 9.3 mg protein/ml, and stirred at room temperature for 1 hour;

c) suspended in 3 mM LIS in 50 mM Tris buffer pH 7.5, at 9.3 mg protein/ml and stirred for 1 hour at room temperature;

d) suspended in 300 mM LIS in 50 mM Tris buffer pH 7.5, at 25 mg protein/ml and stirred at room temperature for 15 minutes.

In all cases, PMSF was added to the extraction buffer at a final concentration of 2 mM. After centrifugation of the suspension at 45,000 g for 30 minutes at 4°C, the supernatant, which contained the extracted membrane proteins, was decanted and dialysed overnight against several changes of 50 mM Tris buffer pH 7.5 in order to remove the extracting agent (LIS). The LIS non-extracted pellet was washed twice in cold distilled water by centrifugation at 45,000 g for 30 minutes at 4°C. Both the dialysed supernatant and the pellet (resuspended in Tris buffer) were tested for biological activity in cohesion assays as described in section 2.7.8. The "control" buffer for such assays consisted of 50 mM Tris buffer pH 7.5 containing 30 mM, 3 mM or 300 mM LIS and 2 mM PMSF, dialysed overnight against Tris buffer at 4°C.

2.7.8. Biological activity assay

To test the effect of a particular soluble

extract on cohesion of various cell populations, a dose--response curve was established as with plasma membranes (see section 2.5.5.). Washed cells were suspended in 17 mM phosphate buffer pH 6.0 at a final concentration of 1 x 10^6 per ml. The extracted membrane proteins were then added to the cell suspension at the desired concentration, and the suspension shaken for 20 minutes. Cohesion was assessed as described in section 2.2.1.

2.8. LIS-solubilized material

Several treatments were performed on the LIS--extracts obtained from log phase cell membranes in order to characterize them.

2.8.1. Heat stability

The stability of the LIS-solubilized material (see section 2.7.7.) against heat was investigated by heating a 200 µlts sample at 100°C for 10 minutes. The heat-treated extract was then tested for biological activity on cohesion of log phase cells as described in section 2.7.8.

2.8.2. Effect of periodate

A 300 ults sample of LIS-solubilized material was incubated overnight with 50 mM sodium periodate (final concentration) in 50 mM Tris buffer pH 7.5 at 4°C in the dark. A control sample without the extracted proteins was run in parallel. The reaction was stopped by adding glucose to a final concentration of 100 mM. As it was found that glucose interfered with the standard cohesion assay used to test the biological activity of a particular extract (see section 2.7.8.), the reaction mixtures were dialysed overnight against 50 mM Tris buffer pH 7.5, and subsequently

used to test their inhibitory activity on log phase cell cohesion as described in section 2.7.8.

2.8.3. Adsorption to log phase cells

LIS-solubilized material (250 ugr protein/ml) was incubated at 22°C with 1 x 10^6 log phase cells in a final volume of 1 ml, and shaken for 30 minutes in phosphate buffer. The cells were removed by centrifugation at 300 g for 5 mins, and 1 x 10^6 fresh log phase cells were suspended in the supernatant. These were shaken for 20 minutes and their cohesiveness assessed as described in section 2.2.1. A control without LIS-extracted material but 50 mM Tris buffer pH 7.5 instead was run in parallel.

For adsorption experiments, the same procedure was followed except that 10 times more cells and extracted proteins were used to adsorb the inhibitory activity to the cells (2.5 mg of LIS-solubilized proteins and 1 x 10^8 log cells per ml), and the cell pellet obtained after adsorption was suspended in 1 ml of phosphate buffer. The cell suspension was then centrifuged at 300 g for 5 minutes and 1 x 10^6 fresh log cells were added to the supernatant. Cohesiveness was assessed in the usual way (see section 2.2.1). A control without LIS-solubilized extract was run in parallel.

2.8.4. SDS-polyacrylamide gel electrophoresis

Proteins can be separated from each other on the basis of differences in (1) size, (2) solubility properties, (3) electric charge, (4) adsorption behaviour, and (5) the biological affinity of a protein for a specific ligand. Separation of proteins on the basis of molecular size can be carried out in different ways, and SDS-polyacrylamide gel electrophoresis (PAGE) provides a comparati-

vely good method for it. The protein mixture is treated with the detergent (SDS) which dissociates them into subunits and completely unfolds each polypeptide chain to form a long rod-like SDS-polypeptide complex. In this complex, the polypeptide chain is coated with a layer of SDS molecules in such a way that their hydrocarbon chains are in tight hydrophobic association with the polypeptide chain, and the charged sulphate ions on the detergent are exposed to the aqueous medium. These complexes contain a constant SDS to protein ratio, and differ only in mass.

When a SDS-treated single chain protein is subjected to an electric field (electrophoresis) in a molecular sieve gel (acrylamide) containing SDS, its rate of migration is determined primarily by the mass of the SDS-polypeptide particle. The electric field simply supplies the driving force for the molecular sieving. To calibrate a given system, marker proteins of known molecular weight are also run for comparison.

Throughout the course of this work, the overall approach for SDS-PAGE of plasma membranes, LIS extract and adsorbed material was that of Fairbanks (1971). Concentrated stock solutions of "running gel" were mixed in the order and proportions given in Table 2-1, and added to 11 cm glass tubes of 6 mm diameter that had been previously siliconized with Repelcote. Each tube was then overlaid gently with distilled water. When polimerization was complete (about 20 minutes at room temperature), the distilled water was carefully removed from the top of the gels, and stock solutions of "stacking gel" (Table 2-1) were mixed and added to the top of the running gels to form a 1 cm stacking gel. Each tube was again overlaid with distilled water and when polimerization was complete, the water was removed and the top of the gels rinsed and overlaid with about 0.5 ml of electrophoresis buffer. The gels were left to stand overnight at room temperature.

Table 2-1

Preparation of 10%-acrylamide gels with 4.5% acrylamide stacking gels

Stock solutions	Running gel	Stacking gel
BIS-acrylamide (0.8-30% w/w)	3.25ml (0.2-10%)*	1.46ml (0.2-4.5%)
750mM Tris,pH 8.8	5ml (375mM)	---
250mM Tris,pH 6.8	---	5ml (125mM)
10% SDS (w/w)	0.1ml (0.1%)	0.1ml (0.1%)
H_2O	1.2ml	2.24ml
1% TEMED** (v/v)	0.25ml (0.025%)	1ml (0.1%)
7.5% Ammonium persulphate (w/v)	0.2ml (0.15%)	0.2ml (0.15%)

*Final concentrations in the gels are shown in brackets.

**TEMED= N,N,N',N'-Tetramethyl ethylenediamine

The protein samples were suspended in an appropriate volume of 125 mM Tris buffer pH 6.8 and solubilized by adding SDS to a ratio SDS/protein=3, 2-mercaptoethanol to a final concentration of 5%. The suspension was heated at 100°C for 10 minutes. Once solubilized, the proteins were subjected to electrophoresis at 1.5 mA/gel for 4 hours. The electrophoresis buffer consisted of 25 mM Tris, 192 mM glycine, 0.1% SDS at pH 8.3. Bromophenol blue was used as tracking dye. The gels were fixed in 25% isopropanol/10% acetic acid overnight, and stained with 0.01% Coomasie blue in 10% isopropanol/ 10% acetic acid for 9 hours. 10% acetic acid was used to destain the gels.

Molecular weight markers were: thyroglobulin (330,000 dalton/subunit), ferritin (220,000 dalton/half unit and 18,500 dalton/subunit), catalase (60,000 dalton/ subunit), lactate dehydrogenase (36,000 dalton/subunit) and bovine serum albumin (67,000 dalton/subunit). These were purchased by Pharmacia Fine Chemicals (calibration kit).

2.9. Antibodies

An enormous number of methods are at present available for raising antibodies against specific antigens. Throughout the course of this work, the production of an antibody against specific antigens involved in cohesion of vegetative Ax-2 cells was considered. The problem was first approached by developing antisera against freeze-dried log phase cells. Since the results obtained with this antiserum were not satisfactory, it was then decided to raise antibodies against isolated plasma membranes from log phase cells. Both immunization procedures are described as follows, as well as the immunological techniques used in this study.

2.9.1. Immunization against freeze-dried log phase cells

Log phase cells were harvested, washed once in distilled water, twice in phosphate buffer and then freeze dried. 10 mg of dried cells were suspended in 0.5 ml of phosphate buffered-saline (PBS). 0.5 ml of complete Freund adjuvent (CFA) was added and the suspension was drawn up into a syringe fitted with a SWG-19 needle, and emulsified by aspiration and ejection strongly through the needle. This was stopped when a drop of the emulsion on cold water formed discrete globules floating just below the surface. The emulsified material was inoculated intramuscularly into each rump of 2 Half-lop rabbits from which a pre-injection blood sample had been previously taken from the marginal ear vein.

After 22 days, 20 mg of freeze-dried log cells, suspended in 1 ml of PBS were inoculated intravenously into each rabbit (two lots of 10 mg into each marginal ear vein). This was followed by a series of alternate injections containing 10 mg and 20 mg of lyophylized cells into the back and the ear vein respectively, on days 29, 36, 43 and 50. Each of these inoculations consisted of 1 ml volume injected into two separate sites.

7 days later a blood sample was collected from the marginal ear vein of each rabbit, so that the antibody could be tested. 2 months later a booster injection was performed on each rabbit. This consisted of 20 mg/ml of freeze-dried log cells in PBS, injected intravenously into the marginal ear veins. A week after the booster injection the rabbits were bled out from the heart. The blood was collected and allowed to clot at room temperature for about 1 hour before being placed at $4^{o}C$. The serum was decanted, centrifuged to remove contaminating cells, and stored at $-20^{o}C$ in 1 ml aliquots.

2.9.2. Immunization against isolated plasma membranes

Plasma membranes isolated from Ax-2 log phase cells (see section 2.5.3) were inoculated into 3 Half-lop rabbits from which a pre-injection blood sample had been previously obtained from the marginal ear vein. 3 mg of plasma membrane protein, emulsified in 1 ml of 1:1 PBS:CFA (see section 2.9.1) were injected in 4 lots of 0.25 ml into each foot, over the metacarpals or metatarsals rather than between them so as to reduce bleeding. 46 days later, a booster injection was performed on each rabbit. This consisted of 3 mg of membrane protein per animal, given in PBS:CFA (a) and in PBS (b):

(a) 9 mg of log phase cell membrane protein were suspended in 1 ml of PBS and 1.5 ml of CFA were added. The suspension was emulsified as described in the previous section, and 0.5 ml of the emulsion was injected in two lots of 0.25 ml into each rump.

(b) 6.7 mg of log phase cell membrane protein were suspended in 4.5 ml of PBS, and 1 ml of the suspension injected into the marginal ear vein per animal.

A week after the booster injection, 30 ml of blood were collected from the marginal ear vein of each animal. The blood was allowed to clot at room temperature for 1 hour before being placed at 4°C. The serum was decanted, centrifuged and stored at -20°C in 1 ml aliquots.

2.9.3. Testing the antibodies

The serum antibody level was qualitatively assessed using precipitin and Ouchterlony double diffusion tests (Ouchterlony & Nilson, 1973). For this purpuse, an antigen solution was prepared by freezing and thawing a suspension containing:

a) 10 mg/ml of freeze-dried cells in PBS plus 0.5% sodium deoxycholate (to test the anti-log cell serum), or

b) 1 mg/ml of membrane protein in PBS plus 0.5% sodium deoxycholate (to test the anti-log membrane serum).

In both cases, fragments were discarded by centrifugation at 30,000 g for 30 minutes. The soluble extracts obtained by extracting log cell membranes with various agents (see section 2.7) were also used as antigens for double diffusion tests.

Serial dilutions of the antigen solution were prepared to use in the precipitin test. 2 mm glass capillaries were filled with a small amount of the undiluted antiserum and a layer of the dilute antigen was then applied on top of the antiserum.

For the double diffusion test Ouchterlony plates were prepared using 1% purified agar (Oxoid, N°1) in 0.15% sodium azide. A 10 mm diameter central well was cut 7 mm from diametric peripheral wells. The antiserum was placed in the central well with the antigens in the peripheries. The plates were incubated at 22°C for at least 48 hours before precipitation lines were visible.

2.9.4. Immunofluorescence

A variety of procedures are used which permit the detection of antibodies. For immunofluorescent work, the antiserum can be used in two ways:

Directly, by using a single layer of fluorescein-labelled specific antibody, or indirectly, where the unlabelled specific antibody is used first and the material is subsequently treated with fluorescent antibody against rabbit

immunoglobulin (gamma globulin). Thus if the first layer employed rabbit antibody, fluorescent antibody against rabbit gamma-globulin would be used in the second layer.

In the present work, the indirect technique was used in order to obtain brighter fluorescence staining of cells (by increasing the number of fluorescent molecules attached to each antigenic site, and also because it was more economical in antiserum use). For this purpuse, the antiserum was often undiluted. The fluorescent antibody conjugate was a commercial antiserum raised in sheep against rabbit gamma-globulin, conjugated with fluorescein isothyocyanate (FITC) (Wellcome Labs.) and it was diluted 1:32 with PBS unless otherwise stated.

The controls used for this procedure were:

a) an autofluorescent control with sheep anti-rabbit conjugate alone as a single layer (direct technique), and

b) a pre-injection control with non-immune sera followed by sheep anti-rabbit conjugate (indirect technique).

The cells were harvested, washed and suspended in PBS at 2 x 10^5 per ml. They were allowed to settle for 0.5-1 hour on round cover-slips and then washed with PBS. The cells were fixed with ice-cold methanol for 3 minutes, and washed immediatly with PBS. A layer of the antiserum was placed on top of the fixed cells and left to stand for 20 minutes. This was followed by a 20 minutes washing in PBS. The sheep anti-rabbit conjugate (1:32 in PBS) was then applied as a layer, and left to stand for 10 minutes, followed by a 20 minute wash in PBS. The cover-slips were then mounted in glycerol:PBS (1:1) and examined under UV light in a Zeiss Photomicroscope II.

When live cells were used, the fixation step was omitted and the antiserum complement inactivated by heating the serum at 56°C for 30 minutes before use.

2.9.5. Absorption of antiserum

In order to obtain a specific serum only reactive with antigenic groups in Ax-2 cells synthesised in log phase of growth, it was found valuable to absorb the anti-log cell serum with Ax-2 stationary phase and cycloheximide-treated cells (see sections 2.1.1 and 2.4.1.). For absorption, the cells were harvested and then washed in cold distilled water and PBS before being packed by centrifugation at 1,500 g for 15 minutes. 1 ml of anti-log cell serum was then mixed with 1.5 ml of packed cells and the suspension ultrasonicated for 10 minutes. After checking that no whole cells were present, the sonicated suspension was incubated for 1.5 hours at 37°C and then overnight at 4°C. Cell fragments were discarded by centrifugation at 30,000 g for 30 minutes at 4°C.

2.9.6. Purification of globulins from the anti-log membrane serum

At some stage during the course of this study, it was necessary to produce a purified globulin preparation from the anti-log membrane serum to be used in cohesion assays. This was performed using the relative insolubility of globulins in 50% saturated ammonium sulphate. An equal amount of saturated $(NH_4)_2SO_4$ was added dropwise to the antiserum, maintained in ice. The solution was left to floculate for 30 minutes and then centrifuged at 2,500 g for 30 minutes at 4°C. The precipitate was washed in 50% saturated $(NH_4)_2SO_4$, recentrifuged and then dissolved in a volume of PBS, half that of the original antiserum. The globulin solution was dialysed overnight at 4°C against several changes of PBS in order to eliminate excess SO^{2-} ions.

2.9.7. Cohesion assays with anti-log membrane serum

To test the effect of the anti-log membrane serum on cell cohesion, a dose-response curve was established. After harvesting, the cells were washed and suspended in 17 mM phosphate buffer pH 6.0 at a final concentration of 1 x 10^6 per ml. The antiserum (or non-immune serum, control) previously heated at 56°C for 30 minutes to inactivate complement was added to the cell suspension at the desire
shaken for 20 minutes. Cell cohesion was assessed as described in section 2.2.1.

2.9.8. Anti-log membrane serum in development studies

To test the effect of the antiserum on cell development, Ax-2 log phase cells were washed and suspended in cold distilled water at a final concentration of 1 x 10^8 per ml. 0.5 ml of the cell suspension was placed over the surface of a 47 mm Millipore filter (see section 2.1.3.) resting on an absorbent pad saturated with 1.6 ml of Millipore solution (see section 2.1.3.) containing different concentrations of heat-inactivated immune or non-immune sera. The Millipore filters were incubated under humid conditions at 22°C and examined after 24 hours of incubation.

Chapter three: Cohesive behaviour of Ax-2 cells in shaken suspension

3.1. Introduction

Previous investigations of the cohesive properties of axenically grown Dictyostelium discoideum cells have shown that cells in the log phase of growth are cohesive when gyrated in suspension, whereas cells in the stationary phase of growth are not cohesive under the same conditions (Swan & Garrod, 1975). Furthermore, the growth medium from stationary phase cultures accumulates a low molecular weight factor which completely inhibits cohesion of log phase cells in phosphate buffer, and partially inhibits the cohesion of aggregation-competent cells (Swan et al., 1977; Garrod et al., 1978).

Gerisch (1961) has shown that the cohesive properties of Dictyostelium discoideum cells change during development. Immunological studies of the cell surface properties have provided evidence for the existence of two independent cohesive mechanisms, contact sites A (CS A), and contact sites B (CS B) (Beug et al., 1970; Beug et al., 1973a). The EDTA-sensitive cohesion of vegetative cells is mediated by CS B and with the development of aggregation-competence EDTA-resistant CS A are added. Swan et al (1977) have suggested that the low molecular weight inhibitory factor found in stationary phase medium may bind to contact sites B.

This chapter is concerned with studies which further investigate the cohesive properties of vegetative Ax-2 cells, as well as the mechanism of action of the inhibitory factor. Evidence is presented for the specific inhibition of contact sites B-mediated log phase cell cohesion by the inhibitory factor, and a working hypothesis is proposed for the study of the molecular basis of cohesion at the earliest phase of the life cycle.

3.2. Results

3.2.1. Change in cohesiveness of Ax-2 cells during the transition from log to stationary phase of growth

Ax-2 cells were harvested at different stages of growth, and their cohesiveness assessed in phosphate buffer as described in section 2.2.1.

Figure 3-1a shows the growth curve obtained for Ax-2 cells, with a generation time of approximately 10 hours. It can be seen from figure 3-1 b that log phase cells, harvested when at a density of 2 x 10^6 per ml (point A in fig 3-1 a) cohered rapidly. As growth proceded into the stationary phase of growth, the cells became progressively less cohesive. The equilibrium number of particles (expressed in terms of percentage of initial count, see section 2.2) was found to increase as the cells were harvested at higher densities and shaken in phosphate buffer for one hour (points B, C, D, and E in figs 3-1 a,b). After 3.5 days in stationary phase, cohesiveness was completely lost (point F in figs 3-1 a,b).

To be sure of working with non-cohesive populations, the stationary phase cells used in subsequent experiments were harvested at or after the stage of growth represented by point E in figure 3-1 a.

3.2.2. Mutual cohesion of log phase and stationary phase cells

Given the above observations, and the fact that stationary phase medium contains a low molecular weight inhibitory factor of log phase cell cohesion (Swan *et al.*, 1977), the possibility of whether stationary phase cells although non-cohesive themselves would form mutual

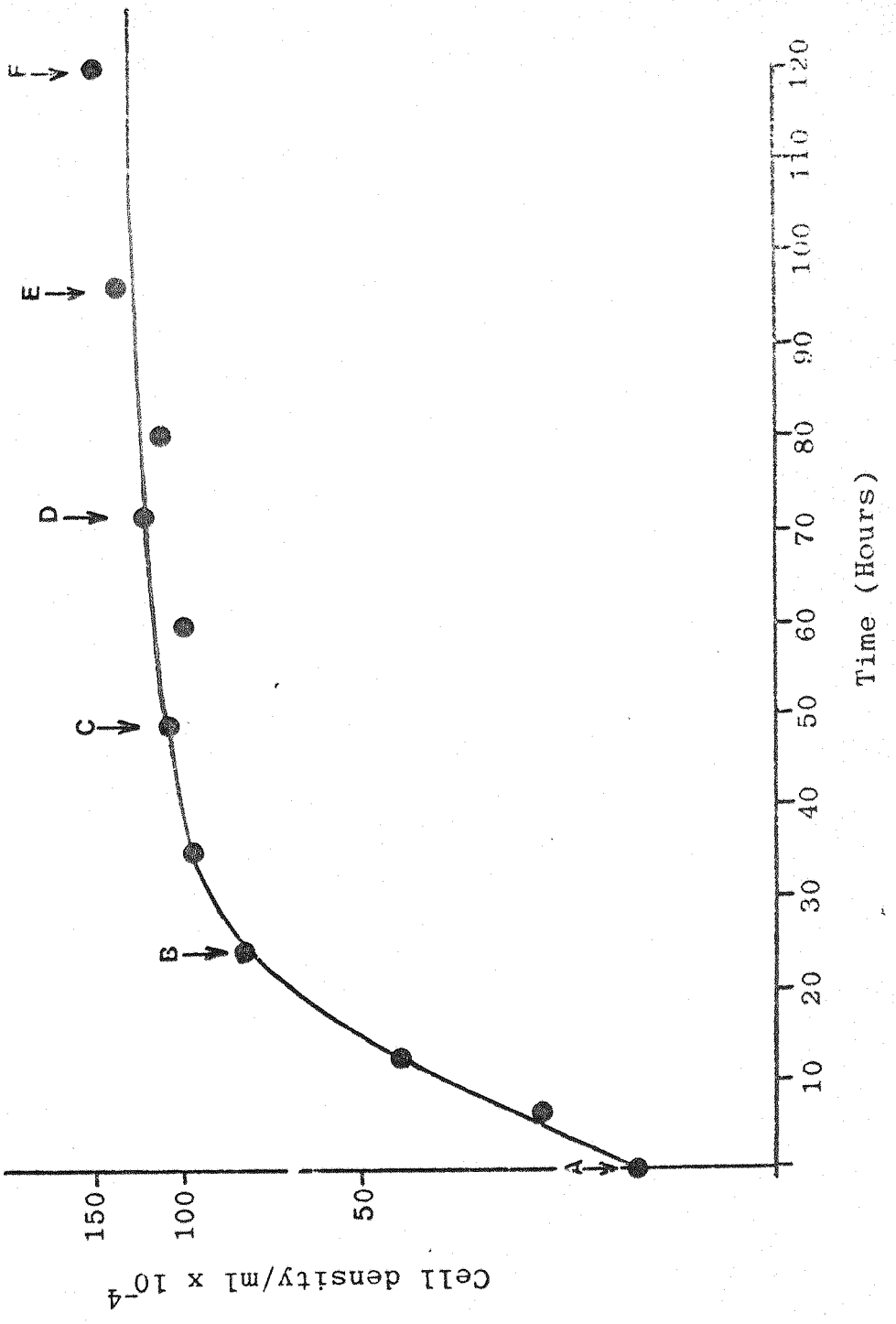

Figure 3-1 a: Growth curve of Ax-2 D. discoideum cells. The arrows indicate the cell densities at which cells were harvested for the experiment in figure 3-1 b.

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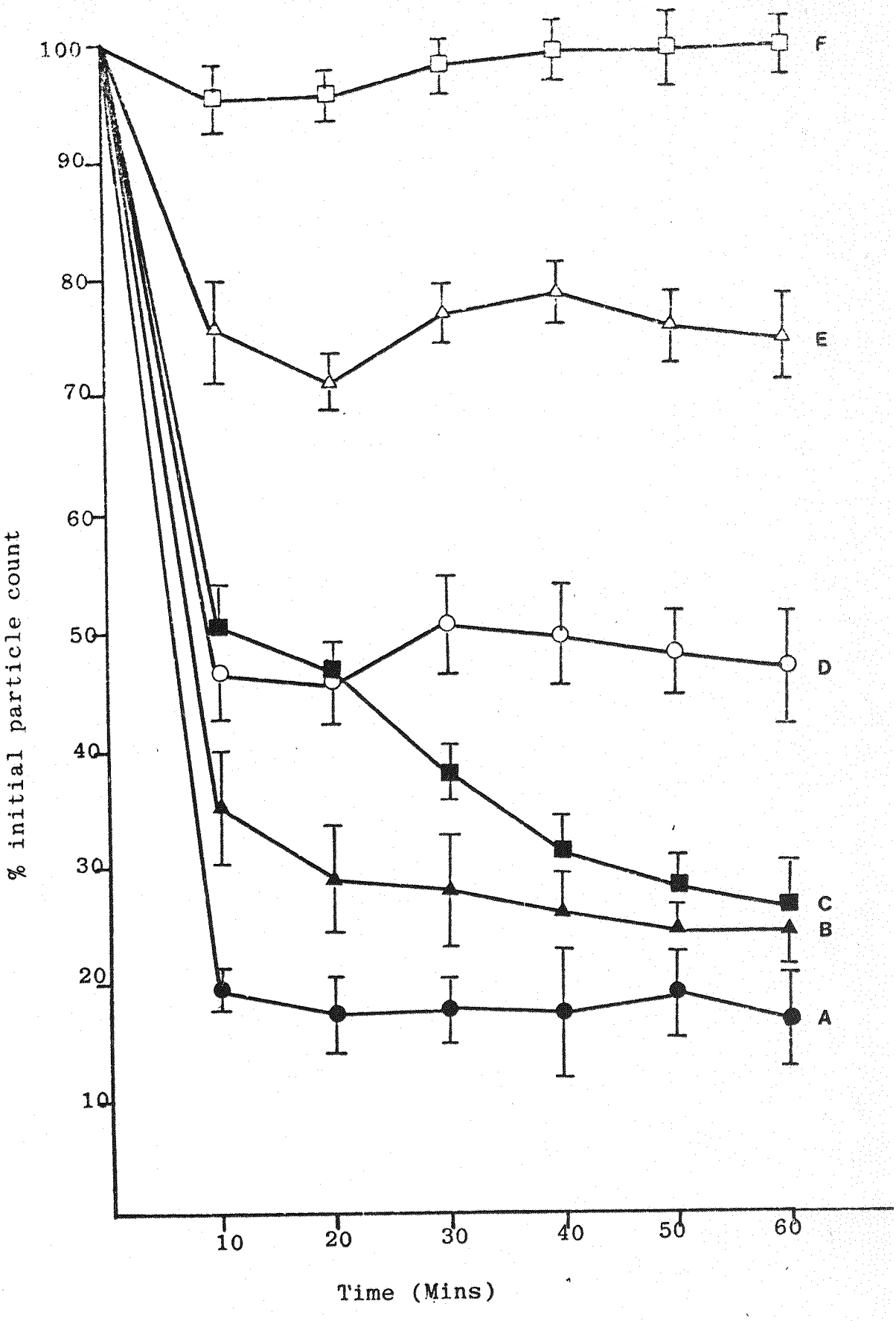
Figure 3-1 b: Cohesion of Ax-2 cells in phosphate buffer at different stages of growth. The letters A to F indicate the stages of growth at which the cells were harvested, and correspond to the points labelled on figure 3-1 a. The points are shown $\pm$ standard deviations.


