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A LIGAND-RECEPTOR MODEL FOR THE COHESIVE BEHAVIOUR OF *DICTYOSTELIUM DISCOIDEUM* AXENIC CELLS

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SUMMARY

Axenically grown cells of *D. discoideum* Ax-2 harvested in the log phase of growth, cohere rapidly when shaken in phosphate buffer. After 3·5 days in the stationary phase of growth, cells become completely non-cohesive. Although they do not stick to each other, stationary phase cells do stick to both log phase cells and aggregation-competent cells. The cohesion of stationary phase cells with these other 2 cell types is inhibited by both EDTA and the low-molecular-weight factor which we have previously demonstrated in stationary-phase growth medium.

There is a decline in the sensitivity of slime mould cell cohesion to the low-molecular-weight inhibitory factor as the cells become aggregation-competent. This effect parallels the developmentally-regulated decline in sensitivity to EDTA. The low-molecular-weight inhibitor is not a chelating agent, however. The effect of the inhibitor seems to be specifically against contact sites-B mediated cohesion. We suggest that the simplest cohesive mechanism which can explain our results, is that the EDTA-sensitive cohesion of log phase cells could be dependent on a ligand-receptor system.

INTRODUCTION

Previous investigations of the cohesive properties of axenically grown cells of the slime mould *Dictyostelium discoideum*, have shown that cells in the log phase of growth are cohesive when gyrated in 17 mM phosphate buffer at pH 6·0, whereas cells in the stationary phase of growth are not cohesive under the same conditions (Swan & Garrod, 1975). Furthermore, growth medium from stationary phase cultures contains a low-molecular-weight factor which completely inhibits the cohesion of log phase cells in phosphate buffer, and partially inhibits the cohesion of aggregation-competent cells (Swan, Garrod & Morris, 1977; Garrod, Swan, Nicol & Forman, 1977).

Gerisch (1961) showed that the cohesive properties of *D. discoideum* cells changed during early development. The cohesion of vegetative cells was inhibited by EDTA, whereas during the acquisition of aggregation-competence (the ability to undergo chemotactic aggregation), EDTA-resistant cohesiveness developed. Immunological studies of the cell surface properties have provided evidence for the existence of 2

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independent cohesive mechanisms, contact sites A and contact sites B (Beug *et al.* 1970; Beug, Katz & Gerisch, 1973). The EDTA-sensitive cohesion of vegetative cells is mediated by contact sites B, and with the development of aggregation competence, EDTA-resistant contact sites A are added. An alternative view is that EDTA-resistant cohesion is mediated by the carbohydrate-binding protein 'discoidin' and its receptor (Rosen & Barondes, 1978). 'Discoidin' and contact sites A appear not to be identical (Bozzaro & Gerisch, 1978; Müller & Gerisch, 1978). We have suggested that inhibition of cohesion by the low-molecular-weight factor found in stationary phase axenic medium may be due to the binding of this factor to contact sites B (Swan *et al.* 1977).

In this paper we further explore the cohesive properties of log and stationary phase cells, and the action of the inhibitory factor. Our results suggest that the cohesion of log phase cells may be mediated by a ligand-receptor mechanism.

MATERIALS AND METHODS

Growth conditions

Cells of the axenic strain Ax-2 of *D. discoideum* were grown in axenic medium, supplemented with 86 mM glucose (Watts & Ashworth, 1970). Log phase cells were harvested when at a density of $1–5 \times 10^6$ cells/ml; stationary phase cells were harvested when the cell count had remained constant at approximately $1–2 \times 10^7$ cells/ml for 3·5 days (at or after point *E* in Fig. 1B), except where otherwise stated. After harvesting, the cells were washed twice by centrifugation with cold distilled water before use.

Acquisition of aggregation-competence

Cells harvested at log phase of growth were suspended in 17 mM phosphate buffer pH 6·0 at a concentration of 1×10^6 cells/ml, and shaken for 8 h in a New Brunswick G86 Rotary Shaker, at 140 rev/min and 22 °C. Cells were then dissociated by washing with cold distilled water and repeated pipetting through a fine-tipped Pasteur pipette before use in cohesion assays.