100
90
80
70
60
50
40
30
20
10
% initial particle count
10
20
30
40
50
60
Time (Mins)
F
E
D
C
B
A

contacts with log phase cells was investigated.

Log and stationary phase cells were mixed in varying proportions and the cohesive curves of the mixed populations were established. The results are shown in figure 3-2. In each case, the cohesion curve for the log--stationary phase cell mixture had the same shape as the curve for log phase cells, and the equilibrium particle number increased slightly with increasing proportion of stationary phase cells in the mixture. However, in the case of the 1:1 log:stationary mixture for example, the equilibrium particle number was much less than would be expected if stationary phase cells were forming no cohesive contacts in the mixture. If that were the case, the equilibrium particle concentration should be greater than 50%, being made up of stationary phase cells which remained single, plus a few aggregates of log phase cells. Since the equilibrium number of particles was considerably below 50% and since stationary phase cells were not cohesive themselves, it was concluded that cohesive contacts must have formed between stationary and log phase cells.

An alternative possibility would be that log phase cells release an aggregation factor into the buffer which promotes cohesion of stationary phase cells. An experiment was performed to exclude this possibility:

Log phase cells, at a final concentration of 5×10^5, 1×10^6 and 1×10^7 per ml, were allowed to cohere in phosphate buffer as described in section 2.2.1 for one hour. The cells were then removed by centrifugation and fresh stationary phase cells were suspended in the supernatant at a final concentration of 1×10^6 per ml. These were shaken and their cohesiveness assessed after 30 mins. No cohesion was observed, indicating that log phase cells did not release an aggregation factor into the medium. The results obtained with log-stationary phase cell mixtures

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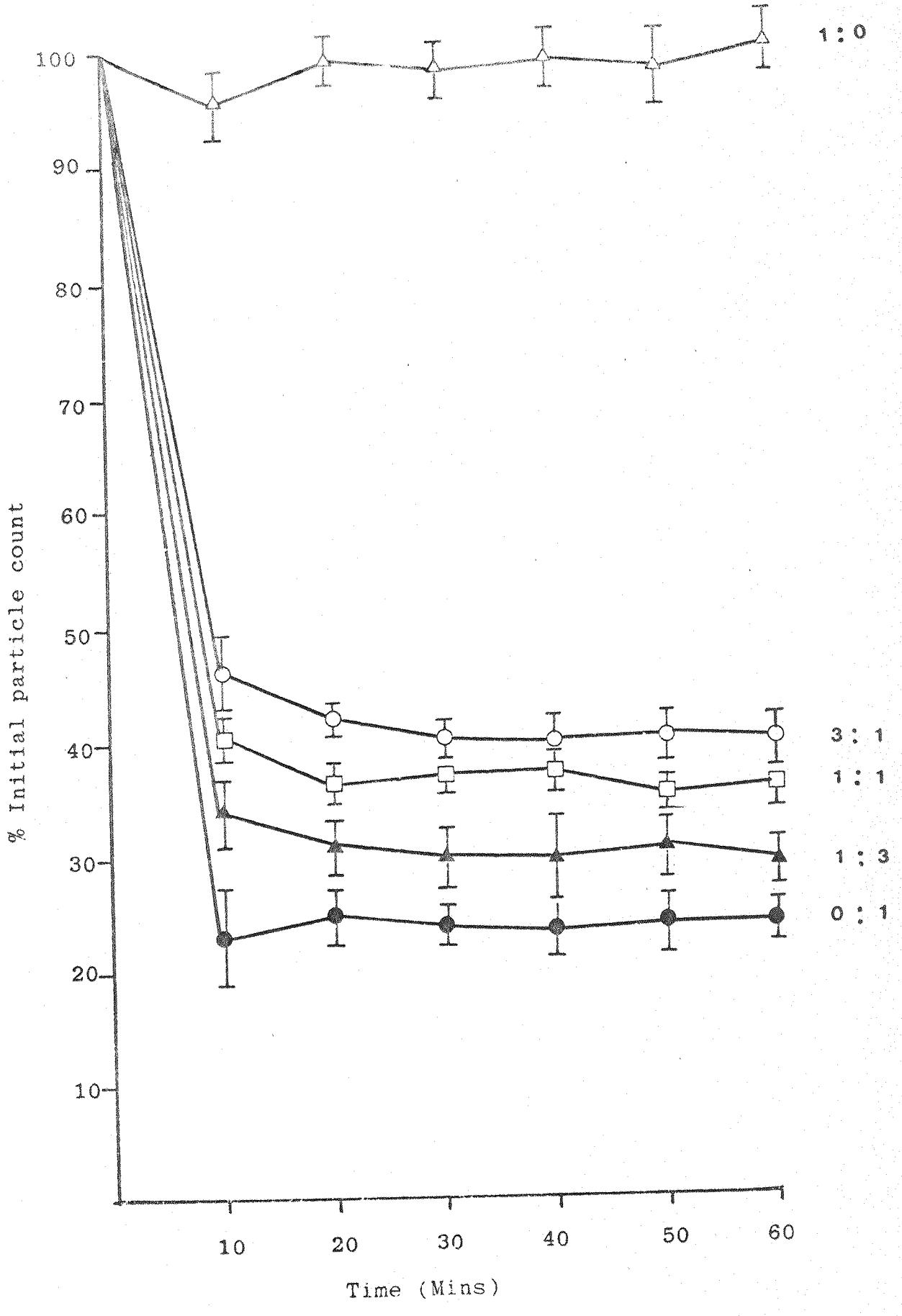
Figure 3-2: Cohesion of mixtures of log and stationary phase cells in phosphate buffer. In each case, the proportion of the two cell types is indicated at the right side of the figure. Points are shown ± standard deviations.

STAT:LOG


1:0
3:1
1:1
1:3
0:1
100
90
80
70
60
50
40
30
20
10
% Initial particle count
10
20
30
40
50
60
Time (Mins)

could therefore be explained in terms of mutual cohesion between log and stationary phase cells.

3.2.3. Mutual cohesion of aggregation-competent and stationary phase cells

The possibility of mutual cohesion between early differentiated cells (aggregation-competent) and stationary phase cells, in gyrated suspension and phosphate buffer was also investigated.

Dissociated aggregation-competent cells were mixed with stationary phase cells in a 1:1 ratio at a final concentration of 1×10^6 cells/ml, and the degree of cohesion in the mixture determined as described in section 2.2.1. Table 3-1 shows that the equilibrium number of particles obtained after shaking the cell mixture for 20 minutes in phosphate buffer was less than would be expected if they were forming no mutual contacts. In this case, the equilibrium particle concentration would be greater than 50%, being made up of a few aggregates of aggregation-competent cells (see Table 3-1) and single stationary phase cells.

These results showed that stationary phase cells and aggregation-competent cells were mutually cohesive.

3.2.4. Effect of cycloheximide on cell cohesion

Hoffman and McMahon (1978) have recently reported that cycloheximide-treated cells (Ax-2) are CS A and CS B defective. The cohesive behaviour of such cells, and their ability to form mutual contacts with log phase, stationary phase and aggregation-competent cells was investigated.

Table 3-1

Effect of EDTA on cohesion of cell mixtures

Cell mixture*	Conditions†	Equilibrium particle number††
Log+Stat	Control	33 ± 2.1
Log+Stat	10mM EDTA	98 ± 3.3
AggC alone	Control	18 ± 2.0
AggC+Stat	Control	47 ± 4.2
AggC+Stat	10mM EDTA	73 ± 5.1

* All cell mixtures were prepared in 1:1 ratios; Log= log phase cells; Stat= stationary phase cells; AggC= aggregation-competent cells.

† Cell mixtures at a final concentration of 1×10^6 per ml were shaken as described in section 2.2.1. Control conditions consisted of 17 mM phosphate buffer pH 6.0; EDTA was used in the same buffer.

†† Obtained after 20 minutes of shaking under the indicated conditions, and expressed as the percentage of the initial count. Standard deviations are shown.

Log phase cells were shaken in suspension in the presence of 500 µgr/ml of cycloheximide as described in section 2.4.1., and the samples were taken at 2 hours intervals, the cells washed to remove cycloheximide, resuspended in 17 mM phosphate buffer pH 6.0 at a final concentration of 1 x 10^6 cells/ml and shaken for 20 minutes. The control samples consisted of cells shaken in the absence of cycloheximide.

It was found (fig 3-3) that the equilibrium particle number increased with the time of shaking. At 8 hours, complete loss of cohesiveness was achieved. However, these cells were found to recover their cohesiveness when incubated overnight in the absence of cycloheximide.

Mutual cohesion between CH-cells (see section 2.4.1) and log phase, stationary phase and aggregation--competent cells was investigated using the same approach as in sections 3.2.2 and 3.2.3. Cell mixtures (1:1) were prepared at a final concentration of 1 x 10^6 per ml in phosphate buffer, and the degree of cohesiveness was determined as described in section 2.2.1. Table 3-2 shows the equilibrium particle number obtained when CH-cells were shaken with log phase cells for 30 minutes (59% of the initial count) was higher than would be expected if they were forming mutual contacts. A similar result was found when aggregation-competent cells were mixed with CH-cells (see table 3-2). In addition it was found that stationary phase cells and CH-cells were not mutually cohesive. In this case the equilibrium particle concentration was found to remain constant at about 100% of the initial count, both cell types being non-cohesive.

Following these results it was concluded that cycloheximide-treated cells did not form mutual contacts with other cell types.

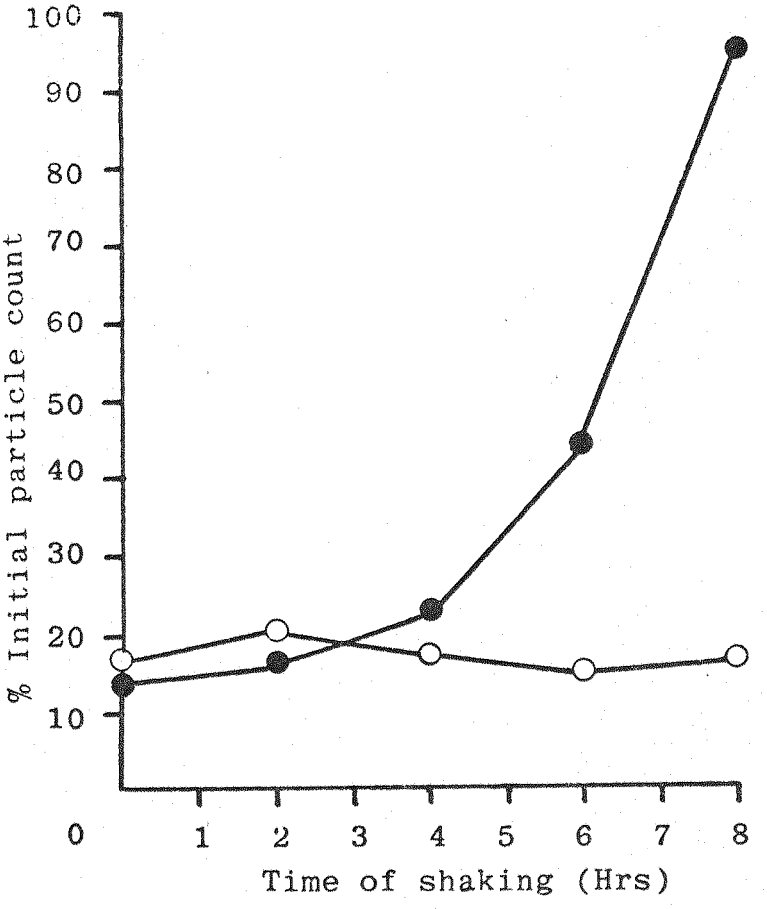


Figure 3-3: Effect of cycloheximide on cohesiveness of log phase Ax-2 cells aged in phosphate buffer. Cells were shaken in the presence (●—●) or absence (○—○) of cycloheximide (500 µg/ml) and at the times indicated their cohesiveness assessed. The equilibrium particle numbers after 20 minutes are shown. Each point represents the average from 3 experiments.

Table 3-2

Cohesive properties of cycloheximide-treated cells

Cell mixture*	Equilibrium particle number †
CH + Log	56 ± 5.2
CH + Stat	96 ± 2.0
CH + AggC	49 ± 3.3

* All cell mixtures were prepared in 1:1 ratios; CH= cycloheximide-treated Ax-2 cells; Log= Log phase cells; Stat= stationary phase cells; AggC= aggregation-competent cells.

† Obtained as in table 3-1.

3.2.5. Effect of EDTA on cohesion of cell mixtures

In order to investigate the possible relationship between the observed mutual cohesion of both log-stationary and aggregation-competent-stationary cell mixtures and the EDTA-sensitive (contact site B-mediated) and EDTA-resistant (contact site A-mediated) cohesive mechanisms proposed by Beug *et al.* (1970; 1973a), the effect of 10 mM EDTA on cohesion of the cell mixtures was determined.

Table 3-1 shows the equilibrium particle numbers obtained when both log-stationary and aggregation-competent-stationary cell mixtures (1:1) were shaken in the presence of EDTA as described in section 2.2.2. In the case of log-stationary phase cell mixtures, complete inhibition of cohesion was obtained, whereas with aggregation-competent-stationary cell mixtures only partial inhibition with EDTA was achieved.

3.2.6. Effect of the low molecular weight inhibitor from stationary phase medium on the cohesion of cell mixtures

The ability of the inhibitory factor found in stationary phase medium to mimic the previously observed effect of EDTA on cohesion of cell mixtures was investigated by assessing the equilibrium particle number obtained after 30 minutes of shaking in the presence of increasing concentrations of inhibitory factor.

Figure 3-4 shows the dose-response curves obtained with both log phase cells and log-stationary phase cell mixtures (1:1). In both cases the equilibrium particle number increased approximately linearly with increasing inhibitor concentration, and reached a maximum

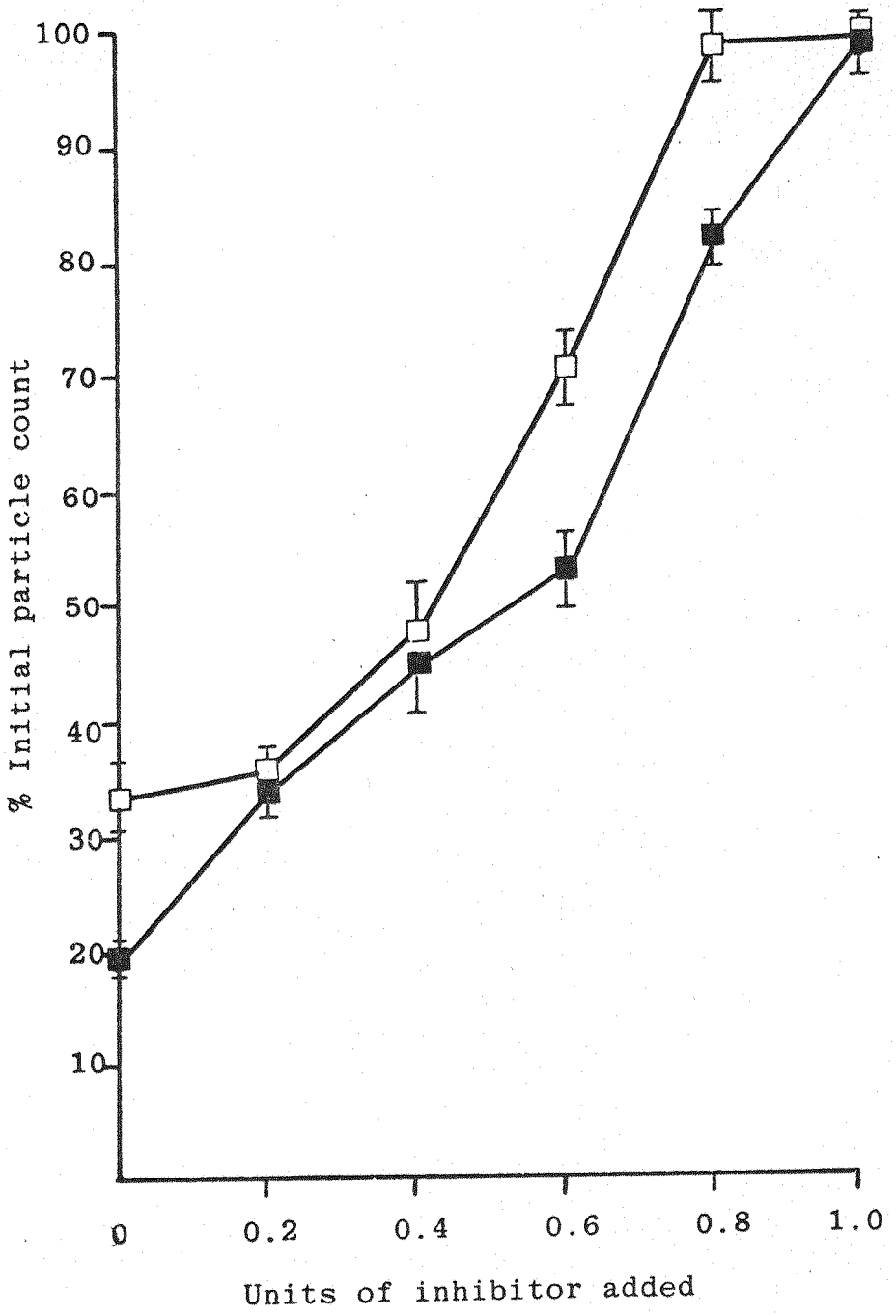


Figure 3-4: Effect of increasing inhibitor concentration on cohesion of log cells (■—■) and log:stat cell mixtures (□—□). Each point indicates the equilibrium particle number obtained after 30 minutes of cohesion in phosphate buffer with the appropiate amount of inhibitor. Points are shown ± standard deviations.

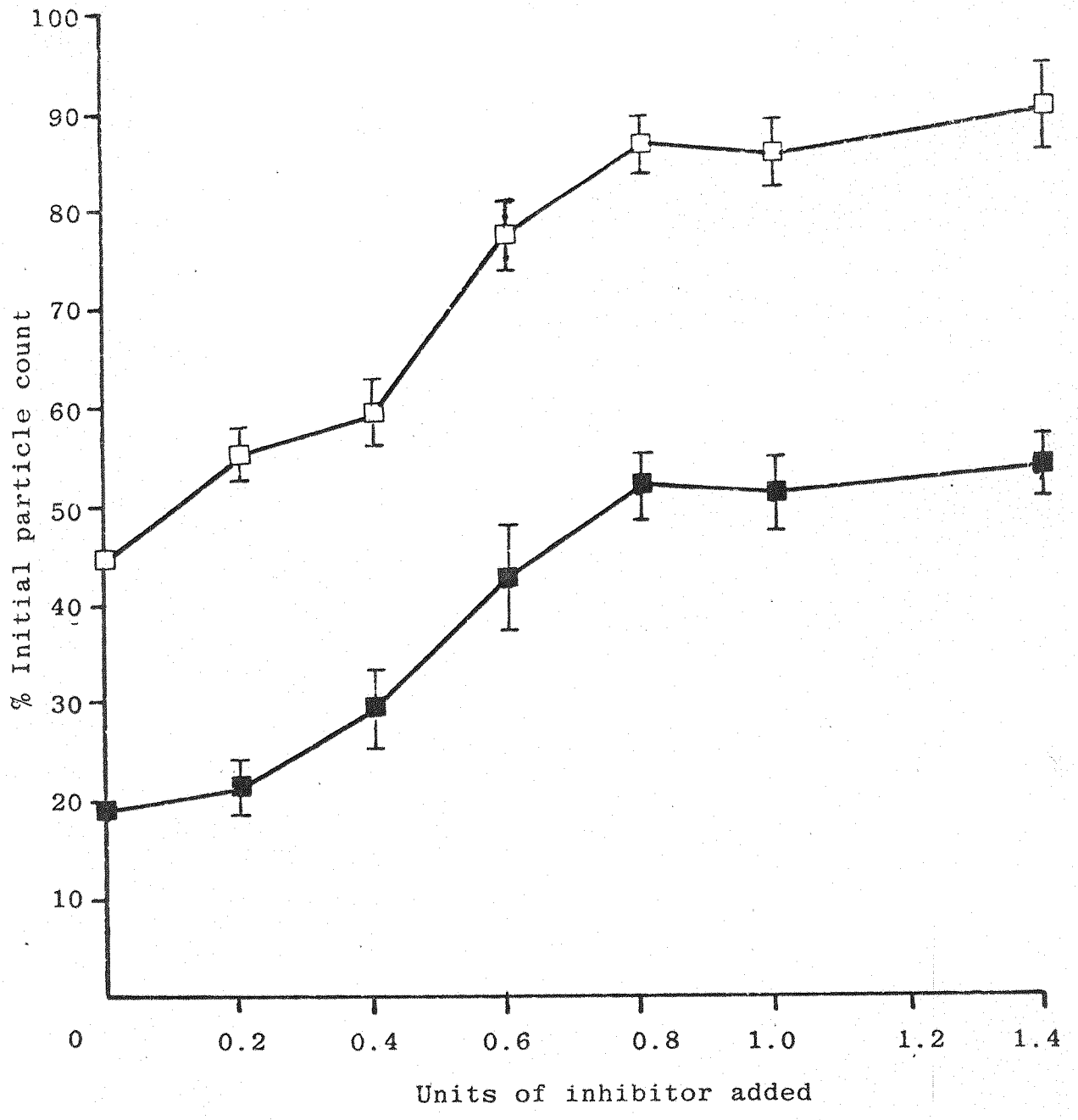


Figure 3-5: Effect of increasing inhibitor concentration on cohesion of aggregation-competent cells (■—■) and aggregation-competent-stationary phase cell mixtures (1:1) (□—□). Equilibrium particle numbers are indicated as in figure 3-4. Points are shown ± standard deviations.

value corresponding to 100% inhibition of cell cohesion.

The results of similar experiments with aggregation-competent cells and 1:1 stationary-aggregation-competent cell mixtures are shown in figure 3-5. In the case of aggregtion-competent cells, less than 50% inhibition of cohesion was obtained even in the presence of 1.5 units of inhibitor (see definition in section 2.3.2. With stationary-aggregation-competent cell mixtures, slightly more than 50% inhibition of cell cohesion was obtained. The equilibrium particle number was about 45% of the initial count in the absence of inhibitor and increased to about 90% at high inhibitor concentrations, however, 100% inhibition of mutual cohesion was never achieved.

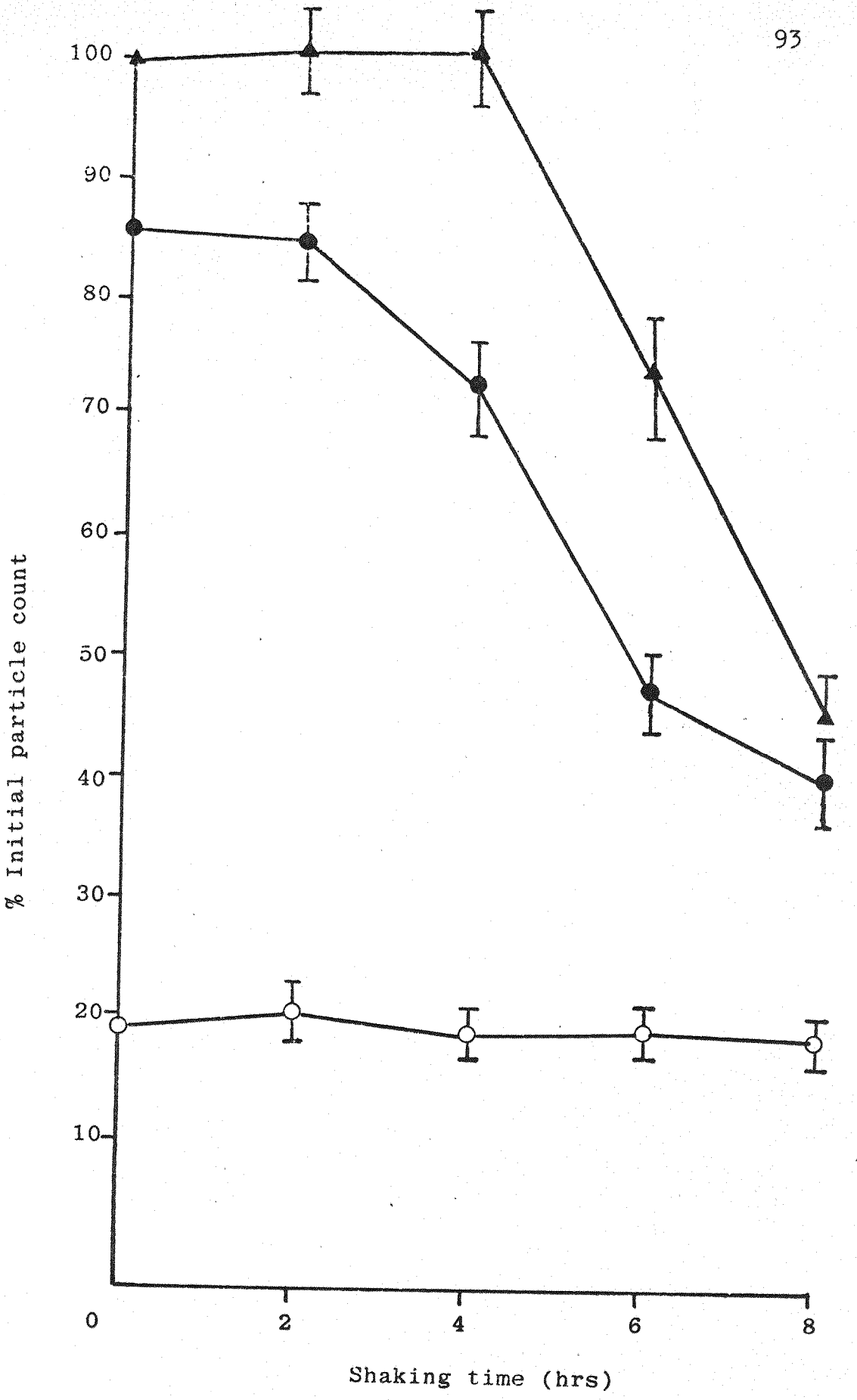
3.2.7. Evidence that the inhibitory factor acts against contact sites B

The previous results showed that the cohesion of log phase cells was completely inhibited by the inhibitory factor found in stationary phase medium, whereas with aggregation-competent cells the maximum obtainable inhibition was less than 50%. From these observations it was found that it would seem that EDTA and the inhibitory factor could have parallel effects. In order to persue this parallel the following experiment was carried out:

Log phase cells were allowed to develop aggregation-competence by shaking them for 8 hours in phosphate buffer (see section 2.1.4). Samples were taken at two hour intervals, the cells were dissociated and their cohesiveness assessed in phosphate buffer containing either 10 mM EDTA or one unit of inhibitory factor, for 20 minutes. Controls were conducted in phosphate buffer alone.

It can be seen from figure 3-6 that there was

Figure 3-6: Comparison of the effects of EDTA and the inhibitor on cohesion at different times during acquisition of aggregation-competence. Equilibrium number of particles obtained after 20 minutes of shaking in 17 mM phosphate buffer alone (○—○), 10 mM EDTA (●—●) and 1 unit of inhibitor (▲—▲) are plotted. Points are shown ± standard deviations. This graph includes some results reported by A. P. Swan (1978).


100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
% Initial particle count
Shaking time (hrs)

indeed a correspondence between the effects of EDTA and the inhibitory factor, a sharp decrease in inhibition occuring as the cells approached the aggregation stage. This suggested that the inhibitory factor, like EDTA, acts against contact sites B but not against contact sites A, as defined by Beug et al. (1970).

3.2.8. Effect of Ca^{2+} on the inhibited cohesion of log phase cells by the cohesion inhibitor

Since EDTA is a chelating agent, the possibility that the inhibitory factor might act in the same way was also considered. In order to investigate this it was attempted to reverse the inhibitory effect of the factor by adding Ca^{2+} ions to the cell suspension, in addition to the factor.

Table 3-3 shows that complete inhibition of log phase cell cohesion was achieved with one unit of inhibitor in the presence of 1 mM and 10 mM $CaCl_2$.

Table 3-3

Effect of Ca^{2+} on activity of cohesion inhibitor

Incubation conditions*	Equilibrium particle number ±
Control	20 ± 1.0
1 mM Ca^{2+}	19 ± 2.0
10 mM Ca^{2+}	18 ± 1.5
1 u. inhibitor	102 ± 2.7
1 mM Ca^{2+} + 1u. inhibitor	97 ± 2.0
10 mM Ca^{2+} + 1u. inhibitor	100 ± 5.0

* In all cases, log phase cells at a final concentration of 1 x 10^6 per ml, were shaken at the indicated conditions according to section 2.2.1. Control conditions were as in table 3-1. $CaCl_2$ and 1 unit (u) of inhibitor were used in phosphate buffer.

± Obtained as in table 3-1. Standard deviations are shown.

3.3. Discussion

The main results obtained in this preliminary study of the cohesive properties of vegetative Ax-2 cells are as follows:

(i) Stationary cells , although they do not form cohesive contacts to each other, are able to cohere both with log phase and aggregation-competent cells.

(ii) This cohesiveness and that of log phase cells is inhibited by both EDTA and the low molecular weight inhibitory factor found in stationary phase cultures (Swan et al., 1977).

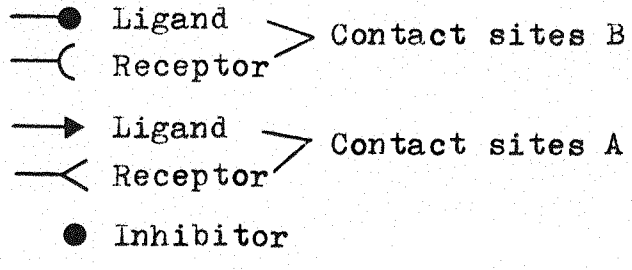
(iii) The effect of the inhibitory factor, like that of EDTA, appears to be specific for the cohesion of vegetative cells. Thus, it seems to act against contact sites B, as defined by Beug et al. (1970).

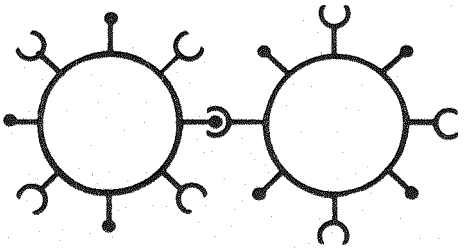
Preliminary analysis of the nature of the inhibitory molecule by thin layer and gas-liquid chromatography have indicated that it is a carbohydrate with an approximate molecular weight of 500 daltons (Swan, 1978) It has also been suggested that the inhibitory factor binds to the surface of log phase cells (Swan et al., 1977).

All these observations led to the proposal of a working hypothesis which seems to be the simplest possible explanation for the cohesive behaviour of Ax-2 vegetative Dictyostelium discoideum cells.

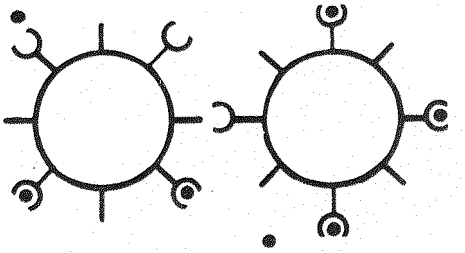
Firstly, the EDTA-sensitive cohesion of log phase cells could depend on the interaction of two complementary molecules, i.e., a ligand and a receptor (see fig 3-7 a). If so, cohesion should be inhibited by the inactivation of either of these components. In terms of this model, the fact that stationary phase cells are not able to cohere to each other, and the progressive decrease of

Figure 3-7: Diagram showing proposed model for the cohesion of axenic slime mould cells and its inhibitor. a) Log phase cells; b) Stationary phase cells; c) Stationary-log cell mixture; d) Log phase cells plus inhibitor; e) Aggregation-competent cells; f) Aggregation-competent-stationary cell mixture.

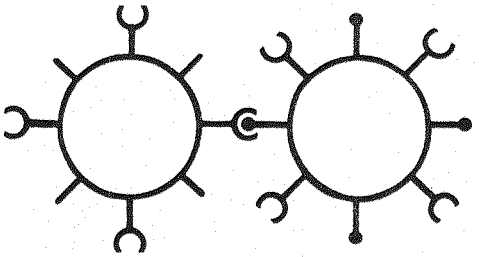




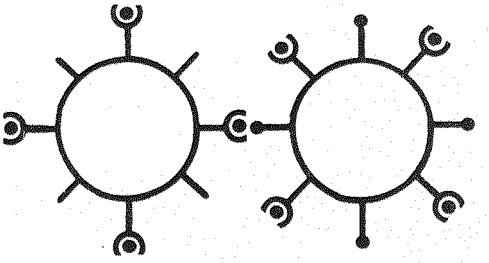
a



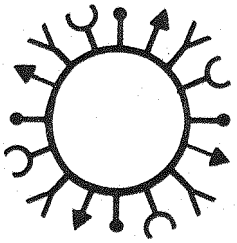
b



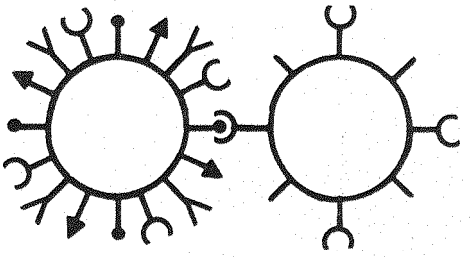
c



d



e



f

cohesiveness observed as cells grow from log to stationary phase (figs 3-1 a,b) would be interpreted as some kind of inactivation of either the ligand or the receptor during the transition from log to stationary phase of growth. In addition, one has to account for the appearance of the low molecular weight inhibitory factor in stationary phase cultures. The accumulation of this factor could be a purely fortuitous consequence of cellular metabolism, however, since it is a carbohydrate in nature the simplest interpretation of the results obtained would be that, as cells enter the stationary phase of growth the terminal portion of a glycoprotein or glycolipid ligand , which normally binds to the receptor on the surface of the cells, in some way becomes detached and is released to the medium. Thus, stationary phase cells would consist of an incomplete ligand-receptor system and therefore would be unable to stick together (fig 3-7 b).

Secondly, when log and stationary phase cells are mixed, cohesive contacts would occur between the receptors on the surface of stationary phase cells and the complementary ligands in log phase cells (fig 3-7c). The terminal fragment of the ligand, released into stationary phase medium would retain the ability to bind to its complementary receptors. Thus, when added to log phase cells it would block the cohesive apparatus by so binding (fig 3-7 d). This terminal fragment of the ligand would be the inhibitory factor. It is expected from this argument that the inhibitory factor would block cohesion between log and stationary phase cells completely, which it does (fig 3-4).

The effect of the inhibitor appears to be specific for the cohesive mechanism of log phase Ax-2 cells. There is a developmentally-regulated decline in sensitivity to the inhibitor which parallels the decline in EDTA sensitivity as aggregation-competence is approached (fig 3-6).

If the proposed model is correct it would suggest that contact sites B are a ligand-receptor system. The reason why the inhibitory factor only partially inhibits the cohesion of aggregation-competent cells could be that they possess two cohesive mechanisms (Beug et al., 1973a) or more than two as suggested by Ray et al.(1979) only one of which is being blocked by the factor (fig 3-7 e). The factor would only partially block cohesion because it would interact specifically with CS B but would have no effect on CS A. Stationary phase cells could therefore stick to aggregation-competent cells by interaction between their B-site ligands and the B-receptors of aggregation--competent cells (fig 3-7 f). In addition, the factor would not cause complete inhibition of cohesion of stationary-aggregation-competent cell mixtures because of the CS A--mediated residual cohesiveness of aggregation-competent cells.

On the other hand, the absence of cohesive sites on the surface would lead to a complete inability of defective cells to form mutual contacts either with log or stationary phase cells, and to be cohesive themselves. Cycloheximide-treated cells have been shown to be completely non-cohesive (fig 3-3) and also unable mutually to cohere with log or stationary phase cells, in contrast with stationary phase cells which do mutually cohere with both log and aggregation-competent cells.

It seems certain that the cohesive mechanism does not involve discoidin, the endogenous lectin described by Rosen and Barondes (1978). This is because the low molecular weight inhibitory factor found in stationary phase cultures has been found to be without effect on the discoidin--mediated agglutination of rabbit erythrocytes (Nossiter & Garrod, unpublished.A sample of discoidin was kindly provided by G. Gerish).

If cohesion of vegetative Ax-2 cells is then dependent upon the binding between a ligand and a receptor, both present at the surface of cohesive log phase cells, it would be expected that plasma membranes isolated from those cells to bind the surface of intact log phase cells and therefore inhibit their cohesion. The next chapter describes the results obtained from the study on the effects of isolated plasma membranes on cell cohesion.

Chapter four: Isolated plasma membranes: Effects on cell cohesion

4.1. Introduction

In the previous chapter, a ligand-receptor model has been proposed to explain the cohesive behaviour of Ax-2 cells in shaking suspension. This model predicts a CS B-mediated cohesive mechanism for vegetative cells and suggests that both ligand and receptor molecules occur on the surface of log phase cells.

Merrell and Glaser (1973) and Gottlieb, Merrell and Glaser (1974) have isolated plasma membrane--enriched fractions from chick embryonic neural retina and optic tectum showing that these fractions were able to interact preferentially with the homologous cells, preventing cell cohesion. Following a similar approach, it was attempted to isolate plasma membranes from axenic log phase cells and show that these membranes have a specific inhibitory effect on log phase cell cohesion.

These studies have also been extended to show lack of species specific cohesion in that Dyctiostelium discoideum plasma membranes inhibit cohesion of four different species of slime moulds.

It should be pointed out that inhibition of cell development by partially purified plasma membranes of D. discoideum has been previously reported (Smart & Tuchman, 1976).

4.2. Results

4.2.1. Purity of isolated plasma membranes

Plasma membranes were isolated according to the method described by Brunette and Till (1971). The aqueous two-phase polymer system employed for the separation procedure (see section 2.5.2) allowed large amounts of plasma membranes to be purified in a relatively short period of time (3 hours).

Two criteria were used to determine the level of purity of isolated plasma membranes: electron microscopy and enzyme marker activities.

(i) Electron microscopy

Figures 4-1 a,b show electron micrographs of the plasma membrane fraction obtained from log phase cells. They show a mixture of membranous sheets and vesicles with a rather heterogenous size distribution. There were no structures recognisable as mitochondria or endoplasmic reticulum.

(ii) Enzymatic characterization

Three particulate enzyme markers were chosen to characterize the isolated plasma membrane fraction . Alkaline phosphatase (a membrane marker), succinate dehydrogenase (a mitochondrial marker) and glucose 6-phosphatase (an endoplasmic reticulum marker) activities were assayed on both the crude particulate fraction from the original cell homogenate (see section 2.5.1) and the pelleted plasma membrane fraction as described by Green and Newell (1974).

Table 4-1 summarizes the specific activity

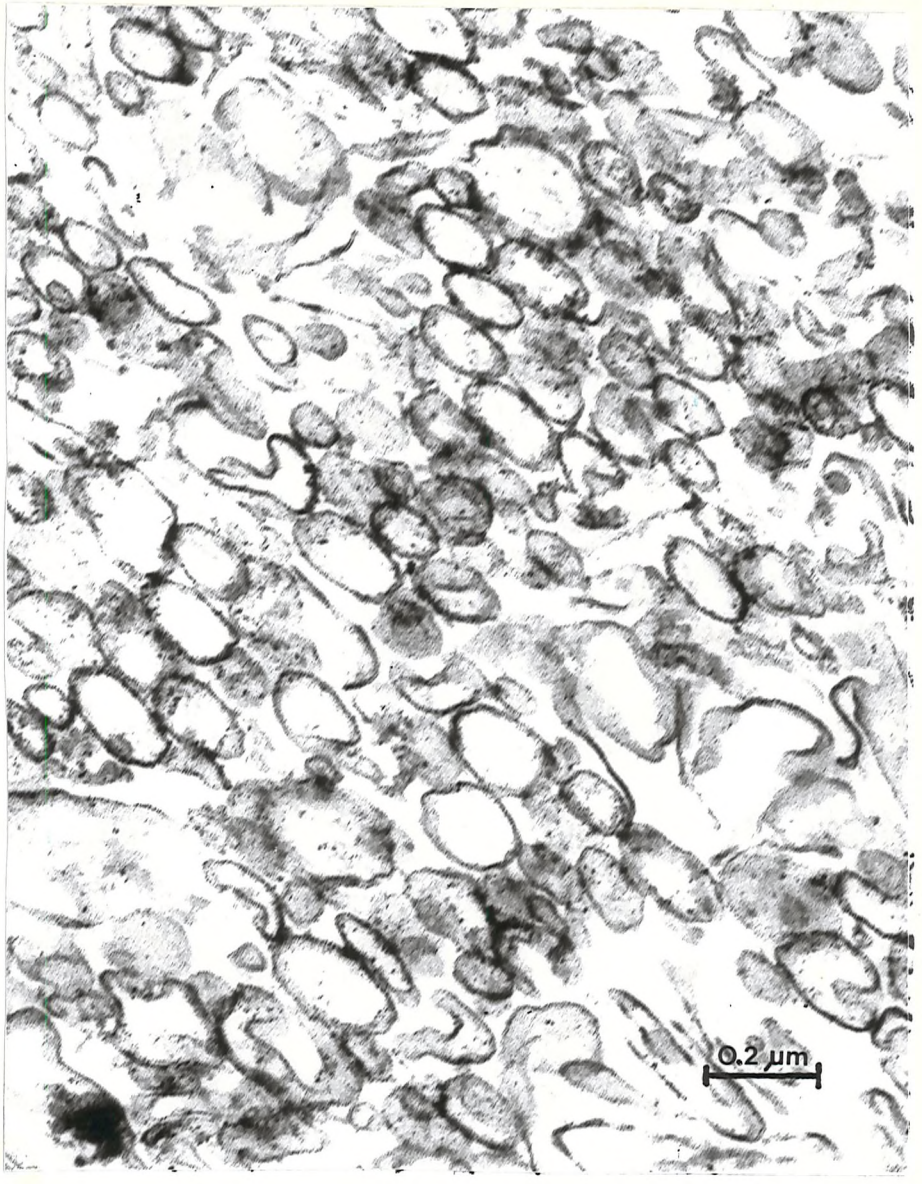


Figure 4-1 a: Electron micrograph of isolated plasma membrane fraction from log phase Ax-2 cells. Thin sections were kindly cut by Carolyn Lawson-Kerr.

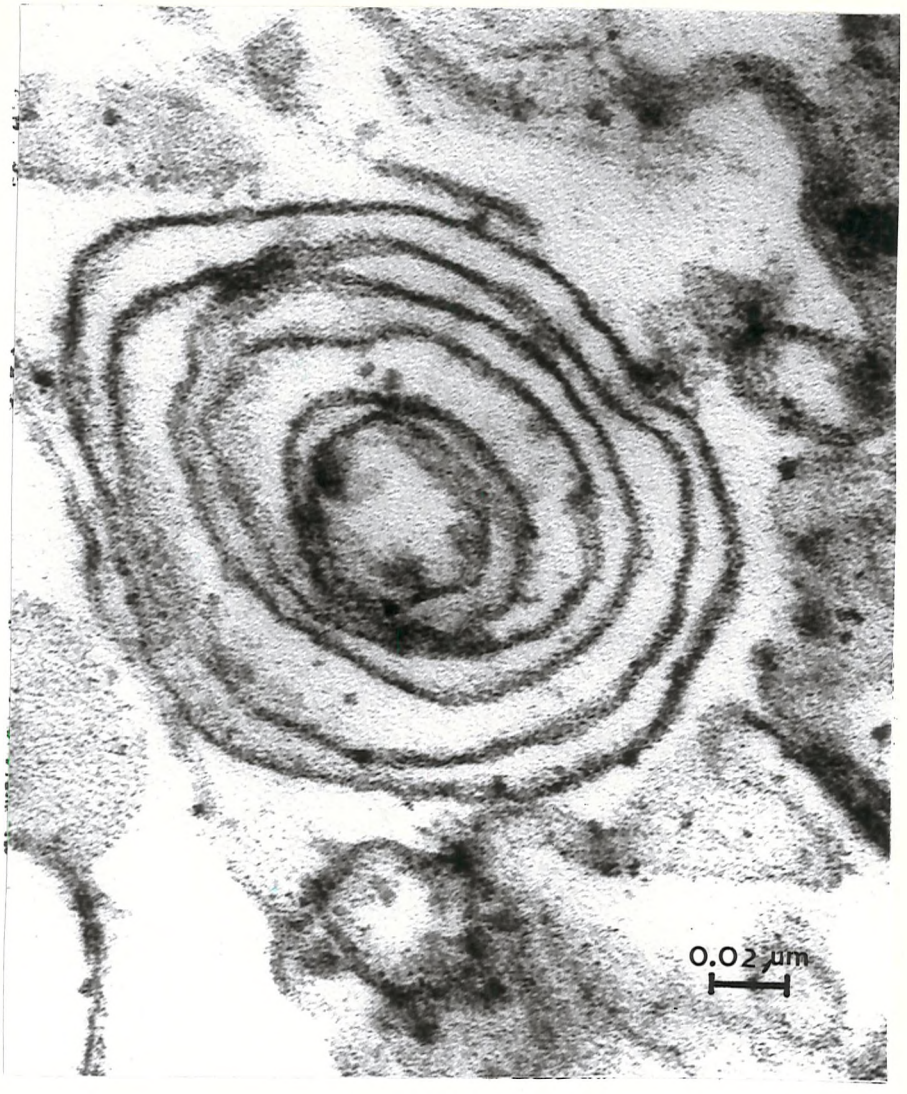


Figure 4-1 b: Electron micrograph of isolated plasma membrane fraction from log phase Ax-2 cells. Thin sections were kindly cut by Carolyn Lawson-Kerr.

Table 4-1

Enzymatic characterization of isolated plasma membranes

Marker Enzyme	Specific activity		Relative Specific Activity**
	Crude Particulate Fraction*	Cell Membrane Fraction	
Alkaline Phosphatase+	0.035	0.331	9.45
Succinate Dehydrogenase+	59.0	0.008	0.00014
Glucose 6-Phosphatase++	0.043	0.001	0.032

* 13,000 xg after washing in 20 mM Tris, pH 7.5 (see section 2.5.1.)

** Ratios of specific activity in the membrane fraction to the specific activity in the crude fraction.

\+ μ mol substrate utilized/min per mg of protein.

++ nmol substrate utilized/min per mg of protein.

Each value represents the average obtained from three assays.

values obtained in each case. The ratios of specific activity in the membrane fraction to the specific activity in the crude fraction (relative specific activity) shows a 9.45 fold enrichment with respect to alkaline phosphatase, whereas the specific activities of succinate dehydrogenase and glucose 6-phosphatase were respectively depleted to 0.0004 and 0.032 of their specific activities in the homogenates.

These results indicated no detectable contamination of either mitochondria or endoplasmic reticulum in the isolated membrane fraction.