Cohesion assay

Washed cells were suspended in 17 mM phosphate buffer, pH 6·0, at a final concentration of 1×10^6 cells/ml, and shaken at 140 rev/min. Cohesion was assessed by sampling shaking suspensions at intervals and counting the number of particles per ml. To test the effect of EDTA on cell cohesion, cells were shaken in presence of 10 mM EDTA in phosphate buffer, pH 6·0 and cohesion assayed in the usual way. Before experiments designed to test the effect of the cohesion inhibitor on cohesiveness, a dose–response curve was established in order to select the minimum concentration of product giving complete inhibition of log cell cohesion (see Fig. 3, p. 162). We have defined one unit of inhibitor as that concentration required to give 100% inhibition of log phase cell cohesion, at 1×10^6 cells/ml in phosphate buffer at pH 6·0, under our standard conditions of shaking.

Isolation of the cohesion inhibitor

The method was that of Swan *et al.* (1977). The inhibitory activity was removed from stationary phase medium by dialysis against distilled water over 24 h at 4 °C, and reduced to 3 ml by evaporation under vacuum. The concentrated product was kept frozen and used within 15 days. There was no loss of activity during this time.

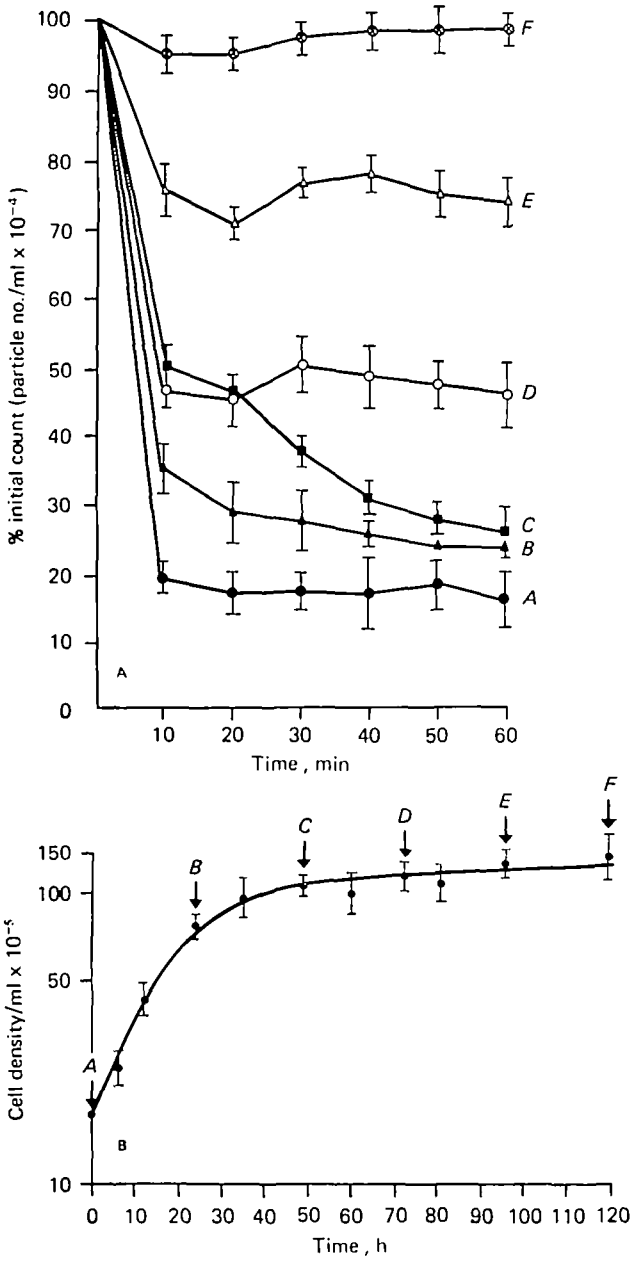

Fig. 1. A. Cohesion in 17 mM phosphate buffer of Ax-2 cells at different stages of growth. The letters *A* to *F* indicate the stages of growth at which the cells were harvested, and correspond to the points labelled on the growth curve in B. The points in this and subsequent figures are shown ± standard deviation.

RESULTS

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

It can be seen from Fig. 1A that log phase cells harvested when at a density of 2×10^6/ml (point *A* in Fig. 1B), cohered rapidly. As growth proceeded into the stationary phase, the cells became progressively less cohesive, until, after about 3·5 days in stationary phase, cohesiveness was lost completely. To be sure of working with non-cohesive populations, the stationary cells used in subsequent experiments were harvested at or after the stage of growth represented by point *E* in Fig. 1B.

Mutual cohesion of log phase and stationary phase cells

Given the above observations and the fact that stationary medium contains a low-molecular-weight inhibitor of log cell cohesion, we investigated whether stationary cells, although non-cohesive themselves, would stick to log cells. Log and stationary cells were mixed in varying proportions and the cohesion curves of the mixed populations were established. The results are shown in Fig. 2.