4.2.2. Effect on log phase cell cohesion

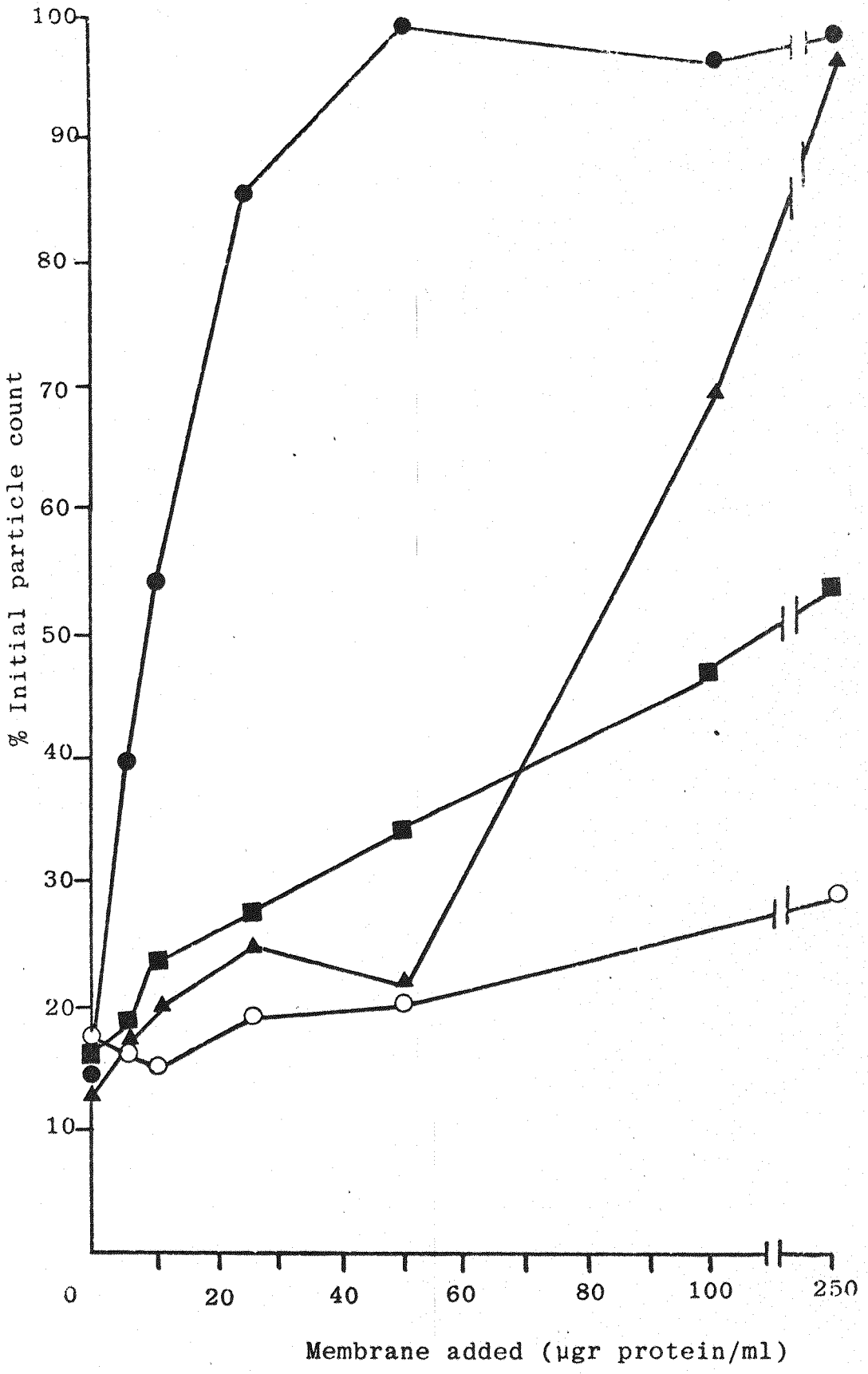
(i) Log phase cell membranes

When Ax-2 log phase cells were titrated with different amounts of plasma membranes isolated from log phase cells, it was found that the equilibrium number of perticles obtained after 20 minutes of shaking increased linearly with increasing membrane protein concentration, and reached a maximum value corresponding to 100% inhibition of cell cohesion (fig 4-2). This value was achieved when 50 µgr/ml of membrane protein were added to the cell suspension as described in section 2.5.5, and maintained at higher concentrations of membrane protein.

(ii) Stationary phase cell membranes

According to the suggestion made in the previous chapter, membranes isolated from stationary phase cells may possess an incomplete ligand-receptor system, and should therefore inhibit log cell cohesion less effectively than plasma membranes from log phase cells.

Figure 4-2: Dose-response curves showing the effect of plasma membranes isolated from log phase cells (●—●), aggregation-competent (▲—▲), stationary phase (■—■) and cycloheximide-treated cells (○—○) on cohesion of log phase Ax-2 cells. Each point indicates the equilibrium particle number obtained after 20 minutes of cohesion in the presence of the indicated concentrations of membrane protein. They represent the average of eight determinations from two experiments using separate batches of membranes.


100
90
80
70
60
50
40
30
20
10
% Initial particle count
0
20
40
60
80
100
250
Membrane added (µgr protein/ml)

It was found that only partial inhibition of log phase cell cohesion was achieved even at membrane protein concentrations that give 100% inhibition of cell cohesion with log cell membranes. Figure 4-2 shows that even in the presence of 250 ugr/ml of membrane protein, the equilibrium number of particles only reached 50% of the initial count.

(iii) Aggregation-competent cell membranes

Bearing in mind that the vegetative cell cohesion mechanism persists on the surface of aggregation--competent cells (Beug et al., 1970), it would therefore be expected that membranes isolated from aggregation--competent cells should inhibit log phase cell cohesion. This was found to be the case, but complete inhibition of cohesion was not achieved until the concentration of aggregation-competent membrane protein exceeded 100 µgr/ml (see fig 4-2).

(iv) Cycloheximide-treated cell membranes

According to the cohesive behaviour of cycloheximide-treated cells (CH-cells, see section 3.2.4) it would be expected that plasma membranes isolated from these cells should have no effect on log cell cohesion.

When plasma membranes from CH-cells were added to log phase cells as described in section 2.5.5, no significant inhibition of cohesion was found, even in the presence of 250 µgr/ml of CH-cell membrane protein (see fig 4-2), a concentration that gave 100% inhibition of log cell cohesion when log cell membranes were used.

4.2.3. Effect of log cell membranes on cohesion of aggregation-competent cells

According to the working hypothesis it would be expected that log cell membranes would inhibit partially the cohesion of aggregation-competent cells, due to the presence of both CS A and CS B on the surface of these cells (Beug et al., 1970; 1973a).

Figure 4-3 shows that cohesion of aggregation-competent cells in fact was only partially inhibited by 50 µgr/ml of log cell membrane proteins, a concentration sufficient to prevent completely the cohesion of log phase cells. The equilibrium particle number never exceeded 46% of the initial count, even at much higher concentrations of membrane protein added to the cell suspension.

4.2.4. Effect of log cell membranes on cohesion of different slime mould species

In order to determine the degree of species specificity among different slime mould species, the effect of log phase membranes on the cohesion of feeding cells of Dictyostelium discoideum (NC-4), Dictyostelium mucoroides, Dictyostelium purpureum and Polysphondylium violaceum was investigated.

Figure 4-4 shows that the dose-response curves for inhibition of cell cohesion versus membrane concentration were very similar for the four different species, complete inhibition of cohesion being achieved in each case, as would be predicted from the interspecific cohesion studies by Nicol and Garrod (1978).

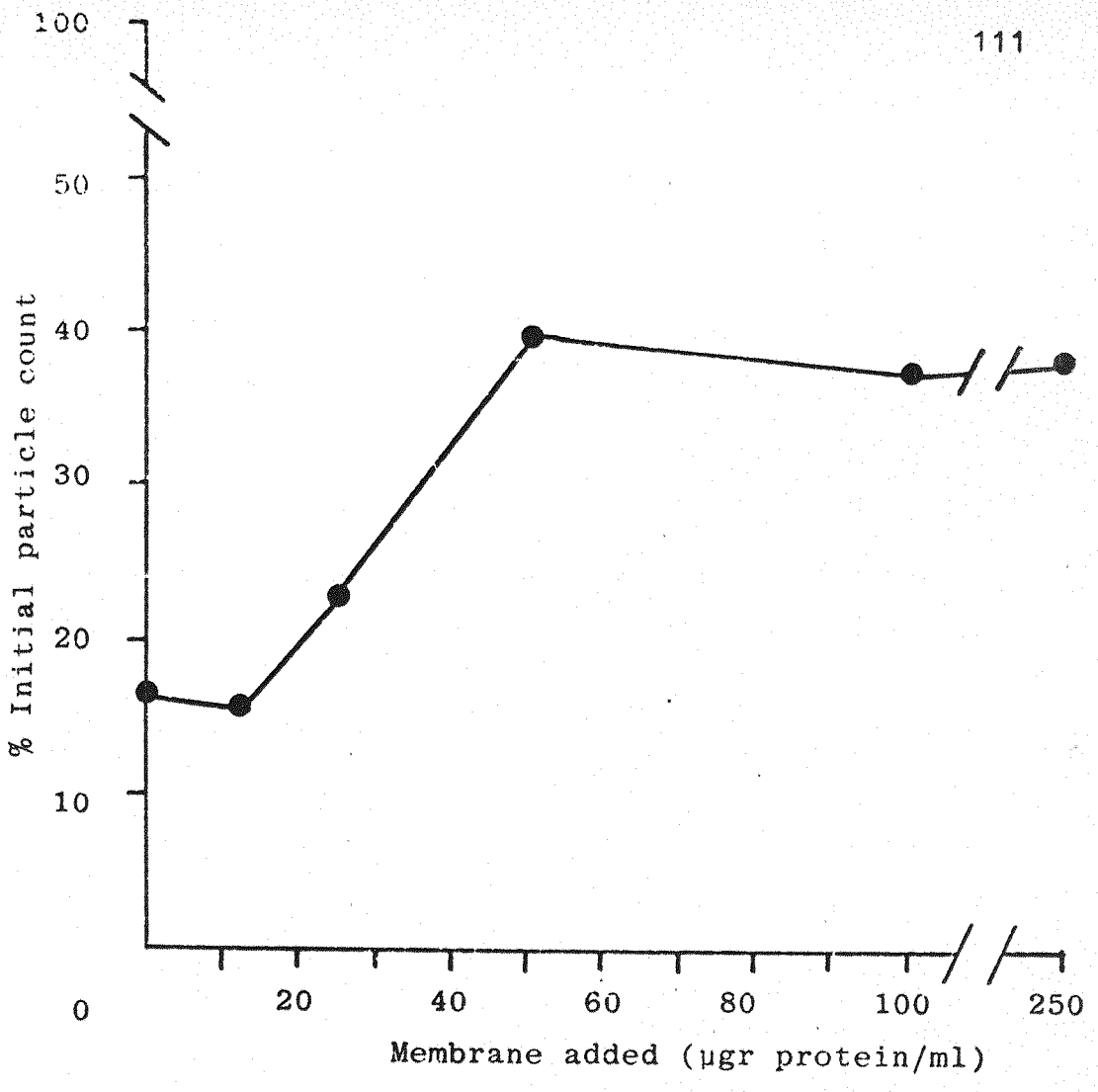
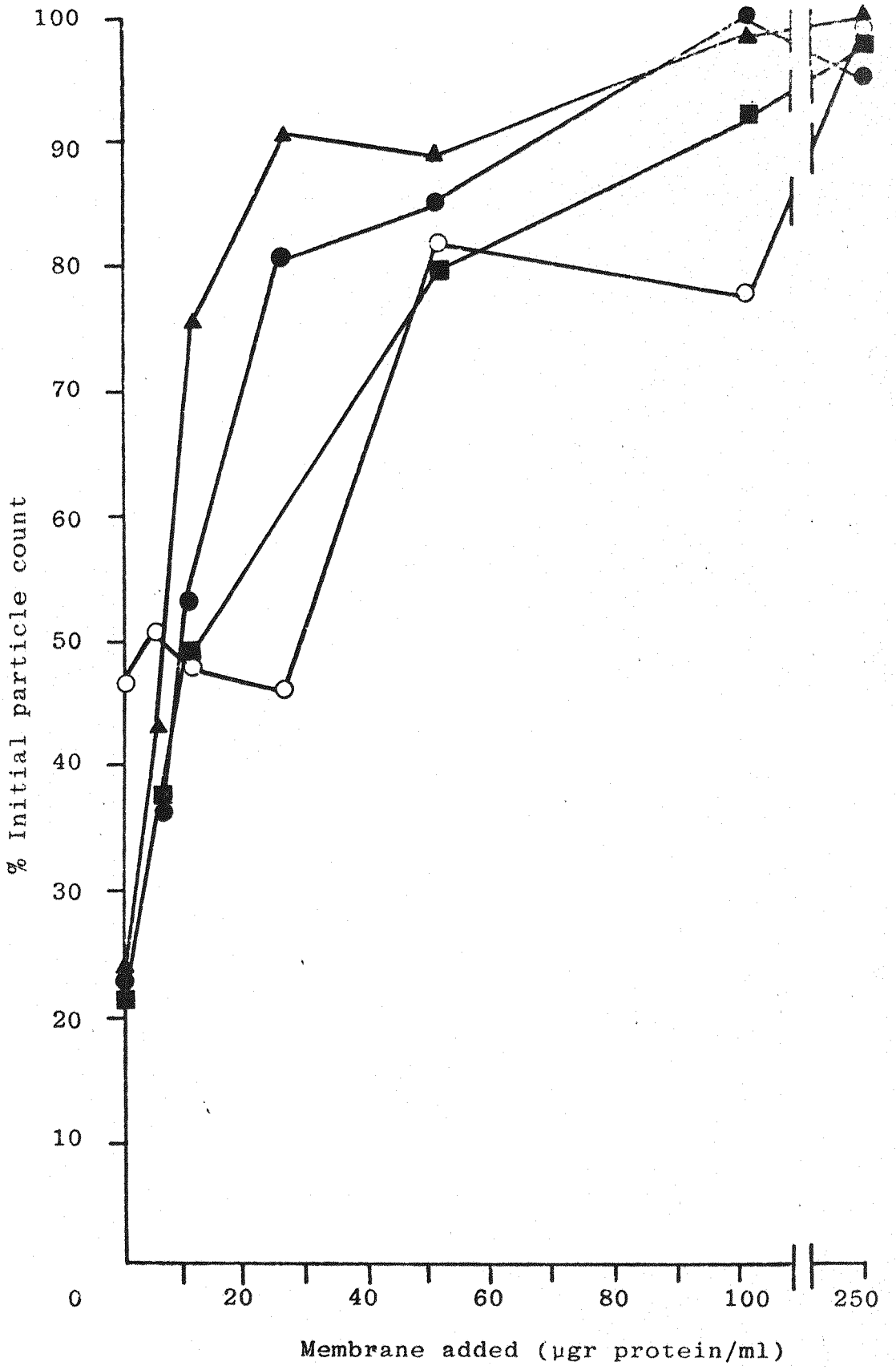


Figure 4-3: Dose-response curve showing the effect of isolated log cell membranes on cohesion of aggregation-competent cells. Each point represents the equilibrium particle count obtained after 20 minutes of shaking in the presence of the indicated amounts of membrane protein; they are the average from 3 experiments using different batches of membranes.

Figure 4-4: Dose-response curves showing the effect on cohesion of feeding cells of different species of slime moulds by plasma membranes isolated from Ax-2 log phase cells. ▲—▲, *Dictyostelium purpureum*; ●—●, *D. discoideum* strain NC-4; ○—○, *D. mucuroides*; ■—■, *Polysphondilium violaceum*. Equilibrium number of particles are shown as in figure 4-2. Each point represents the average of eight determinations from two experiments using separate batches of membranes.


100
90
80
70
60
50
40
30
20
10
0
% Initial particle count
20
40
60
80
100
250
Membrane added (µgr protein/ml)

4.2.5. Effect of periodate on biological activity of log cell membranes

Figure 4-5 shows the curves obtained by adding different amounts of periodate-treated membranes isolated from log phase cells, to a log phase cell suspension shaken for 20 minutes in phosphate buffer as described in section 2.5.5. It can be seen that the equilibrium particle count obtained in the presence of 50 µgr/ml of 10 mM periodate-treated log cell membrane protein, was only 70% of the initial count, whereas that obtained with the same amount of 50 mM periodate-treated log cell membranes was 25% of the initial count, which is approximately the same value obtained when no membranes were added to the cell suspension. These results were compared with those obtained from non-treated log cell membranes (control, see fig 4-5) and were interpreted to indicate inhibition of biological activity of log cell membranes by treatment with 50 mM sodium periodate as described in section 2.5.6.

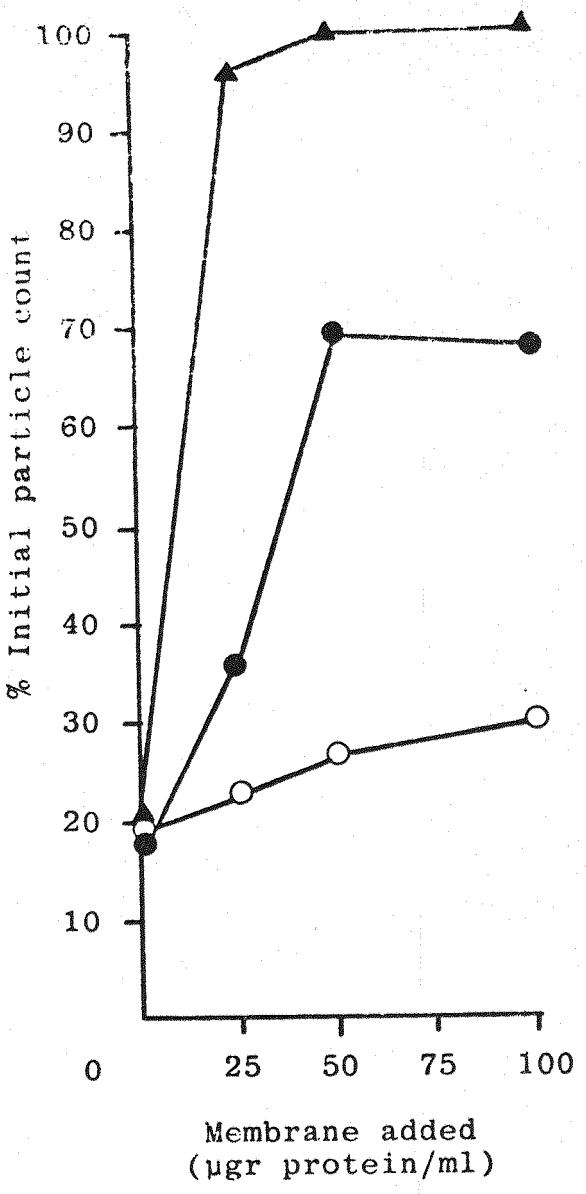


Figure 4-5: Effect of periodate on the inhibitory activity of log cell membranes on cohesion of homologous cells. The points indicate the equilibrium particle count obtained after 20 minutes of cohesion in the presence of the indicated protein concentration of non-treated log cell membranes (▲—▲), 10 mM periodate-treated (●—●) and 50 mM periodate-treated log cell membranes (○—○).

4.3. Discussion

The aim of the previous work was to investigate the feasibility of the working hypothesis proposed in chapter 3 for the cohesive behaviour of vegetative Ax-2 Dictyostelium discoideum cells, by analysing the effects of isolated plasma membranes on cell cohesion. According to the proposed ligand-receptor model, it would be predicted that plasma membranes isolated from log phase cells should bind to the surface of intact log phase cells by complementary interaction, thus inhibiting log phase cell cohesion.

The main results obtained in this study are as follows:

(a) Plasma membranes were isolated in an "active" form by the two-phase polymer system developed by Albertson (1971).

(b) Electron microscopy revealed the absence of any recognizable subcellular organelles other than membranous sheets and vesicles with a rather heterogenous size distribution.

(c) Enzymatic characterization showed no detectable contamination of either mitochondria or endoplasmic reticulum in the isolated plasma membrane fraction which could contribute to the biological activity of those membranes observed in subsequent experiments.

(d) Plasma membranes isolated from log phase cells were able to completely inhibit the cohesion of log phase cells.

(e) Plasma membranes isolated from stationary phase, aggregation-competent and cycloheximide-treated cells were either less effective or ineffective in terms of inhibition of log phase cell cohesion.

(f) Plasma membranes isolated from log phase cells produced partial inhibition of aggregation-competent cell cohesion.

(g) The inhibitory activity of log cell membranes was partially and completely abolished by treating the membranes with 10 mM and 50 mM sodium periodate respectively.

(h) Cohesion of D. discoideum NC-4, D.mucoroides, D. purpureum and Polysphondylium violaceum vegetative amoeba inhibited with equal facility by isolated plasma membranes from Ax-2 D. discoideum log phase cells.

The present results are consistent with the ligand-receptor hypothesis proposed for vegetative cell cohesion mechanism of Ax-2 cells. It is, therefore, reasonable to expect that plasma membranes isolated from cells which have incomplete (stationary phase), different (aggregation--competent) or absent (cycloheximide-treated) molecular cohesion mechanisms were either less effective or completely ineffective to inhibit log phase cell cohesion, whereas membranes isolated from log phase cells, which possess a complete ligand-receptor system, completely inhibited log phase cell cohesion.

On the other hand, the specificity of the inhibitory effect of log cell membranes was demonstrated by examining their behaviour with aggregation-competent cells. The degree of inhibition produced by these membranes on aggregation-competent cell conesion was found to be almost identical to that obtained with the low molecular weight inhibitory factor found in stationary phase medium, previously shown to be specific for log phase cell cohesion, i.e., contact sites B (see section 3.2.7). This result emphasised the possibility that both the inhibitory factor and log phase cell membranes could interact with the same molecular cohesive sites on the surface of the cells.

The results obtained with cycloheximide--treated cell membranes support the contention of Hoffman and McMahon (1978) that these cells lack contact sites.

In section 3.3 it was suggested that cycloheximide-treated cells were not able to form mutual contacts with either log or stationary phase cells because they were contact sites B-defective. This is again confirmed by finding complete absence of inhibitory activity of cycloheximide-treated cell membranes on log phase cell cohesion (see fig 4-2).

The fact that log phase cell membranes, treated with 10 mM and 50 mM periodate were partially and completely ineffective to inhibit log phase cell cohesion could be interpreted in terms of a carbohydrate dependence for such inhibition, which is consistent with the idea of a glycoprotein or glycolipid molecule involved in cell contactes. The progressive oxidation of the carbohydrate residue of this molecule would cause, according to the proposed model, an also progressive loss of inhibitory activity of the membranes.

Figure 4-6 shows diagramatically the proposed interactions between different types of membranes and the log phase cell surface, according to the results obtained in this chapter.

It should also be pointed out that autoclaved plasma membranes isolated from aggregating-phase cells of Dictyostelium discoideum (strain Ax-3) have been demonstrated to inhibit chemotactic aggregation and developmentally-regulated enzyme synthesis (Smart & Tuchman, 1976; Tuchman, Smart & Lodish, 1976). These authors claim that "membranes appear to act preventing cells from becoming competent to aggregate, rather than by simply blocking the formation of intercellular bonds." however, they also suggest that "the formation and maintenance of specific cell contacts may play a critical role in the control of slime mould development". The present work suggests that the inhibitory effect of plasma membranes on development could be partly attributed

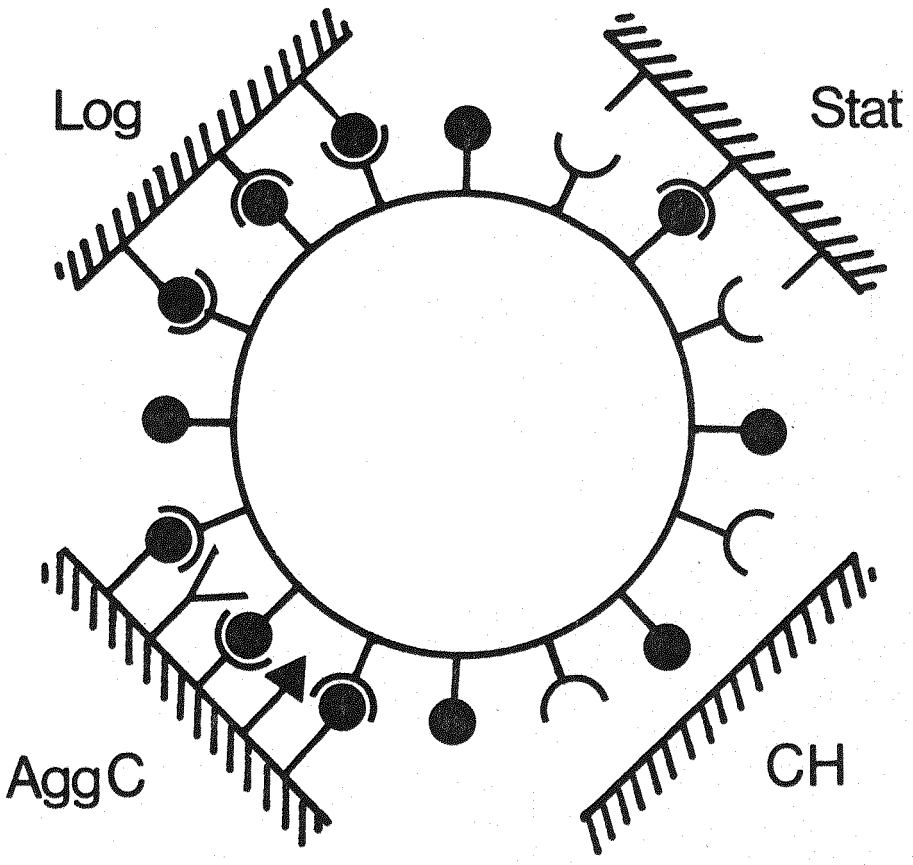


Figure 4-6: Diagram showing the proposed interactions of plasma membranes isolated from log-phase cells, stationary phase cells, CH cells and aggregation-competent cells, with the surface of log phase Ax-2 cells. —●— Contact sites B; —➤— Contact sites A.

to the demonstrated inhibition of cell cohesion. This is emphasised by the fact that the low molecular weight cohesion-inhibitory factor found in stationary phase medium is specifically active against contact sites B and also reversibly inhibits development (Swan et al., 1977; Garrod et al., 1978; Jaffé et al., 1979).

Finally, Nicol and Garrod (1978), Springer and Barondes (1978), Bozzaro and Gerisch (1978) and Sternfeld (1979) have recently shown mutual cohesion between different species of slime moulds. The present results clearly suggest that contact sites B-mediated cohesion is not species-specific, because cohesion of D. discoideum NC-4, D. mucoroides, D. purpureum, and Polysphondylium violaceum vegetative cells was inhibited with equal facility by membranes isolated from Ax-2 log phase cells. This is also consistent with the fact that the low molecular weight inhibitory factor from stationary phase cultures, blocks cohesion of the same species (Swan et al., 1977).

At this stage, there were two pieces of evidence, which seemed to be consistent with the proposed ligand-receptor model for vegetative cell cohesion mechanism: the low molecular weight inhibitory factor from stationary phase medium (Swan et al., 1977; Garrod et al., 1978; Jaffé et al., 1979) and isolated plasma membranes from log phase cells, both produce 100% inhibition of log phase cell cohesion. Following these observations, it was attempted to isolate the inhibitory activity from log phase cell membranes, that is, the cell surface components involved in vegetative cell cohesion, in a soluble and "active" form.

Chapter five: Solubilization of the active components

5.1. Introduction

In the previous chapter it was shown that plasma membranes isolated from log phase cells inhibited completely the cohesion of log phase cells. This inhibitory effect was also shown to be specific for vegetative cells, and consistent with the ligand-receptor system proposed in chapter 3.

According to these observations, an active inhibitory component should be extractable after solubilization of log phase cell membranes. Various possibilities were considered:

a) If only one of the constituent molecules on the complementary ligand-receptor systems, i.e., the ligand or the receptor, were extracted, it should be able to interact with the complementary molecule the surface of log phase cells, therefore, inhibiting its ability to cohere,

b) If both ligand and receptor were solubilized from log phase cell membranes, complete inhibition of log phase cell cohesion would be expected by the addition of these molecules to the cells, only in the case that they remained in the dissociated state. By contrast, if the solubilized molecules remained associated as a ligand-receptor complex, no interaction between the complex and the surface of log phase cells could take place and therefore, no inhibition of cell cohesion would be observed.

c) If the solubilization procedure involved the denaturation of the ligand-receptor system, no inhibitory activity would be found even after being solubilized from log phase cell membranes.

Since even by the most gentle extraction at least some of the membrane proteins are liable to be

irreversibly inactivated, the choice of an appropriate solubilization agent was also considered. Ionic detergents, although more effective than non-ionic detergents, are usually more harmful to biological activities (Ne'eman, 1971; 1972). The milder non-ionic detergents, are the most succesful in achieving controlled solubilization of biomembranes to lipoprotein particles, retaining integrated biological activities (Loach, Sekura, Hadsell & Stemer, 1970). However, the non-ionic detergents are difficult to remove from the solubilized material and frequently interfere with protein determination (Ne'eman, 1971). Organic solvents, on the other hand, although they may very rapidly destroy the biological activity of membrane proteins, they still have certain advantages over detergents (Zahler & Weibel, 1970). The chaotropic effects of certain ions containing aromatic groups on aqueous suspensions of plasma membranes, have also been used for solubilizing membrane proteins (Robinson & Jenks, 1965).

Müller and Gerisch (1978) have obtained a glycoprotein-enriched fraction by extracting plasma membranes from aggregation-competent Dictyostelium discoideum cells with n-butanol. Further purification of this fraction by DEAE-cellulose chromatography and centrifugation on a sucrose gradient yielded a material that reversed the cell dissociation produced by univalent Fab (obtained from antibodies raised against aggregation-competent cells). This was identified as contact site A, a 80,000 dalton Concanavalin A-binding glycoprotein, which acts as the target site of adhesion, blocking Fab in aggregating Dictyostelium discoideum cells.

Proteins responsible for the specific inhibition of cell aggregation have also been solubilized from plasma membranes of neural retina and optic tectum chick embryonic cells, by delipidation with acetone followed by

extraction with lithium diiodosalicylate (LIS) (Merrell et al., 1975). The inhibitory activity of these extracts was shown to be trypsin-sensitive and represented a 20-fold increase compared to whole membranes. This activity was suggested to be due to at least one active component of the cell surface recognition system of neural retina and optic tectum embryonic cells.

This chapter is concerned with the solubilization of an active fraction from log phase Ax-2 cell membranes, and the demonstration of its specific inhibitory effect on log phase cell cohesion. Non-ionic and ionic detergents, chaotropic agents and chelating agents were used for this purpose. The protein concentration, temperature and time of extraction were varied in order to obtain the optimal solubilization yield with minimum loss of biological activity.

5.2. Results

5.2.1. Solubilization in chloroform:methanol

Plasma membranes isolated from log phase cells were extracted with chloroform:methanol (2:1, v/v) in order to investigate whether their inhibitory activity on log phase cell cohesion was associated with lipids (see section 2.7.1).

After extraction, a precipitate was formed in the interface between the methanol:water (upper) and the chloroform (lower) phases. This precipitate (chloroform insoluble material) contained 35% of the total amount of membrane protein, whereas the chloroform phase contained 53% of the total extracted membrane proteins (chloroform-soluble material).

Figure 5-1 shows the dose-response curves obtained when different amounts of both chloroform-soluble and chloroform-insoluble materials were added to a log phase cell suspension as described in section 2.7.8. It can be seen that most of the inhibitory activity was found to be present in the chloroform-insoluble fraction. The dose-response curve obtained in this case was almost identical to that found when non-extracted log phase cell membranes were added to the cell suspension as a control (see fig 5-1). By contrast, the chloroform-soluble material did not show any significant activity when tested in a similar way (see fig 5-1). These results clearly showed that the inhibitory activity observed in log phase cell membranes (see previous chapter) was not associated with lipids.

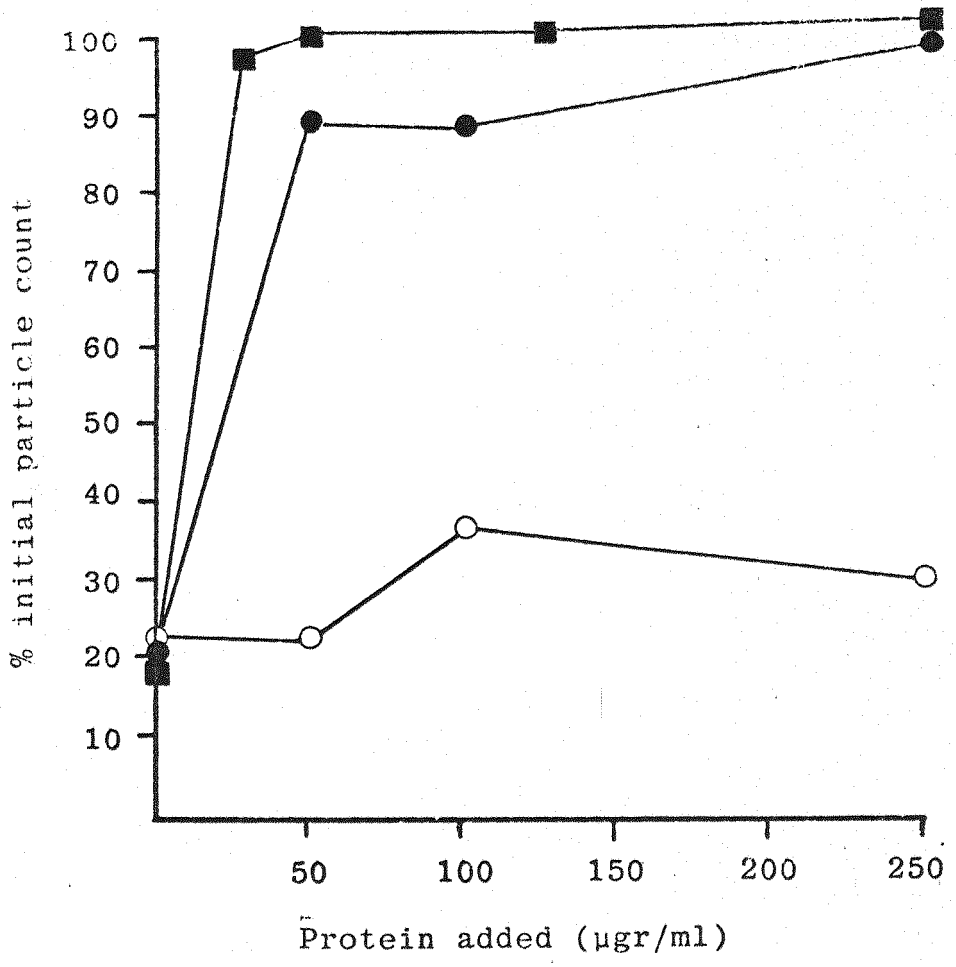


Figure 5-1: Dose-response curves showing the effects of log cell membranes (■—■), chloroform-extractable (○—○) and chloroform insoluble (●—●) log cell membrane fractions on cohesion of log phase cells. Each point indicates the equilibrium particle count obtained after 20 minutes of cohesion in the presence of the indicated concentration of protein.

5.2.2. Sodium deoxycholate extraction

Sodium deoxycholate (NaDOC) was the first ionic detergent used in an attempt to solubilize log cell membrane proteins. Table 5-1 shows that 47% of the total membrane proteins were solubilized by NaDOC (see section 2.7.3). However, only 54% of the initial particle count was achieved by adding 200 µgr/ml of NaDOC-extracted protein to log phase cells suspended in phosphate buffer and shaken for 20 minutes as described in section 2.7.8.

Table 5-1 also shows the results obtained when log phase cells were shaken for 20 minutes in the presence of "control buffer" (see section 2.7.3). This consisted of 1% NaDOC in 50 mM Tris buffer and 2 mM PMSF dialysed extensively against Tris buffer. The equilibrium number of particles, expressed as the percentage of the initial count, was found to be identical to that obtained with phosphate buffer alone.

5.2.3. Sodium dodecyl sulphate extraction

According to the previous results, another ionic detergent was tried in order to improve both yield and biological activity of extracted membrane proteins. Sodium dodecyl sulphate (SDS), used as described in section 2.7.4 was capable of extracting 53% of the total log cell membrane proteins.

Table 5-1 shows that no significant inhibitory effect was found in this extract, even at concentrations of 200 µgr/ml of extracted proteins.

As the removal of SDS from the solubilized proteins is not easy, a control in which log phase cells were suspended in dialysed SDS-Tris buffer (see section 2.7.4) was tested. Table 5-1 shows that the equilibrium

Table 5-1

Inhibitory activity yields obtained by solubilizing log phase cell membranes with various agents

Solubilizing agent	Extraction yield (%)	Extract activity*	Control activity**
NaDOC	47	54	21
SDS	53	23	19
Triton X-100	34	31	18
Nonidet P-40	40	35	21
EDTA	13	16	15

* Corresponds to the percentage of initial particle count obtained after shaking log phase cells (1×10^6 /ml) in the presence of 200 µgr/ml of extracted protein as described in section 2.7.8. In the case of EDTA and Nonidet P-40, 100 µgr/ml and 250 µgr/ml were used instead.

** Corresponds to the percentage of initial particle count obtained after shaking log phase cells (1×10^6/ml) in the presence of equivalent volumes of "control buffers" as indicated in sections 2.7.3, 2.7.4, 2.7.5, 2.7.6 and 2.7.2 for each solubilizing agent.

number of particles obtained after shaking the cells for 20 minutes in the presence of the "control buffer" was 19% of the initial particle count.

As the SDS-solubilized proteins were totally ineffective to inhibit log cell cohesion, and the tested control showed no inhibitory effect, it was difficult to decide if either the presence of residual amounts of SDS in the solubilization medium, the denaturation of the inhibitory activity of log cell membranes, or the lack of efficency in solubilizing the activity were the factors responsable for such effects.

5.2.4. Triton X-100 extraction

Triton X-100, a non-ionic detergent, was also used in order to investigate if the biological activity of log cell membranes was able to be solubilized by disrupting hydrophobic interactions with the detergent.

34% of the total membrane proteins were extracted with Triton X-100 as described in section 2.7.5. The detergent was succesfully removed from the solubilized medium by adsorption onto Bio-Beads SM2, a neutral porous styrene-divinyl benzene copolymer (Holloway, 1973; see section 2.7.5). The Triton-free soluble extract was found to inhibit partially the cohesion of log phase cells when 200 µgr/ml of extracted proteins were added to the cell suspension and the cells shaken for 20 minutes (Table 5-1). A control containing the same amount of log phase cells suspended in Triton-free solubilization buffer (see section 2.7.5) was also tested. This was found to reproduce the cell behaviour in phosphate buffer.

5.2.5. Nonidet P-40 extraction

Nonidet P-40 (NP-40), another non-ionic detergent was also tried for the solubilization of log phase cell membranes. 40% of the total membrane proteins were extracted with NP-40, and the detergent removed in the same way as described for Triton X-100 in the previous section.

The soluble extract was not able to give any significant inhibitory effect on log phase cell cohesion. Table 5-1 shows that at concentrations as high as 400 μgr/ml of extracted protein, only 40% of the initial particle count was achieved after shaking log phase cells in the presence of the solubilized proteins for 20 minutes. The control consisted of log phase cells shaken in NP-40--free solubilization buffer (see section 2.7.6) gave no inhibition of log phase cell cohesion, as would be expected.

5.2.6. EDTA extraction

Following the argument described in section 5.1, EDTA was used as a membrane solubilizing agent. It was found that only 13% of the total log cell membrane proteins were extracted by 10 mM EDTA as described in section 2.7.2. This extract, however, did not show any inhibitory activity when tested with log phase cells as indicated in table 5-1. When log phase cells were shaken in EDTA-free solubilization buffer (see section 2.7.2), a normal cohesive response of these cells was obtained indicating that the chelating agent had been removed from the solubilization buffer in which the extracted proteins were present (see table 5-1, EDTA control).

5.2.7. Lithium diiodosalicylate extraction

Studies in which lithium diiodosalicylate (LIS) has been used as a solubilizing agent were prompted by a report of Robinson and Jenks (1965) which showed that LIS was an effective way to dissociate model peptide complexes. In addition to its ability to disrupt plasma membranes, a property shared by many denaturating agents, LIS seems to release glycoproteins from membrane fragments (Marchesi & Andrews, 1971).

Different attempts were made to solubilize log cell membrane proteins with LIS. Table 5-2 shows the results obtained when the concentration of LIS, the concentration of membrane protein and the time of extraction were varied. It was found that 300 mM LIS at 25 mg/ml of membrane protein were the best conditions for extraction in terms of yield and biological activity of the extract. The soluble extracts obtained when log cell membranes were extracted with 3 mM and 30 mM LIS failed to show complete inhibition of log cell cohesion. However, in all cases, a considerable fraction of the total inhibitory activity was found in the LIS-non-extracted material, even when 84% of the total membrane proteins had been extracted (table 5-2).

In all cases, LIS was removed from the solubilized proteins by dialysis as described in section 2.7.7. A control containing log phase cells suspended in LIS-free solubilization buffer (control buffer, see section 2. 7.7) was found to give 20% of the initial particle count after shaking the cells for 20 minutes.

Figure 5-2 shows the dose-response curves obtained when the LIS-extracted soluble fraction and the LIS-insoluble material were added to a log phase cell suspension at different concentrations and the cells shaken for 20 minutes. A progressive increase can be seen

Table 5-2

Extraction of log cell membranes with lithium diiodosalicylate

Conditions*	Extraction yield(%)	Soluble activity**	Insoluble activity**
3mM LIS, 9.3mg protein/ml, 1 hr, pH 7.5, R.T.	47	79	58
30mM LIS, 9.3mg protein/ml, 1 hr, pH 7.5, R.T.	1	60	51
30mM LIS, 9.3mg protein/ml, 15mins, pH 7.5, R.T.	37	59	52
0.3M LIS, 25mg protein/ml, 15mins pH 7.5, R.T.	84	100	50

* Described in detail in section 2.7.7.

** Refers to the percentage of the initial particle count obtained after 20 minutes of shaking 1 x 10^6 log phase cells/ml in the presence of 200 µgr/ml of extracted protein (average values are shown).

R.T.= Room temperature

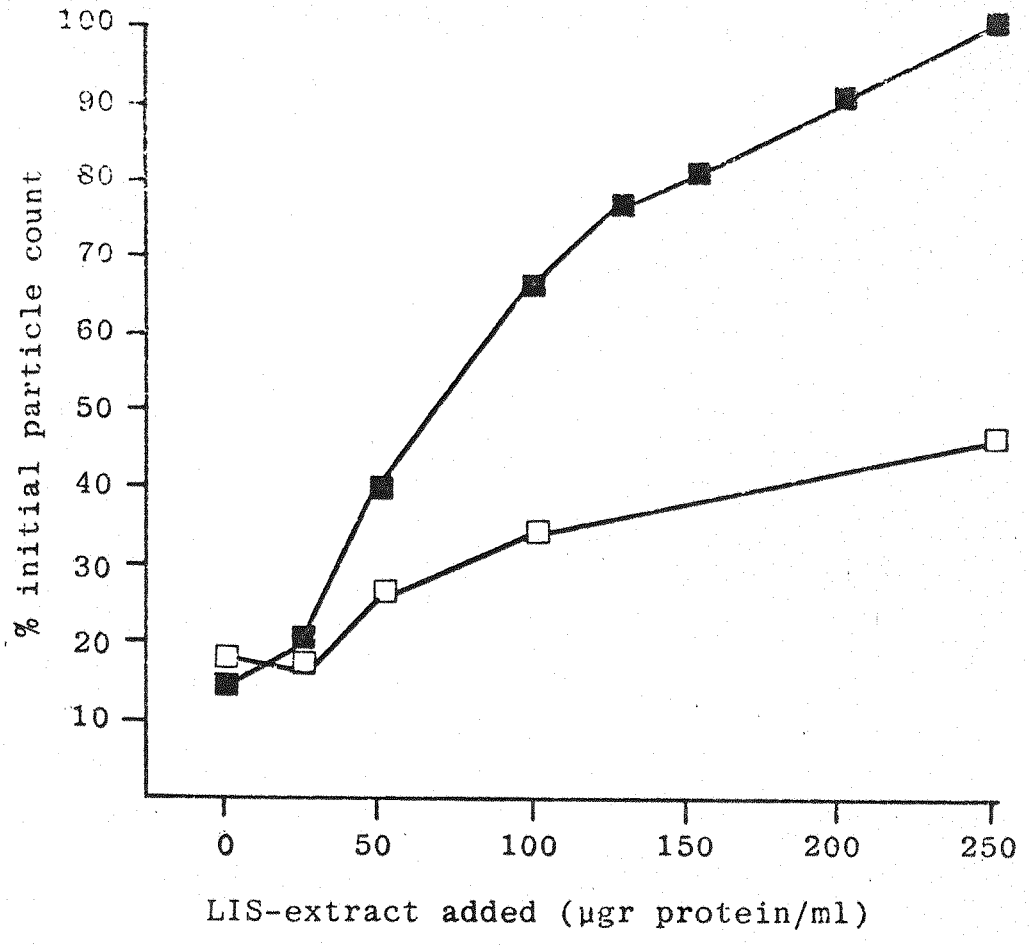


Figure 5-2: Dose-response curves showing the effects of LIS-extractable material (■—■) and LIS-non-extractable material (□—□) on cohesion of log phase Ax-2 cells. Each point indicates the equilibrium particle count obtained after 20 minutes of cohesion in the presence of the indicated concentration of protein.

in the inhibitory activity (expressed as the percentage of the initial particle count) in both cases, as the concentration of added protein increased. At 250 µgr/ml, the LIS-soluble material showed complete inhibition of cohesion of log phase cells. However, the insoluble non-extracted proteins also gave a certain amount of inhibition, indicating that although most of the inhibitory activity had been extracted with LIS, some activity was still present in the insoluble material.

A control containing 10 mg/ml of rabbit non-immune serum was also included as a control, in order to eliminate the possibility that the inhibitory effect could be due to the presence of protein. Log phase cells (1×10^6 per ml) were shaken for 20 minutes with 10 mg/ml of rabbit serum and cohesion assessed as described in section 2.7.8. No inhibition of log phase cell cohesion was observed in this case.

5.2.8. Evidence that the LIS-extracted material contains a specific inhibitory activity against contact sites B

The previous results demonstrated that the LIS-extracted material obtained from log phase cell membranes completely inhibited log phase cell cohesion. It would seem that this material, and the inhibitory factor described in chapter 3 could have parallel effects. In order to investigate this possibility, the inhibitory activity of the soluble extract was tested against aggregation-competent cells.

Figure 5-3 shows the degree of inhibition (expressed as the percentage of the initial particle count) obtained after shaking the cells in the presence of the indicated amounts of solubilized protein for 20 minutes

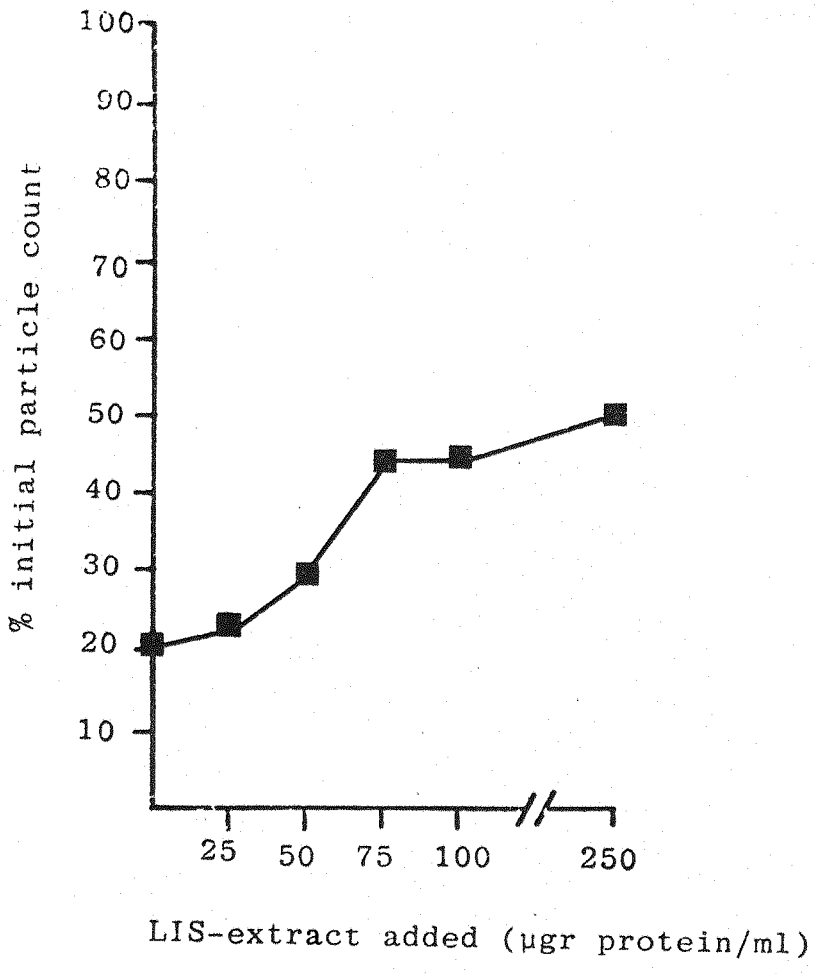


Figure 5-3: Dose-response curve showing the effect of LIS-extracted material on cohesion of aggregation-competent cells. Equilibrium particle numbers are plotted as in figure 5-2. Each point represents the average from 3 experiments.

as described in section 2.7.8. It can be seen that the maximum obtainable inhibition of aggregation-competent cell cohesion was less than 50%, even at concentrations which gave 100% inhibition of log cell cohesion (250 µgr/ml, see fig 5-2). This suggested that the LIS-extracted material contained a specific inhibitory component against contact sites B but not against contact sites A as defined by Beug et al., 1973a).

5.3. Discussion

The present work was designed to solubilize the active component(s) from log phase cell membranes, and demonstrate its(their) specific inhibitory activity on log phase cell cohesion. According to the working hypothesis proposed in chapter 3, it would be predicted that solubilized log cell membranes would contain the inhibitory activity described in the previous chapter and, therefore, would be able to inhibit completely log phase cell cohesion.

As the chemical nature of this inhibitory activity was not known, several methods were tried in order to obtain a soluble extract which retained the biological activity and also provided a reasonable amount of material for future work.

The main results obtained in this study are as follows:

(a) The inhibitory activity in log phase cell membranes was found to be insoluble in chloroform, indicating that it was not associated with lipids.

(b) Ionic detergents were used as solubilizing agents. Sodium deoxycholate (NaDOC) was capable to extract 54% of the inhibitory activity of log cell membranes , but the extraction yield was only 47% of the total membrane proteins. Sodium dodecyl sulphate (SDS) gave a better extraction yield but the soluble extract obtained failed to inhibit log phase cell cohesion, even at concentrations of 200 µgr/ml of extracted protein.

NaDOC, despite being ionic, resembles the non-ionic detergents in its low denaturating effect (Ne'eman, Kahane & Razin, 1971). As it does not bind to membrane proteins in significant quantities, it also causes far less

conformational changes than strongly ionic detergents like SDS (Philippot, 1971; Allan & Crumpton, 1971). SDS binds in considerable quantities to proteins (up to 2 gr of detergent per gr of protein; Nelson, 1971). The absence of biological activity in the SDS extract could be due to the binding of the detergent to the active sites in the solubilized "cohesive" proteins, or to an extremely rapid denaturation of those proteins by the detergent.

(c) Triton X-100 and Nonidet P-40 were used as non-ionic detergents. Neither of them was capable of extracting more than 31-35% of the inhibitory activity from log phase cell membranes, and more than 60% of the total membrane proteins remained in a particulate form after extraction with these detergents. However, despite the common difficulties in removing these detergents from the solubilized membrane material (Rottem, Stein & Razin, 1968; Gaylor & Delwiche, 1969), they were succesfully separated by adsorption onto Bio-Beads SM-2 as described in section 2.7.5.

(d) EDTA was found to be a very poor solubilizing agent of log phase cell membranes. The yield of extraction was only 13% of the total membrane proteins, and the soluble extract obtained failed to inhibit log cell cohesion. These effects could be due to the very low concentration of chelating agent used.

(e) At high concentration of log cell membrane proteins (25 mg/ml), lithium diiodosalicylate (LIS) was found to be the most suitable solubilizing agent. The soluble extract obtained gave complete inhibition of log phase cell cohesion, and a very high extraction yield (84%) was achieved. The LIS-extracted material was also able to inhibit partially the cohesion of aggregation-competent cells. This indicated that it contained a specific component(s) acting against contact

sites B but not against contact sites A.

A major obstacle to a complete understanding of the cohesive mechanism of vegetative slime mould cell cohesion has probably been the inability to obtain a suitable method for the solubilization of vegetative cell plasma membranes. The present study demonstrates the feasibility of using log phase cell membranes for extraction of large amounts of solubilized proteins. These proteins could be effectivelly extracted with lithium diiodosalicylate, a reagent which has also been used to extract the major human erythrocyte glycoprotein (Marchesi & Andrews, 1971) and the carcinoembryonic antigen (Rosai et al., 1970) in high yields. It may therefore be considered as of general use for extraction of glycoproteins from plasma membranes.

The LIS-solubilized material possessed the ability to inhibit completely the cohesion of log phase cells. Furthermore, the specificity of the solubilized inhibitory activity from log phase cell membranes was demonstrated by examining its behaviour with aggregation-competent cells. The degree of inhibition obtained in this case was found to be almost identical to that obtained with the low molecular weight inhibitory factor accumulated in stationary phase cultures (chapter 3), and the log cell membranes (chapter 4), both previously shown to be specific for log phase cell cohesion, i.e., contact sites B. These findings support the possibility that both the inhibitory factor and the solubilized component(s) from log phase cell membranes could be acting via a common mechanism.

On the other hand, the fact that the chloroform-soluble material from log cell membranes was completely ineffective inhibit log phase cell cohesion, whereas most of the inhibitory activity remained in the chloroform-insoluble material, clearly discriminated the possibility of a

glycolipid molecule involved in log phase cell cohesion, as it was first suggested in chapter 3.

All these results are consistent with the ligand-receptor hypothesis formulated for vegetative cell cohesion. It is therefore reasonable to expect that once the inhibitory activity of log phase cell membranes has been solubilized, the soluble proteins would still show a specific inhibitory effect on log cell cohesion, if their biological activity has been preserved through the solubilization procedure. However, it was very difficult at this stage to decide unequivocally whether the solubilized active material consisted of either the ligand, the receptor or both.

The next problem to approach was the purification of the active component(s) involved in log phase cell cohesion.

Chapter six: Partial characterization of the solubilized inhibitory activity from log phase cell membranes

6.1. Introduction

In the previous chapter it was demonstrated that the LIS-solubilized extract obtained from Ax-2 log phase cell membranes contains a specific inhibitory activity on log phase cell cohesion. It was then suggested that if the ligand-receptor model proposed in chapter 3 for the cohesive mechanism of vegetative cells is correct, the LIS extract from log cell membranes should contain one or more of the components involved in log cell cohesion, and by binding to the complementary structure on the intact surface of log phase cells, prevent their cohesion.

However, it was not possible at this stage to rule out the possibility that the specific interaction of a "ligand" in the extract with the cell surface, inhibited log phase cell cohesion by changing the properties of the receptors on the cell surface topography. Due to this fact, it was considered that the chemical modification of the solubilized inhibitory activity, either by periodate or heat, should eliminate these possibilities. According to the proposed model, treatment with periodate should abolish the inhibitory activity of the extract, by inactivating carbohydrate "sites" on the active components present in the extract.

On the other hand, it was thought that if the active component(s) present in the LIS extract from log cell membranes binds to the surface of log phase cells, therefore, inhibiting cell cohesion, then a depletion of inhibitory activity in the extract would be observed after binding. Merrell *et al*. (1975) have shown that the interaction of chick embryonic optic tectum and neural retina cells with a LIS extract obtained from homologous cell membranes resulted in depletion of the aggregation-inhibitory activity present in the original extract.

Furthermore, if the binding of inhibitory activity to log phase cells could be demonstrated, then log phase cells would be used as an affinity medium through which the activity could be purified.

The present study attempted to :

(1) Demonstrate that the extracted inhibitory activity from log phase cell membranes had the same properties as the inhibitory factor in terms of heat stability, and the same properties as log phase cell membranes inhibitory activity in terms of periodate sensitivity; and

(2) Attempt a step of purification in which log phase cells would be used to adsorb the activity and from which the activity would be released and eventually characterized.

6.2. Results

6.2.1. Effect of heat on the biological activity of LIS-solubilized extract from log cell membranes

According to the fact that the low molecular weight inhibitory factor accumulated in stationary phase cultures has been demonstrated to be heat-stable (Swan et al., 1977), and the working hypothesis proposed in chapter 3, it would be expected that LIS-solubilized inhibitory activity from log cell membranes would also be heat-stable.

Table 6-1 shows that the cohesion of log phase cells (1×10^6 per ml) was in fact completely inhibited by addition of 250 ugr/ml of LIS extracted proteins which had been heated at 100°C for 10 minutes as described in section 2.8.1. The concentration of heat-treated LIS extract protein used in these experiments was identical to that found to be the minimal inhibitory concentration of LIS extract during the titration experiments (see fig 5-2 in chapter 5).

6.2.2. Effect of periodate on the biological activity of LIS-solubilized extract from log cell membranes

When LIS-solubilized extract was pre-incubated with sodium periodate as described in section 2.8.2, and then added to a log phase cell suspension and shaken for 20 mins in phosphate buffer, no inhibition of log phase cell cohesion was observed (table 6-2). It can be seen from table 6-2 that the equilibrium particle number obtained in the presence of 250 ugr/ml of periodate-treated LIS extracted proteins was only 13% of the initial count, whereas that obtained with the same amount of non-treated LIS extract was 100% of the inital count. These results were compared with those obtained when an equivalent volume of "control buffer" (50 mM Tris, pH 7.5, 50 mM periodate, 100 mM glucose, dialysed overnight

Table 6-1

Heat stability of LIS-extracted inhibitory activity

Conditions	Concentration (µgr protein/ml)	% Initial Count *
17 mM phosphate buffer, pH 6.0	---	14 ± 0.7
LIS extract	250	98 ± 3.0
Heat-treated LIS extract	250	92 ± 8.3

Experimental conditions are described in section 2.8.1.

*Measured as the equilibrium particle number obtained after shaking 1 x 10^6 log cells/ml at the indicated conditions for 20 minutes, and expressed as the percentage of the initial count (t=0); 100%=complete inhibition. Standard deviations are shown.

Table 6-2

Effect of periodate on LIS-extracted inhibitory activity

Conditions	Concentration (µgr protein/ml)	% Initial Count*
17 mM phosphate buffer, pH 6.0	---	13
LIS extract	250	100
Periodate-treated LIS extract	250	13
Control**	---	12

Experimental conditions are described in section 2.8.2.

*As in table 6-1.

**Refers to an equivalent volume of control sample (see section 2.8.2) added to a log cell suspension (1×10^6 /ml) and shaken for 20 minutes.

against Tris buffer) was added to the cell suspension. In this case, no inhibition of log phase cell cohesion due to the presence of reacting substances was observed (see table 6-2).

These findings were interpreted to indicate that the LIS-solubilized inhibitory activity could be totally eliminated by pre-treatment with periodate, and supported the assumption that the carbohydrate portion of a glycoprotein must be directly involved in cohesion of log phase Ax-2 cells.

6.2.3. Evidence that LIS-solubilized inhibitory activity binds to the surface of log phase cells.

Bearing in mind that the LIS-solubilized inhibitory activity occurs on the surface of log phase cells, it would therefore be expected that log phase cells should bind the inhibitory component(s) so that the inhibitory activity in the extract would be depleted. This possibility was investigated by incubating 1 x 10^7 log phase cells per ml with LIS extract (250 µgr of protein/ml) in gyrated suspension, for 30 minutes at 22°C (controls without LIS extract). The cells were then removed by centrifugation. The supernatant was used in a standard cohesion assay with fresh log phase cells (1 x 10^6/ml), and after 20 minutes the equilibrium number of particles measured as decribed in section 2.2.1.

Figure 6-1 shows that in fact the interaction of log phase cells with the LIS extract resulted in depletion of the inhibitory activity present in the intact extract. It can also be seen that an excess of log phase cells (1 x 10^7/ml) was used to remove the minimal inhibitory concentration of extracted proteins which gave 100% inhibition of cohesion of 1 x 10^6 log phase cells (see

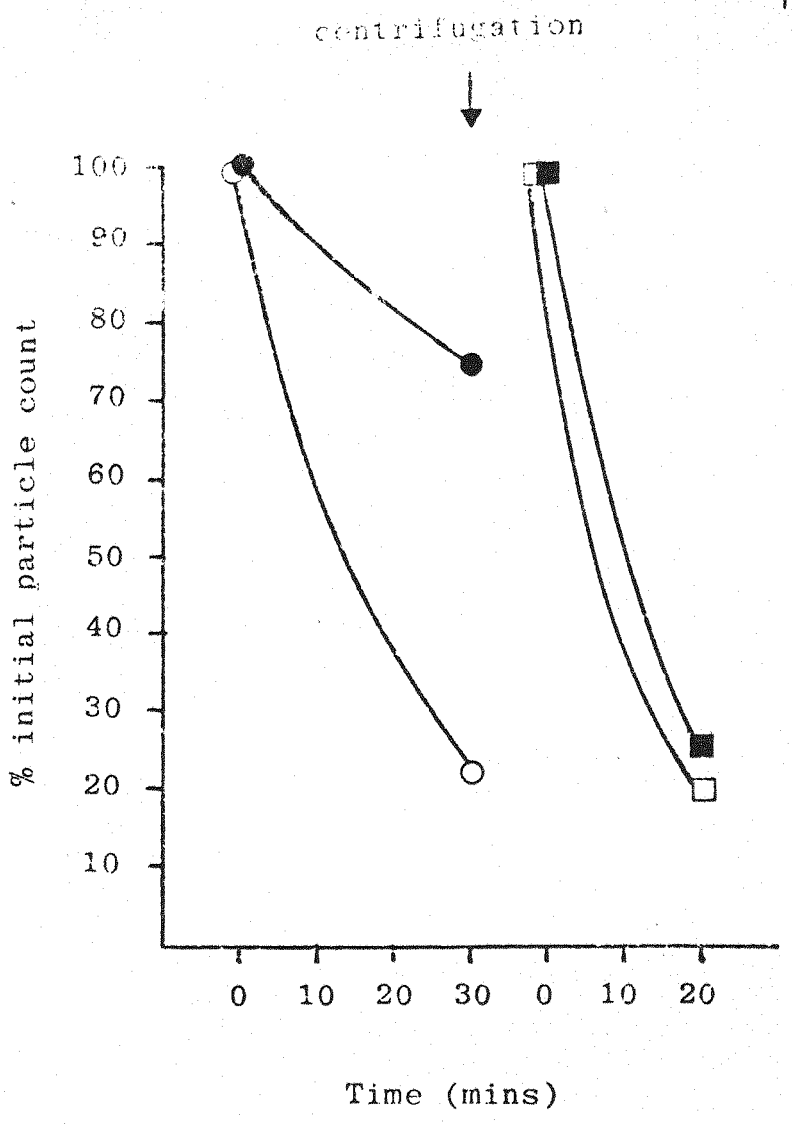


Figure 6-1: Cohesion of Ax-2 log phase cells in the presence (●—●, test) and absence (○—○, control) of LIS extract (250 µgr protein per ml). The arrow indicates the time at which the cells were centrifuged and 1 x 10^6 fresh log phase cells were suspended in test (■—■) and control (□—□) supernatant to assay cohesion for 20 minutes.

fig 5-2 in chapter 5), and therefore, only 75% of inhibition was achieved.

The fact that the inhibitory component(s) in the LIS extract obtained from log phase cell membranes could be adsorbed by homologous cells, was used to approach the purification of the active component(s) using log phase cells as an affinity medium.

6.2.4. Adsorption and release of the inhibitory activity using log phase cells as an affinity medium

Following the previous observations, an attempt was made to purify the active inhibitory component(s) present in the LIS extract from log phase cell membranes, by adsorbing the activity on log phase cells as described in the previous section, and then releasing it from the cells.

2.5 mg of LIS-solubilized protein were adsorbed with 1 x 10^8/ml log phase cells for 30 minutes at 22°C in gyrated suspension. The cells were then separated by centrifugation at 300 g for 5 minutes, and suspended in phosphate buffer (1 ml) by repeated pipetting. The cell suspension was centrifuged again and 1 x 10^6 log phase cells were suspended in the supernatant and shaken for 20 minutes to assess cohesiveness in the usual way (see section 2.2.1). The cell pellet was suspended in 1 ml of phosphate buffer, and cohesiveness assayed in a similar way.

Figure 6-2 shows the inhibitory effect of the supernatant on cohesion of log phase cells. It can be seen that after 20 minutes of shaking, the equilibrium particle number was 72% of the initial count, whereas 20% was obtained when the cells were shaken in the supernatant obtained from control cells (non-adsorbed). The cell pellet obtained after releasing the inhibitory activity to the supernatant was proved to behave as control cells.

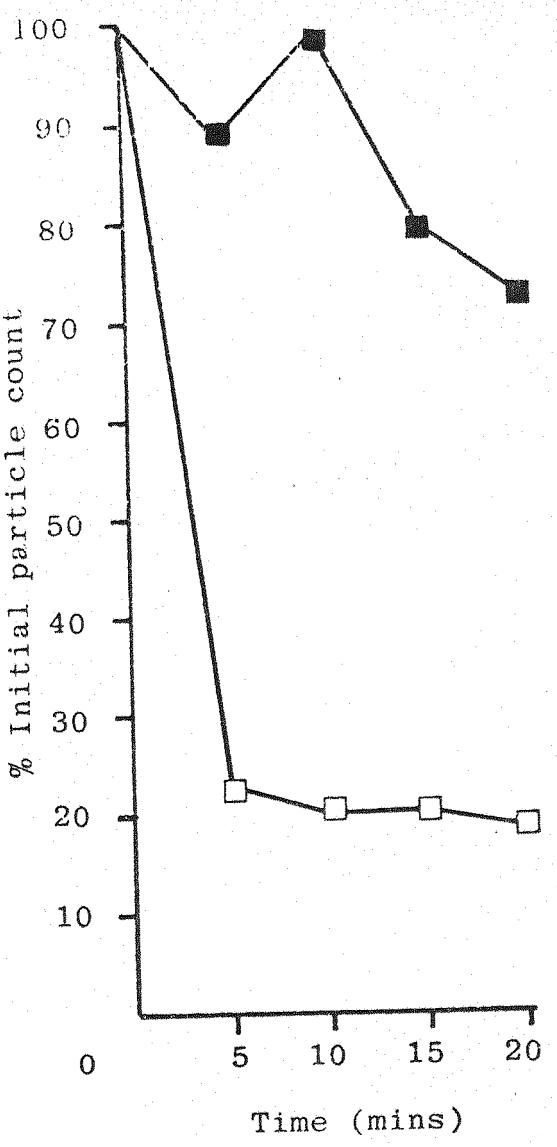


Figure 6-2: Inhibitory effect on cohesion of log phase cells of material released from the surface of log cells after incubation in LIS extract (■—■). Control supernatant from cells incubated in phosphate buffer alone (□—□). Experimental details are described in section 6.2.4.

On the other hand, it was found that 64% of the original amount of LIS-extracted proteins used in adsorption experiments was released by log phase cells after adsorption. Thus, a reasonable amount of inhibitory activity from LIS extract could be adsorbed by (see fig 6-1) and released from (see fig 6-2) log phase cells, and the composition of the released material could therefore be investigated by SDS-polyacrylamide gel electrophoresis.

6.2.5. SDS-polyacrylamide gel electrophoresis of the adsorbed inhibitory activity from LIS extracts of log phase cell membranes

Having found that the cohesion-inhibitory activity found in LIS extracts of log phase cell membranes could be adsorbed by and then released from log phase cells, the next step to consider was the degree of purity of the adsorbed material by SDS-polyacrylamide gel electrophoresis (PAGE).

Figure 6-3 shows the electrophoretic patterns obtained when log phase cell membranes (gel A), LIS extract from log cell membranes (gel B), and the adsorbed and released material (gel C) were electrophoresed. It can be seen that although the LIS extraction of log phase cell membranes reduced the number of proteins present in intact membranes, the adsorbed material still contained a large number of polypeptides. Some of these did not appear in the LIS extract and might be considered to be secreted from the cells during the "release" procedure (see previous section), or a contamination from the cell pellet (see section 6.2.3). However, a band corresponding to a molecular weight of 51,000, present in log phase cell membranes and the LIS extract, also appeared on the adsorbed material (gel C, arrow). This band seems to have been concentrated

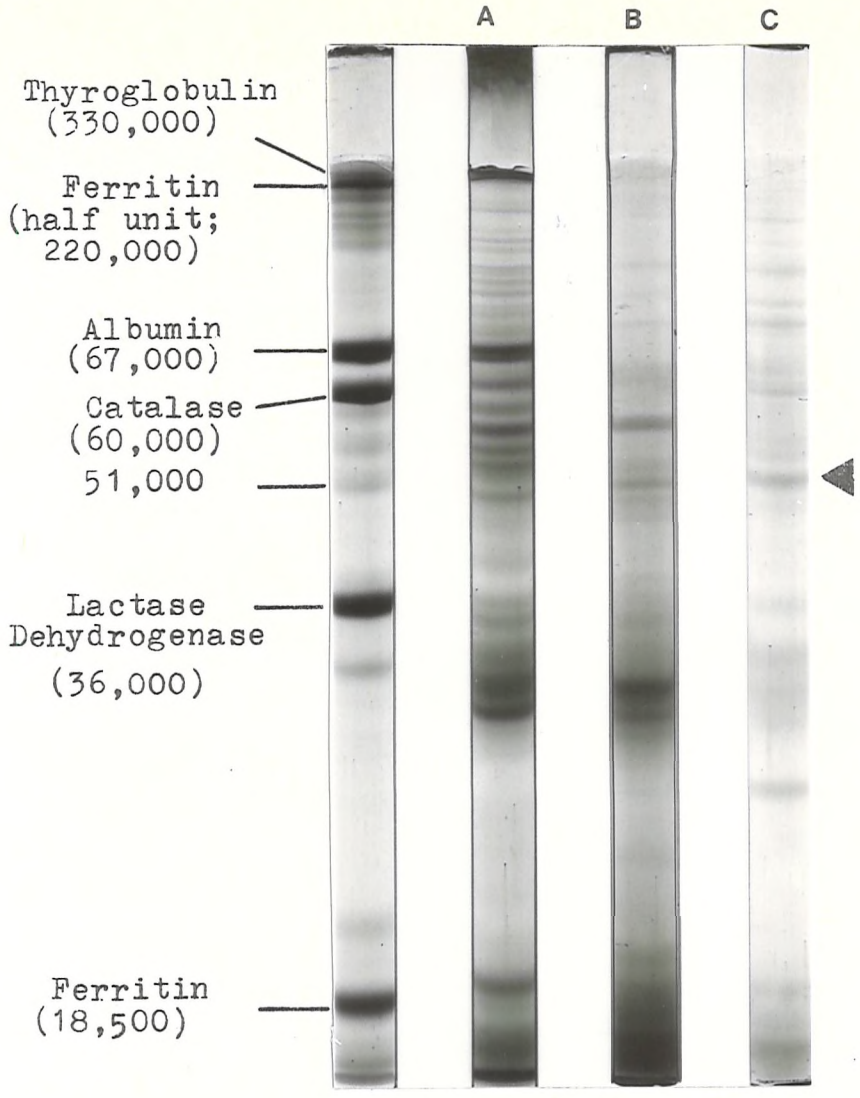


Figure 6-3: SDS-polyacrylamide gel electrophoresis of A, plasma membranes derived from log phase Ax-2 cells (50 µgr protein/gel); B, LIS extract of log cell membranes (50 µgr/gel); C, absorbed fraction of LIS extract by log phase cells (30 µgr/gel). Gels stained with Coomassie blue are shown.

during the adsorption procedure .However, there was no indication to suggest that this particular polypeptide was associated with the inhibitory activity of the adsorbed material on cohesion of log phase cells (see previous chapter).

According to this preliminary analysis, it was concluded that cell adsorption as described in section 6.2.4, although it provides indirect evidence for the binding of the inhibitory activity on the surface of log phase cells, does not appear to be a suitable technique to purify the activity from the LIS extract.

6.2.6. Concanavalin A-binding activity

A preliminary experiment was performed in order to investigate whether the LIS inhibitory activity contained Concanavalin A-binding activity. LIS extract (500 µgr protein) was incubated at 22 °C with 1 ml of a suspension containing ConA-bound agarose (11 mg lectin/ml) previously equilibrated with 50 mM Tris buffer pH 7.2, for 30 minutes at 200 r.p.m. A control with 500 ugr of bovine serum albumin was run in parallel.

The agarose beads were removed by centrifugation and 1 x 10^6 log phase cells were suspended in the supernatant to assess cohesiveness as described in section 2.2.1. It was found that the inhibitory activity present in the original extract had been adsorbed to the Con A-agarose, since the equilibrium particle count obtained after 20 minutes of shaking the cells in this supernatant was identical to the control (approx. 15% of the initial count).

The beads were incubated with 0.2M alpha-methyl-D-mannoside for 30 minutes at 22°C and 200 r.p.m. They were removed by centrifugation and the supernatant

dialysed overnight against 50 mM Tris buffer pH 7.2. 0.5 ml of the dialysed supernatant was added to a log phase cell suspension (1 x 10^6 cells per ml) in order to test its effect on cell cohesion as described in section 2.2.1. 60% of the initial count was obtained after 20 minutes of shaking the cells in the presence of the dialysed material, whereas only 15% of the initial particle count was obtained when the control (albumin) was tested. This indicated that the inhibitory activity present in the LIS extract had been adsorbed by Con A-bound agarose beads, and then released by addition of alpha-methyl-D-mannoside to the Con A-agarose -protein complex.

6.3. Discussion

As demonstrated in the previous chapter, an inhibitory activity of log phase cell cohesion was extracted from log phase cell membranes, which appeared to act specifically against cohesion of vegetative cells. This supported the ligand-receptor model proposed in chapter 3. The immediate questions to this findings were as follows:

a) Is the extracted activity sensitive to heat and periodate?

b) Does the inhibitory activity actually "binds" to the surface of log phase cells?

c) If so, could it be purified by using the cells as a specific adsorbent medium?

The present work was designed to answer these questions. The main results obtained are as follws:

1- The inhibitory activity present in LIS extracts from log phase cell membranes, was completely abolished by pre-treatment with sodium periodate, and not affected by heating the extract at 100°C for 10 minutes. These results, and the fact that 10 mg/ml of non-immune rabbit serum protein were unable to inhibit log phase cell cohesion (see previous chapter) suggested that the inhibitory activity extracted from log phase cell membranes was not changing the receptor properties or the surface topography, but rather interacting specifically with a receptor on the cell surface.

On the other hand, the fact that periodate abolished completely the inhibitory effect of LIS extracts on cohesion of log phase cells, was consistent with the complete lack of inhibitory activity found with periodate-treated log cell membranes (see chapter 4), and supported

the suggestion that a carbohydrate residue is responsible for the inhibitory activity of the extracted material.

Furthermore, the demonstrated heat stability of the LIS extract obtained from log cell membranes, was also found to be consistent with the heat stability observed in the low molecular weight cohesion inhibitor accumulated in stationary phase cultures (Swan et al., 1977).

If the model proposed in chapter 3 is correct, log phase cell cohesion would be mediated by the complementary interaction between a carbohydrate-binding protein (receptor) and a glycoprotein (ligand) on the surface of adjacent cells. It has been also proposed that the carbohydrate residue of the glycoprotein would be the low molecular weight inhibitor found in stationary phase medium. The previous results suggested a great similarity between the inhibitory factor and the inhibitory activity present in log phase cell membranes and extracted from them with LIS:

a) The three activities inhibited completely the cohesion of vegetative cells, and partially the cohesion of aggregation-competent cells (see chapters 4 and 5);

b) The membrane activity and the LIS-extracted activity were abolished by pre-treatment with periodate;

c) The factor activity and the LIS-extracted activity were both stable to heat (see Swan et al., 1977).

However, no direct evidence has been obtained to prove that these inhibitory activities are identical.

2- The interaction of log phase cells with the LIS extract obtained from log phase cell membranes resulted in depletion of the inhibitory activity of the extract. This indicated

that the inhibitory activity in the extract had been adsorbed by the cells, and provided further evidence for the surface localization of the cohesive "sites". This finding led to the thought that once the inhibitory activity has been adsorbed by the cells, it could be possible to release it from the cells by simply suspending the cell pellet in phosphate buffer. This was performed, and the released material was found to contain the activity when tested on log phase cell cohesion. This result was very encouraging since by using log phase cells as an affinity medium, the LIS-extracted activity could then be fractionated from the rest of the proteins.

3- The electrophoretic analysis of the adsorbed and subsequently released material showed a large amount of polypeptides present in the sample, some of them absent in the LIS extract from which they were being fractionated. This was interpreted to indicate that:

a) Protein secretion from log phase cells could be taking place during the release of the inhibitory activity, in which case, a control with "secreted proteins" from the cells should have been also electrophoresed, or

b) Contamination with non-adsorbed LIS extracted proteins could also occur. Since the non-adsorbed material was separated from the cells by centrifugation (see section 6.2.4), the cells might have still contained much of the extracted proteins in the intercellular spaces, and once suspended in phosphate buffer to release the inhibitory activity, any material trapped by the cells would also be released.

On the other hand, a band corresponding to a molecular weight of 51,000, present in log phase cell membranes and the LIS extracts, appeared concentrated in the adsorbed material. This protein, although unidentified,

be a dimer of discoidin I (molecular weight 52,000; Frazier *et al.*, 1975). However, no direct evidence is presented to associate this protein with the glycoprotein responsible for the adsorbed inhibitory activity on cohesion of vegetative Ax-2 cells.

4- The cohesion-inhibitory activity present in the LIS-extracted material, was capable of binding to Concanavalin A-bound agarose beads, and released by addition of alpha--methyl-D-mannoside, a specific sugar to which Con A binds. This result, although preliminary, suggested that Con A binding affinity chromatography could be used as a suitable method to purify the activity from the rest of extracted membrane proteins. Having found that the inhibitory activity could bind Con A-agarose beads, the possibility that at least one of the molecules involved in vegetative cell cohesion would be a glycoprotein, seemed likely. The periodate-sensitive behaviour, and the Con A-binding activity, support this contention.

Chapter seven: Antibodies

7.1. Introduction

Beug et al. (1973a) have proposed the existence of two different classes of contact sites on the surface of Dictyostelium discoideum cells: contact sites A (CS A), responsible for end-to-end contacts in aggregating cells, and contact sites B (CS B), present in growth phase cells (see chapter 1). The fact that the surface of aggregation-competent cells appeared to share certain antigenic sites with growth phase cells, and additionally contained new specific ones, was used by Beug and co-workers to obtain a specific antibody against aggregation-competent cell CS A. This was performed by absorption of univalent antibody fragments (Fab) obtained against aggregation-competent cells with vegetative cells. The new aggregation-specific Fab was shown to block CS A-mediated cohesion of aggregation-competent cells (Beug et al., 1973a). Fab fragments prepared against vegetative cells were found to block cohesion of those cells but failed to affect the EDTA-resistent cohesion of aggregation-competent cells.

Since CS B are present at all stages of growth and development, there was no suitable material with which to absorb the anti-vegetative cell serum in order to obtain an antibody specific for CS B. For this reason, CS B have not been characterized to the same extent as CS A.

According to the ligand-receptor model proposed in chapter 3 for vegetative AX-2 cell cohesion, stationary phase cells would possess half of the ligand-receptor system on their surface. Thus, if an antiserum was prepared against log phase cells, and then absorbed with stationary phase cells, a specific antibody against the cohesive ligands should be obtained. Stationary phase cells would then constitute a suitable material with which to absorb the antiserum. On the other hand, if the antiserum

raised against log phase cells was absorbed with cycloheximide-treated cells, which appear to lack CS A and CS B on their surface (Hoffman & McMahon, 1978) and also are unable to stick together (see section 3.2.4), all the non-specific serum would be absorbed and a purified anti--CS B serum would be obtained. This would enable CS B to be isolated.

The first approach to this study was to raise antiserum against freeze-dried log phase cells. As the results obtained with this serum were not satisfactory, it was decided to raise a second antiserum, this time against isolated plasma membranes from Ax-2 log phase cells. Antisera prepared against purified plasma membranes of transformed baby hamster kidney (BhK) cells have been shown to be of great value in identifying surface glycoproteins involved in substrate adhesion of these cells (Wylie, Damsky & Buck, 1979). Rutishauser et al. (1978) have also made use of anti-membrane serum to investigate the function of a cell adhesion molecule (CAM) involved in the in vitro aggregation of neural cells from chick embryos.

After preparing the anti-log membrane serum, the following questions were asked:

1- Is the anti-log membrane serum a specific marker of the log phase cell surface?,

2- How does it react to antigens prepared from stationary phase, aggregation-competent and cycloheximide-treated cell membranes?

3- Does it inhibit log cell cohesion and, if so, is the inhibition specific to vegetative cells?,

4- Does it inhibit development?

7.2. Results

7.2.1. Properties of an antiserum raised against freeze-dried log phase cells

As it was explained in the previous section, an antiserum from freeze-dried log phase Ax-2 cells was first raised as described in section 2.9.1. Double diffusion in agar plates (see section 2.9.3) was used to test the specificity of the antiserum against different antigen preparations. These antigens were obtained from solubilized extracts of freeze-dried log phase and stationary phase cells (see section 2.9.3). The antiserum against freeze-dried log phase cells showed significant precipitation with both antigens. However, when the precipitation lines were resolved by immunoelectrophoresis, more lines were found with the stationary phase cell antigen than with the log phase cell antigen.

In addition, it was found that the reactivity of this antiserum was completely eliminated after absorption of the antiserum with stationary phase cells or with cycloheximide-treated cells. These results eliminated the possibility of using such antiserum as a potential marker of specific antigenic groups involved in log phase cell cohesion. A second antiserum, against log phase cell membranes, was raised, and the immunisation protocol changed at the suggestion of Prof. G.T. Stevenson (Tenovus Laboratories, Southampton General Hospital).

7.2.2. Properties of an antiserum raised against log phase cell membranes

An antiserum against isolated plasma membranes from log phase cells was obtained as described in section 2.9.2. This antiserum showed a titre (the highest dilution of log cell membrane antigen showing detectable precipitation)

of 1/312.

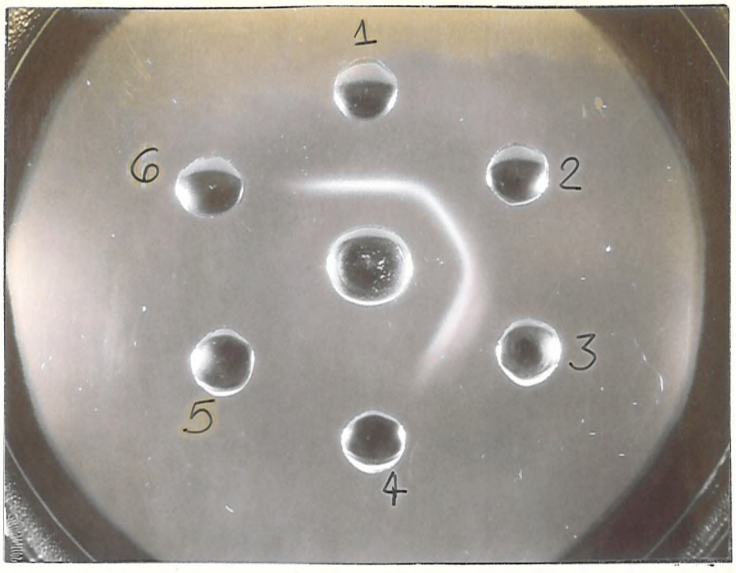
(i) Double diffusion patterns with various antigen preparations

Figure 7-1a shows the reactivity of three antisera raised under identical conditions against log phase cell membranes. A strong line of precipitation was observed when these antisera were reacted with log-membrane antigen (prepared as described in section 2.9.3), whereas the pre-injection serum showed no reactivity.

The appearence of a single precipitation line (within the sensitivity of the technique) first suggested the monospecific nature of this antiserum. However, when tested with antigens prepared by solubilizing stationary phase, aggregation-competent and cycloheximide-treated cell membranes as described in section 2.9.3, the same line of precipitation was observed in each case (fig 7-1b). The band in figure 7-1b for stationary-membrane antigen was weaker than those found with the other antigens. These results eliminated the possibility of dealing with a monospecific antiserum to contact sites B.

On the other hand, when the anti-log membrane serum was tested with log phase cell membrane extracts, obtained as described in sections 2.7.2, 2.7.3, 2.7.4, 2.7.5, 2.7.6 and 2.7.7, a common broad line of precipitation was found with the Nonidet P-40, sodium deoxycholate (NaDOC), sodium dodecyl sulphate (SDS) and lithium diiodosalicylate (LIS) extracts. However, an additional precipitation line was observed with the LIS extract. The EDTA and Triton X-100 extracts showed very weak precipitation lines with the antiserum (see fig 7-2a).

Figure 7-2b shows the double diffusion pattern obatined when the antiserum was reacted with different



a

b

Figure 7-1: Double diffusion patterns obtained after 2 days from reacting (a) three anti-log membrane sera (1,2,3) and their corresponding non-immune sera (4,5,6) with log-membrane antigen, and (b) anti-log membrane serum with four antigen preparations: 1, log-memb; 2, CH-memb; 3, stat-memb; 4, AggC-memb.

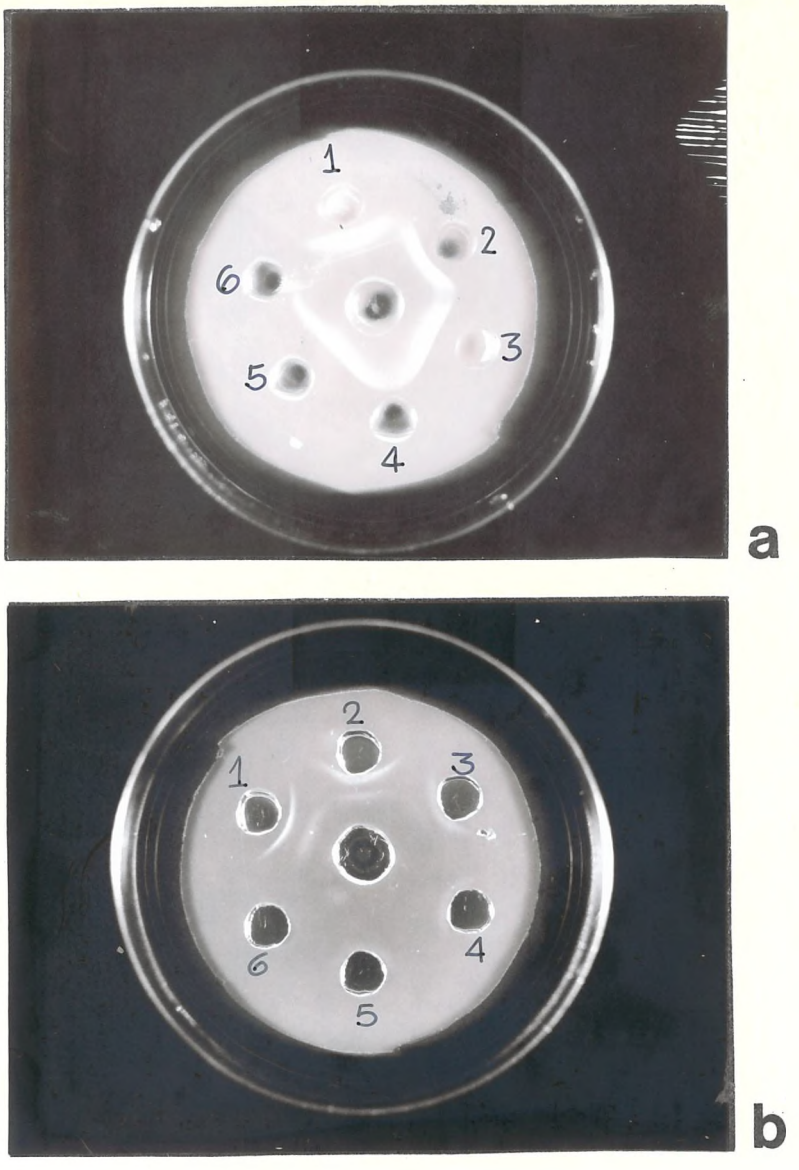


Figure 7-2: Double diffusion patterns obtained after 2 days from reacting (a) 1, LIS-extracted; 2, NP-40-extracted; 3, NaDOC-extracted; 4, Triton X-100 -extracted; 5, SDS-extracted; 6, EDTA-extracted log cell membranes with anti-log membrane serum, and (b) anti-log membrane serum with different dilutions of LIS extract: 1, 1/2; 2, 1/4; 3, 1/8; 4, 1/16; 5, 1/32; 6, undiluted.

dilutions of the LIS extract which had been previously demonstrated to inhibit completely log phase cell cohesion (see section 5.2.7). In this case, 2 main precipitation lines were found, although higher dilutions of the antigen (1/16 and 1/32) failed to react with the antiserum. These results suggested that the serological activity of the LIS extract differed from that of the rest of extracts.

(ii) Indirect immunofluorescent staining of log phase cells

In order to investigate the localization of the antigenic groups in log phase cells which reacted with the anti-log membrane serum, indirect immunofluorescent staining of log phase cells with the antiserum was carried out as described in section 2.9.4.

A bright surface staining was observed on methanol-fixed log phase cells (fig 7-3) indicating surface localization of the relevant antigens. As it was found that a 1/32 dilution of the sheep anti-rabbit conjugate gave a bright non-specific staining, further dilutions were carried out. At 1/64 dilution, the non-specific staining due to the conjugate was eliminated, and controls with a single layer of conjugated appeared unstained under UV light. Cells stained with non-immune serum, showed weak cytoplasmic staining which could not be totally eliminated by diluting the conjugate, but did not show specific surface staining.

7.2.3. Effect of anti-log membrane serum on cell cohesion

The ability of the anti-log membrane serum to inhibit cell cohesion was investigated by assessing the equilibrium particle number obtained after 20 mintes of shaking in the presence of increasing concentrations of anti-serum protein. When log phase cells were shaken in the pre-sence of antiserum, the equilibrium particle number increased

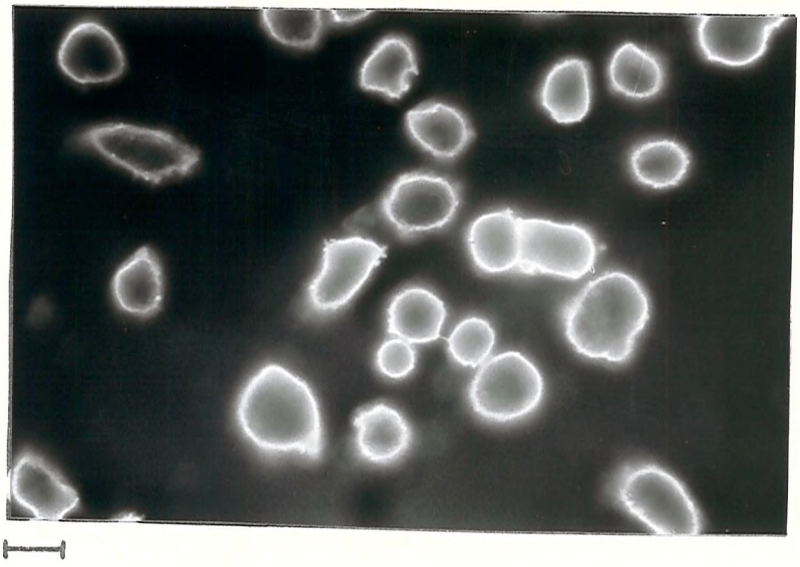


Figure 7-3: Log phase cells fixed and stained with anti-log membrane serum by indirect immunofluorescent techniques (described in section 2.9.3); shown under UV light. Magnification x 700; scale line, 10 μm.

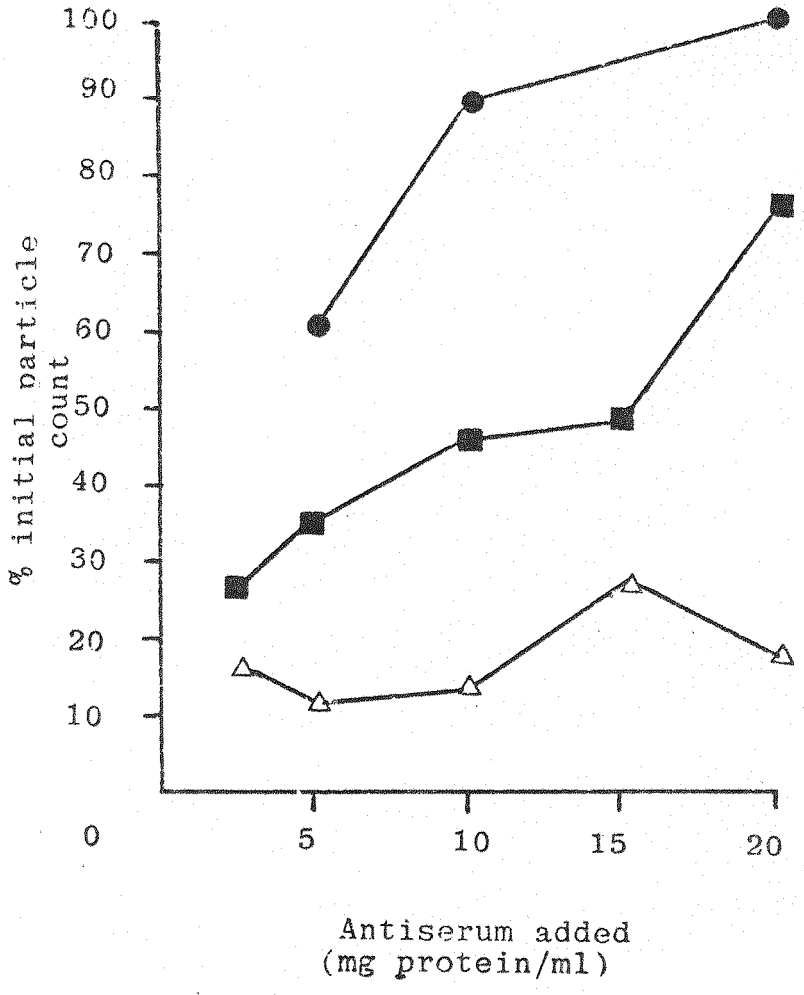


Figure 7-4: Effects of anti-log cell membrane serum on cohesion of log phase cells (■—■) and aggregation-competent cells (●—●), and effect of pre-injection (non-immune) serum on cohesion of log phase cells (△—△). Each point indicates the equilibrium particle count obtained after 20 minutes of shaking in the presence of the indicated concentration of serum protein. They represent the average values from 2 experiments.

as the concentration of antiserum protein increased, and reached an average value corresponding to 80% inhibition of cohesion at 20 mg/ml of added antiserum protein. The cohesion of log phase cells was not affected by the presence of the same concentration of pre-injection (non-immune) serum (see fig 7-4). These results were compared with those obtained when aggregation-competent cells were shaken with increasing concentrations of antiserum and cohesion assessed as with log phase cells. Figure 7-4 shows a maximum average value corresponding to 100% inhibition of aggregation-competent cell cohesion at 20 mg/ml of antiserum protein added to the cell suspension.

The fact that a bivalent antibody was able to inhibit cohesion of both log phase and aggregation-competent cells suggested that the inhibitory effect was not directed against specific sites on vegetative cells. However, the purification of globulins from the antiserum was considered as a possibility to further investigate the inhibitory effects on cohesion.

Figure 7-5 shows the double diffusion pattern obtained when the purified immunoglobulin fraction (obtained as described in section 2.9.6) was reacted against antigens prepared by solubilizing log phase, stationary phase, aggregation-competent and cycloheximide-treated cell membranes as described in section 2.9.3. In each case, the same strong precipitation line obtained with non-purified antiserum (see fig 7-1b) was observed.

When log phase or aggregation-competent cells were shaken in the presence of increasing concentrations of purified globulins, the same inhibitory effect was obtained. No significant differences were detected when both crude and purified antisera were compared for their inhibitory effect on cohesion of log phase or aggregation-competent cells.

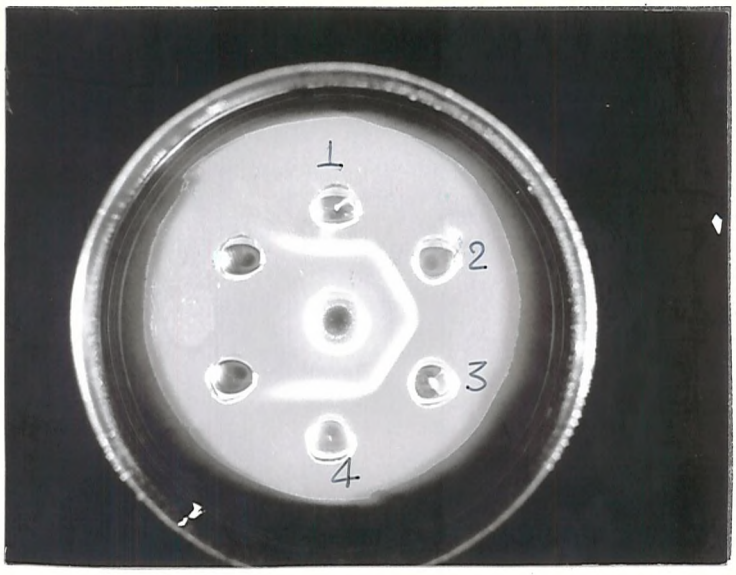


Figure 7-5: Double diffusion pattern obtained after 2 days from reacting a purified globulin fraction obtained from anti-log membrane serum with: 1, log-membrane antigen; 2, stat-membrane antigen; 3, CH-membrane antigen, and 4, AggC -membrane antigen.

7.2.4. Effect of anti-log membrane serum on development

The effect of anti-log membrane serum on development was investigated as described in section 2.9.8. At 20 mg/ml of antiserum protein, development was totally abolished after 24 hours of incubation, whereas with pre--injection (non-immune) serum (20 mg/ml), a mixed non-synchronous population of grexes, migrating slugs and fruiting bodies was observed. Control plates (containing only Millipore solution on the pads, see section 2.9.8) gave a synchronous population of fruiting bodies after 24 hours of incubation. At this time, the pads containing test solutions were replaced by new ones, soaked in Millipore solution, and the Millipores incubated again at 22°C. 17 hours after removing the antiserum, migrating slugs were found. Fruiting bodies were present in those plates which had been pre-incubated with pre-injection serum. Control plates showed fruiting bodies. These results indicated that:

1- at 20 mg/ml, the antiserum had an inhibitory effect on development, and

2- the inhibitory effect was reversible.

7.3. Discussion

The present work was designed to investigate the possible preparation of an antiserum which would react specifically with cell surface antigenic groups involved in vegetative cell cohesion.

According to the ligand-receptor model proposed in chapter 3 for cohesion of vegetative Ax-2 cells, these antigenic groups should be present on the surface of log phase cells, incomplete on the surface of stationary phase cells, and absent on the surface of cycloheximide-tr ated cells. Furthermore, they should contain the inhibitory activity on cohesion of log phase cells demonstrated for the low molecular weight factor from stationary phase cultures (see chapter 3), plasma membranes from log phase cells (chapter 4) and LIS extracts from log phase cell membranes (chapter 5). As it has been suggested in chapter 3, the low molecular weight inhibitor might be the carbohydrate portion of a glycoprotein (the ligand), and the inhibitory activity shown by log cell membranes and LIS extracts on log cell cohesion, might be associated with this glycoprotein. This molecule could in turn be the antigenic group against which an antibody was attempted to be prepared.

The main results obtained are as follows:

1- Antiserum raised against whole freeze-dried log phase cells was not satisfactory since all its activity could be absorbed by stationary phase cells, as judged by cell staining and Ouchterlony test. Cycloheximide-treated cells also appeared to absorb all the activity of the anti-log cell serum.

It should be pointed out, that although Hoffman and McMahon (1978) have reported that cycloheximide-treated cells do not possess contact sites A or B on their surface, no direct evidence has been provided to undoubtly ensure this.

SDS-polyacrylamide gel electrophoresis analysis of plasma membranes derived from cycloheximide-treated cells (Hoffman & McMahon, 1978) have shown a greater number of polypeptide bands that that obtained when vegetative cell membranes were analysed, however, the serological activity of these polypeptides was not demonstrated.

It could be possible that cycloheximide-treated cells, despite being non-cohesive (demonstrated in chapter 4), could be antigenically similar to log phase cells. However, the results obtained with anti-log cell serum indicated that this serum could not be used as a surface marker for specific antigens involved in log phase cell cohesion.

2- A second antiserum, raised against plasma membranes from log phase cells, reacted strongly with a log membrane antigen, stationary membrane antigen, aggregation-competent antigen and cycloheximide membrane antigen. The antigenic group was found to be located on the surface of log phase cells when these were stained by indirect immunofluorescent techniques with the anti-log membrane serum.

3- The LIS extract obtained from log cell membranes, and demonstrated to possess an inhibitory effect on cohesion of log phase cells (see chapter 5), also appeared to contain two major antigens, one of which was present in non-active extracts (SDS, NP-40). The additional antigen on LIS extracts could be associated with the inhibitory activity on cohesion of log phase cells, but direct evidence is still lacking.

4- Anti-log membrane serum inhibited log phase and aggregation-competent cell cohesion, when used at concentrations of 10-30 mg protein/ml as whole antibody. Pre-injection serum by contrast, did not show any inhibitory activity on cohesion of either log phase or aggregation-competent cells when used

at the same concentrations. In addition, a purified globulin fraction obtained from the antiserum, had identical inhibito effects on cell cohesion to those demonstrated for the crude antiserum.

Since Beug et al. (1973a) have shown immunoagglutination of aggregation-competent cells (1×10^7/ml) by addition of 0.2 mg/ml of a purified IgG fraction obtained from anti-aggregation-competent cell serum, the inhibitory effects observed with the anti-log membrane serum and the purified globulin fraction obtained from it, on cohesion of both log phase and aggregation-competent cells, could be attributed to a "covering effect" by which the cohesive sites on the surface of adjacent cells would not be able to promote cell contacts. Additional data on the properties of the anti-log membrane serum (Ellison & Garrod, unpublished) have proved the lack of capping of antisera molecules on the surface of log phase cells. This would also support that the cohesion--inhibitory activity shown in this serum, could be due to the blockage of the specific sites of intercellular adhesion, by covering the cell surface.

5- Anti-log membrane serum was also able to inhibit development of Ax-2 cells on Millipores, when used at concentrations which inhibited cell cohesion in suspension. However, once the antiserum was removed from the Millipore pads, i.e., from the cells, the cells started to differentiate normally. These results could be interpreted to indicate that aggre gation, the first stage of differentiation, could be inhibited for the same reason cohesion in suspension was inhibited (see previous paragraph) and therefore the subsequent steps of development would be inhibited.

Although it was not tested, it would be interesting to show the effect of the antiserum on development, after the aggregation stage . Swan et al. (1977) showed

that the low molecular weight inhibitory factor from stationary phase cultures was able to inhibit development. However, when cells were exposed to the factor after they had reached the aggregation stage, no inhibitory effect on further development was observed. Thus, it would seem that the anti-log membrane serum could mimic the low molecular weight factor in terms of development-inhibitory activity, however, further investigation is required in order to define the nature of this inhibitory activity.

Much evidence is now available to support that some type of contact-mediated cell-cell recognition is required for the continued development of D. discoideum cells beyond the aggregation stage:

a) Cells which are shaken under conditions which prevent the formation of contacts, have been found capable to become aggregation-competent but failed to form pre-spore vesicles (Forman & Garrod, 1977; Garrod & Forman, 1977).

b) Cell contact has also shown to be required for the accumulation of certain developmentally regulated enzymes after the aggregation stage (Grabel & Loomis, 1977).

c) Moreover, it has been demonstrated that the period of propagation of chemotactic waves through fields of aggregating cells changes from 4 minutes to 1.5-2.5 minutes during aggregation. At the same time, the accumulation of cell-bound phosphodiesterase stops and the synthesis of uridine diphosphate glucose pyrophosphorylase begins (Gross et al., 1977). Studies on a mutant which does not undergo these changes suggested the requirement of end-to-end contacts for these changes.

d) It has been found that alkaline phosphatase ,an enzyme which normally accumulates late in development, can be synthesised by cells in suspension and pulsed with cyclic AMP (Rickenberg et al., 1977). Garrod et al. (1978) have suggested that a combination of cell-contact formation

and cyclic-AMP pulsing may be required for continued development after aggregation.

From this evidence it would seem probable that the inhibitory effect of the anti-log membrane serum (and the low molecular weight inhibitory factor) on development, could be due to the inhibition of cell cohesion.

Chapter eight: General Discussion

The most evident fact raised by this work concerns the feasability of trying to reduce cellular behaviour to molecular terms, particularly based on reactions within cells which leads to changes in cell state or behaviour. That is, starting from simple observations on the way D. discoideum vegetative cells behave in axenic medium (Swan & Garrod, 1975), their cohesive behaviour at different stages of growth and development (Swan et al., 1977), in the presence or absence of specific substances which affect their normal cohesiveness (see chapter 3), a ligand-receptor model for the mechanism of cell cohesion has been proposed as a working hypothesis to provide a framework within which the molecular basis of cell cohesion at the earliest phase of the life cycle could be understood and investigated.

According to this model, intercellular adhesion was suggested by interaction between a carbohydrate-binding protein and a glycoprotein, both located at different sites of adjacent cell surfaces. Moreover, the carbohydrate residue of the glycoprotein was suggested to be the low molecular weight inhibitory factor accumulated in stationary phase cultures (Swan et al., 1977; Garrod et al., 1978), thus being the active portion of the molecule to which its receptor (the carbohydrate-binding protein) would complementary bind. As contact sites B have been proposed by Beug et al. (1973a) to be mediators of vegetative cell EDTA-sensitive cohesion, this hypothesis also considered the possibility that contact sites B would be a ligand-receptor system such as that described.

Does the biochemical evidence presented in this work fit with the original model?

1- The fact that plasma membranes isolated from log phase Ax-2 cells were capable of inhibiting completely the cohesion

of homologous cells (see chapter 4), seems to be consistent with the prediction that plasma membranes isolated from those cells should bind to the surface of intact log phase cells by complementary molecular interaction, thus inhibiting cohesion. In addition, plasma membranes isolated from stationary phase, aggregation-competent and CH-treated cells, were found to be less or completely ineffective in terms of inhibition of log phase cell cohesion (see chapter 4). These results are also consistent with the proposed model since it would be expected that plasma membranes isolated from cells which possess an incomplete (stationary), different (aggregation-competent) or absent (cycloheximide) ligand-receptor system, would not be as effective as membranes from cells with the complete system (log phase cells) in inhibiting log phase cell cohesion.

2- Aggregation-competent cell cohesion was demonstrated to be only partially inhibited by log cell membranes (see chapter 4). The extent of inhibition was found to be almost identical to that obtained with the low molecular weight inhibitory factor, shown to be specific against log cell cohesion (see chapter 3). This enphasises the possibility that the inhibitory factor and log cell membranes interact with the same molecular cohesive sites, as predicted by the ligand-receptor model proposed in chapter 3.

3- The inhibitory activity of plasma membranes derived from log phase cells on cohesion of homologous cells has been shown to be completely abolished by pre-treating the membranes with periodate. The carbohydrate nature of the low molecular weight inhibitory factor has been reported by Swan (1978). The fact that periodate eliminates the cohesion-inhibitory activity of log cell membranes, is consistent with the possibility that a carbohydrate could be involved in cell contacts by binding to its carbohydrate-binding receptor, as it was predicted by

the model in chapter 3.

4- The inhibitory activity of log cell membranes could be successfully extracted with lithium diiodosalicylate (LIS), a chaotropic agent which seems to extract glycoproteins preferentially (Robinson & Jenks, 1965). This activity was proved to be chloroform-insoluble. These results emphasised the glycoprotein nature of the cohesion-inhibitory actvity on the surface of log phase cells, and ruled out the possibility of a glycolipid, as it was first proposed in chapter 3.

5- The activity extracted with LIS, was proved to be specific against log phase cell cohesion, heat-stable, periodate-sensitive and possessed a Con A-binding activity. It was also demonstrated that this activity could be absorbed by the surface of log phase cells. These results supported the ligand-receptor system proposed in chapter 3, since the active cohesive components extracted from log phase cell membranes were expected to bind to the surface of intact homologous cells, thus inhibiting cohesion. The glycoprotein nature of at least one of the constituents of the system was confirmed by the periodate-sensitive nature of the extracted activity, also predicted from studies on isolated membranes (see chapters 4 and 5) and by the demonstrated Con A-binding activity (see chapter 6). The similarity between the extracted material and the low molecular weight inhibitory factor in terms of heat stability was also consistent with the proposed hypothesis.

6- The fact that both log cell membranes and the soluble extract obtained from them, showed a specific inhibitory effect on cohesion of vegetative cells and not against aggregation-competent cells, supports the contention that the proposed ligand-receptor model could be related to contact

sites B.

Are other interpretations possible?

It has been suggested that the attainment of stationary phase may not necessarily be due to a depletion of nutrients, but rather to the accumulation of metabolites, such as growth inhibitors (Hänish, 1975; Yarger et al., 1974) and transcription inhibitors (Yarger & Soll, 1975). Assuming that the low molecular weight inhibitory factor accumulated in stationary phase cultures were the end-product of metabolic activity, that is, a metabolite secreted in some way by the cells, it still does seem likely that the decreased cohesiveness of stationary phase cells is due to an alteration of cell surface properties. Rossomando et al. (1974) have demonstrated morphological surface differences between stationary phase cells and log phase cells. Weeks (1973) has shown that log phase cells are more sensitive to Con A agglutination than stationary phase cells. On the other hand, although the role of the inhibitory factor in stationary phase medium still remains obscure, the fact that β-N-acetyl-hexosaminidase, α-glucosidase, α-mannosidase and β-glucosidase are secreted into the growth medium by vegetative cells (Ashworth & Quance, 1972; Every & Ashworth, 1973), supports the possibility that the inhibitory factor could be enzymatically cleaved from cell surface components, causing changes in the cohesive behaviour of the cells.

The only way of determining whether the low molecular weight factor is in fact the carbohydrate portion derived from the hydrolytic cleavage of a glycoprotein on the surface of log phase cells, or a non-specific carbohydrate accumulated in stationary phase medium after being secreted from the cells, would be the chemical identification of the molecule, the identification of the relevant enzymes in axenic medium during the growth phase and the purification

and characterization of the cohesion-inhibitory activity extracted from log phase cell membranes. These molecules should have to be compared in terms of structure and cohesion-inhibitory activity.

Figure 8-1 outlines four possibilities of ligand-receptor models, chosen for reasons of simplicity. In all cases, the low molecular weight inhibitory factor is considered as a carbohydrate accumulated by some mechanism in stationary phase medium.

(i) Homodimer formation

The model shown in figure 8-1 b, predicts that log phase cell cohesion would be mediated by the interaction of two identical bivalent complementary molecules. These molecules would contain both the glycoprotein and the carbohydrate-binding sites. It would therefore be expected that such interaction would be inhibited by inactivation of either of these sites on the same molecule.

If the carbohydrate portion of the glycoprotein site were removed in some way from the surface of the cells during growth, as it was proposed in the original model (see chapter 3 and figure 8-1 a), stationary phase cells would therefore be unable to form cell-cell contacts due to the lack of one of the active sites for cohesion. According to this, stationary phase cells would be capable of forming mutual contacts with both log and aggregation-competent cells which it was found to occur (see chapter 3). Moreover, plasma membranes isolated from log phase cells would possess the ability of inhibiting homologous cell cohesion by complementary binding of identical molecules in the membranes and the surface of the cells. The periodate oxidation of either log cell membranes or the soluble extract obtained from them would inactivate the carbohydrate site of the glycoprotein, allowing log phase cells to stick together in the

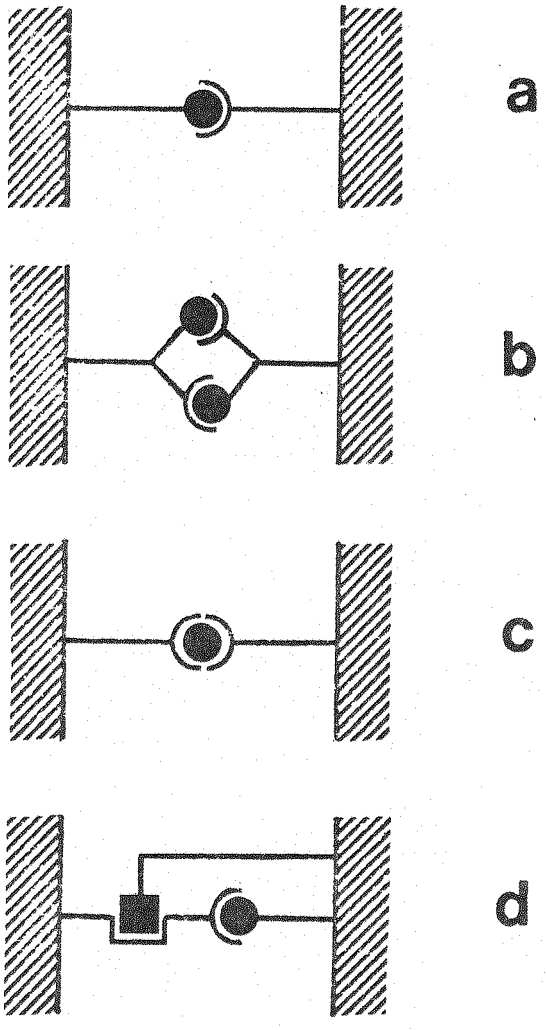


Figure 8-1: Diagram showing alternative ligand-receptor models for cohesion of axenic log phase cells. The membranes of adjacent cells are thought to be held together by a) heterodimer formation; b) homodimer formation; c) interaction with an aggregation factor; d) heterodimer formation regulated by an "activator". —● ligand, —(receptor, ● carbohydrate, —■ activator.

presence of periodate-treated membranes or extract, which they do (see chapters 4 and 5).

However, once extracted from log phase cell membranes, these bivalent molecules could form homodimers, which would reduce or completely abolish the cohesion-inhibitory activity demonstrated when the molecules are attached to membrane vesicles (see chapter 4). It would be reasonable to explain why the minimal inhibitory concentration of log membrane proteins on homologous cell cohesion is 5 times less than that obtained with the LIS extract (see chapters 4 and 5). However, this should also be considered to be reasonable if cell cohesion were mediated by transmembrane heterodimer formation (fig 8-1 a) since complementary binding between single ligand and receptor in solution, could also occur. Therefore, it should be pointed out that the fact that homodimer formation in solution could reduce the fraction of free molecules interacting with their complementary sites on the surface of the cells, and therefore the inhibitory activity of the extract, does not provide evidence to distinguish between models in figure 8-1 a and b.

The fact that after adsorbing the LIS extract obtained from log phase cell membranes with Con A-bound Agarose beads, all the inhibitory activity disappears from the extract (see chapter 6), supports the model in figure 8-1 b. According to this model, both the glycoprotein and the carbohydrate-binding sites are present in the same molecule, so, if the glycoprotein site is allowed to bind to Con A, the carbohydrate-binding site would therefore be indirectly attached to the agarose beads, and no inhibitory species would be left in the extract. On the other hand, if the model in figure 8-1 a were operating, the glycoprotein ligand would be attached to Con A, leaving the complementary receptor in the extract, provided it has been solubilized in an active form. Therefore, after adsorbing the LIS extract to Con A-bound agarose, the extract would still be able to

inhibit log phase cell cohesion by complementary interaction between free receptors and the ligands on the surface of the cells, which it does.

The availability of the purified contact site material would distinguish between models in figures 8-1 a and b.

(ii) An aggregation factor

If log phase cell cohesion were due to the interaction of identical ligands (carbohydrate-binding proteins) mediated by an aggregation factor (a carbohydrate) (see fig 8-1 c), it would be expected to isolate this aggregation factor from axenic medium. As has been suggested by Swan (1978), the concentration of the low molecular weight inhibitory factor in stationary phase medium increases from log to stationary phase where it reaches a maximal value. It could happen that at low concentrations, in the log phase of growth, this factor acts as an aggegation factor, allowing cell-cell contacts to form, but as its concentration in the medium increases, the ligands on the surface of the cells might become saturated, and therefore, the cells would not be able to stick together (stationary phase cells). This model would account for the different cohesive behaviour between log phase and stationary phase cells in axenic medium (Swan & Garrod, 1975) but does not explain the decrease in cohesiveness shown by cells harvested at different stages during growth in phosphate buffer (see chapter 3).

This model seems unlikely, since it seems probable that a glycoprotein is involved in vegetative cell cohesion. It has been demonstrated that log phase cells do not release any aggregation factor when washed in phosphate buffer (see chapter 3). In addition, according to this model, plasma membranes isolated from log phase cells would be expected to

be without effect on homologous cell cohesion, since no aggregation factor would mediate the interaction between the ligands in the membranes and the ligands on the surface of the cells. This was not found to be the case (see chapter 4).

(iii) An activator

A more complicated model like the system shown in figure 8-1 d, makes allowance for the possibility that log phase cell cohesion, mediated by the heterodimer formation between a ligand (glycoprotein) and a receptor (carbohydrate-binding protein), might require the activation by a third component, the activator. If this were the case, log cell cohesion would be inhibited either by inactivation of the recognition sites (ligand and receptor) or the regulatory site (the third component). This model would predict that the free ligand or receptor would inhibit cohesion by competing for their complementary binding sites on the surface of the cells.

It would seem unlikely that once solubilized from the cell membrane, the activator component would maintain its regulatory activity on the surface of intact log phase cells. If this were the case, the inhibition of log phase cell cohesion by solubilized log cell membranes would be limited to the binding of free ligand and/or free receptor to their complementary sites on the surface of the cells.

According to the evidence presented in this thesis, it would be difficult to distiguish this model from those shown in figures 8-1 a and b. Moreover, if the interaction between the activator and its regulatory site were mediated by Ca^{2+} ions, log cell cohesion would be inhibited by EDTA, which it is (Beug *et al.*, 1970; see

chapter 3). Therefore, the parallel inhibitory effects of both EDTA and the low molecular weight inhibitory factor on cohesion of log phase cells (see chapter 3) could be interpreted to indicate the inactivation of the regulatory site by depletion of Ca^{2+} ions, and the inactivation of the receptor site by complementary binding to the inhibitory factor. The fact that the low molecular weight factor is not a chelating agent (see chapter 3) could be interpreted to indicate that EDTA and the factor act on different sites of the same molecule. If this were the case, contact sites B as defined by Beug et al. (1973a) would consist of a three component system containing a recognition site, sensitive to a carbohydrate factor accumulated in stationary phase medium, and a regulatory site, sensitive to EDTA.

What can be suggested as future work?

The evidence presented leads to a number of crucial points which require experimentation in order to understand cell adhesion in slime mould cells at the earliest stage of their life cycle.

1- The chemical nature of the low molecular weight inhibitory factor accumulated in stationary phase cultures would have to be investigated. Preliminary analysis on gas-liquid chromatography (Corina & Garrod, unpublished) have indicated that the partially purified material can be fractionated into 4 fractions. One of these fractions has shown the biological activity against cohesion of log phase cells, but further purification of this fraction has led to the inactivation of the relevant molecule.

In addition, the mechanism by which this factor becomes dettached from the cell surface during the transition from log to stationary phase of growth, has to be considered.

2- The cohesion-inhibitory activity in the LIS extract obtained from log phase Ax-2 cell membranes, will require purification. Once demonstrated that at least one of the molecules involved is a Con A-binding glycoprotein (see chapter 6), Con A binding affinity techniques could be used for that purpose. Its chemical nature, i.e., carbohydrate composition and amino-acid composition, would characterise its structure. Treatment of the purified activity with proteolytic enzymes should demonstrate the role of the protein portion of this molecule in cell cohesion. The identification of the molecule would allow comparisons with other molecules such as discoidin and contact sites A, and will provide information about the role of this molecule in mediating cell contacts at the vegetative stage.

3- The relationship between contact sites B as defined by Beug et al. (1973a) and the ligand-receptor model proposed from the evidence presented in this thesis, has to be investigated. Immmunological studies on the antigenic properties of the extracted inhibitory activity from vegetative cell membranes (see chapter 7) failed to define such relationship.

The immunospecificity of the extracted material could be demonstrated by reacting the purified inhibitory activity with univalent Fab fragments directed against growth phase cell membranes, such as those prepared by Beug et al. (1973 a). If contact sites B and the purified inhibitory activity are identical species, the inhibitory activity would be precipitated by these antibodies, so the remaining antibody would not be able to block cohesion of log phase cells. On the other hand, if purified contact sites B were reacted with Fab fragments directed against aggregation-competent cell membranes (without pre-absorption with vegetative cells) such as those prepared by Beug et al. (1973 a), and then tested with log phase cells, no inhibition of cell cohesion would

be observed because of the absence of contact sites A on the surface of log phase cells. However, if tested with aggregation-competent cells, cell cohesion would be partially inhibited by contact sites B-absorbed Fab, since contact sites B would still be operating on the surface of these cells.

On the other hand, binding studies such as those performed by Beug et al. (1973 b) on contact sites A should provide information about the distribution and amount of contact sites B on the log phase cell surface. This should be approached either by labelling the low molecular weight inhibitory factor or the Fab fragments obtained from an appropiate antiserum against log cell membranes. Electron microscopy analysis of bound material would further demonstrate the localization of these sites.

4- If receptors for carbohydrate-binding proteins like discoidin were to function as contact sites B, it would be expected that Fab fragments obtained against this lectin should inhibit log phase cell cohesion. Furthermore, the haemagglutination activity of the purified LIS-extractable inhibitory activity from log cell membranes should be investigated in order to decide whether contact sites B and discoidin are identical species or have similar funtions. However, if the model proposed in chapter 3 is correct, it would seem very unlikely that contact sites B and discoidin were identical species, since it has been demonstrated that the low molecular weight inhibitory factor from stationary phase cultures does not inhibit the discoidin-induced agglutination of formalinized erythrocytes (Nossiter & Garrod, unpublished). Furthermore, the appearence of native lectins on the surface of several slime mould species occurs only after 4-6 hours of starvation. However, Ax-2 cells accumulate considerable amounts of discoidin in the log phase of growth (Gerisch, 1977). Therefore, if the low molecular weight inhibitory

factor were acting by blocking the lectin system, it might be expected to be without effect on feeding stage cells of D. discoideum NC-4, D. mucuroides, D. purpureum and Ppolysphondilium violaceum. Since these cells are susceptible to both the inhibitory factor (Swan et al., 1977) and isolated plasma membranes from Ax-2 log phase cells (see chapter 4), it seems very unlikely the relationship between discoidin and the proposed ligand-receptor model for vegetative cell cohesion.

5- It would also be very interesting to investigate how the cohesion-inhibitory activity extracted from log phase Ax-2 cell membranes changes during development. A LIS extract from plasma membranes derived from cells at different stages of development should be obtained, and its inhibitory activity on cohesion of log phase cells assessed. With the help of Fab fragments directed aginst the purified activity from log phase cell membranes, the distribution of the relevant antigenic sites could be investigated during development.

6- The role of the inhibitory activity from extracted log phase cell membranes in cell adhesion to the substratum should also be investigated. Preliminary studies have demonstrated that log cell membranes, EDTA and an anti-log membrane serum (see chapter 7) are not able to inhibit adhesion of log phase cells to glass (Nossiter & Garrod; Ellison & Garrod, unpublished). However, inhibiton of log phase cell adhesion to glass has been reported with the low molecular weight inhibitory factor (Swan et al., 1977). According to these results, it would seem that adhesion to glass f llows a different mechanism than cell-cell adhesion. If so, what is the mechanism by which Ax-2 vegetative cells adhere to glass?

7- According to the results obtained with plasma membranes isolated from log phase cells (see chapter 4), the LIS extractable material would also be expected to inhibit cohesion of other slime mould cell species. Therefore, a similar set of experiments should be performed with the cohesion-inhibitory extract in order to investigate if the ligand-receptor model proposed for cell cohesion of Ax-2 cells holds for cohesion between other species.

Implications of the model in relation to morphogenesis

It seems clear that some type of cell-cell -mediated recognition is required for differentiation beyond the aggregation stage of D. discoideum. It has been demonstrated that cell contacts are necessary for the expression of certain enzyme activities involved in the morphogenetic changes leading to the formation of a fruiting body (Gross et al., 1977). Alkaline phosphatase, for example, which normally accumulates late in development, can be synthesised by cells maintained in suspension and pulsed by cyclic AMP (Rickenberg et al., 1977). Cell contacts have also been shown to be required for the formation of prespore vacuoles (Forman & Garrod, 1977; Garrod & Forman, 1977). More recently (Kay et al., 1979) it was demonstrated that cell contacts appear to be a specific requirement for entry into the prespore pathway of gene expression ("Y interaction"), by showing that single cells provided with cyclic AMP, synthesise reduced amounts of UDP-galactose polysaccharide transferase (a prespore-specific enzyme) and normal amounts of glycogen phosphorylase ("pathway-indifferent" enzyme). Pronase was found to inhibit the Y interaction, i.e., the in vitro spore differentiation of mutants capable of spore formation, without preventing chemotactic aggregation or stalk cell formation in the presence of cyclic AMP. The authors have

suggested that this type of interaction "becomes operative shortly after the transition to post-aggregation enzyme synthesis".

On the other hand, cells which are already orientated to aggregation-competence, have demonstrated be dissociated when exposed to the low molecular weight inhibitory factor described by Swan et al. (1977). In the present work, this factor is suggested to have an important role in relation to the ligand-receptor model proposed for vegetative cell cohesion. Furthermore, if aggregated cells are exposed to this factor, morphogenesis is not inhibited and fruiting bodies are formed normally (Swan et al., 1977). It has been suggested (Smart & Tuchman, 1976; Tuchman et al., 1976) that autoclaved membranes derived from aggregation phase cells of D. discoideum cells, strain Ax-3, inhibit chemotactic aggregation and the synthesis of developmentally regulated enzymes. Since the cyclic AMP system is essential for the ordered series of morphogenetic changes which leads to succesful development (Town & Gross, 1978), and the inhibitory factor does not affect these later stages of development, it seems probable that a unique cell-cell interaction must be operating before the aggregation stage is reached. In addition, there is no evidence to suggest that cyclic AMP has an important role in the cohesive behaviour of vegetative cells (Born & Garrod, 1968). The same argument could be applied to explain why aggregation cell membranes, which might contain an additional cohesive system (CS A?) are able to promote inhibition of these later stages of development.

If one assumes that the interaction of a ligand and a complementary receptor on the surface of adjacent cells, such as that proposed in this thesis, may trigger cytoplasmic events leading to differentiation, what is the mechanism for such triggering?, what is the biological

significance of contact sites A appearing at aggregation-competence?, what is the role of contact sites B in stalk/spore differentiation?, why are contact sites B needed in the growth stage of D. discoideum life cycle, where cell-contacts do not appear to occur?. The isolation of the ligand-receptor system would provide a potential tool to answer these questions. Developmental events could then be studied in which the aggregation stage is controlled or completely abolished.

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