In each case the cohesion curve for the log-stationary mixtures had the same shape as the curve for log cells alone, and the equilibrium number of particles increased slightly with increasing proportion of stationary cells. However, considering for example, the 1:1 log:stationary mixture, the equilibrium particle concentration was much less than would be expected if the stationary 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 cells which remained single, plus a few aggregates of log cells. Since the equilibrium number was considerably below 50% and since stationary cells did not stick to each other, we concluded that cohesive contacts must have formed between stationary and log phase cells. An alternative possibility would be that log cells release an aggregation factor into the buffer, which promotes cohesion of stationary cells. However, we now report an experiment which excludes this possibility.

Log phase cells at concentrations of 5×10^5, 1×10^6 and 1×10^7 cells/ml were allowed to cohere in shaken suspension, in phosphate buffer for 1 h. The cells were then removed from the buffer by centrifugation, and fresh stationary phase cells were suspended in the supernatants, at 1×10^6 cells/ml. These were returned to the shaker, and their cohesion assessed after 30 min. No cohesion was observed. We concluded that log phase cells did not release an aggregation factor into the medium, and that the results obtained with log-stationary mixtures could, therefore, be explained in terms of mutual cohesion between log and stationary cells.

Mutual cohesion of aggregation-competent cells and stationary phase cells

Dissociated aggregation-competent cells were mixed with stationary phase cells in a 1:1 ratio, and the degree of cohesion in the mixture determined. The results (Table 1) showed that aggregation-competent and stationary phase cells were mutually cohesive.

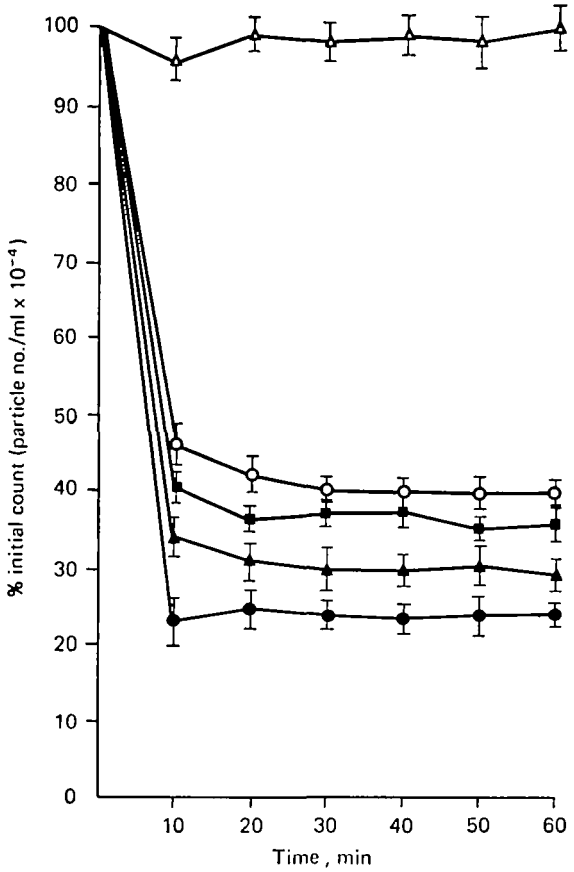

Fig. 2. Cohesion of mixtures of log and stationary phase cells. △, stat:log = 1:0; ○, 3:1; ■, 1:1; ▲, 1:3; ●, 0:1.

Table 1. *Effect of EDTA on cohesion of cell mixtures*

Cell mixture*	Conditions†	Equilibrium particle no.‡
Log + Stat	Control	33 ± 2·1
Log + Stat	10 mM EDTA	98 ± 3·3
AggC alone	Control	18 ± 2·0
AggC + Stat	Control	47 ± 4·2
AggC + Stat	10 mM 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 cells/ml were shaken as described in Methods. Control condition consisted of 17 mM phosphate buffer, pH 6·0; EDTA was used in the same buffer.

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

Effect of EDTA on cohesion of cell mixtures

The effect of 10 mM EDTA on the cohesion of mixtures of stationary phase cells with both log phase and aggregation-competent cells was determined. The results (Table 1) show that in the case of log-stationary mixtures, complete inhibition of cohesion was obtained, whereas with aggregation-competent-stationary mixtures, partial inhibition with EDTA was achieved.

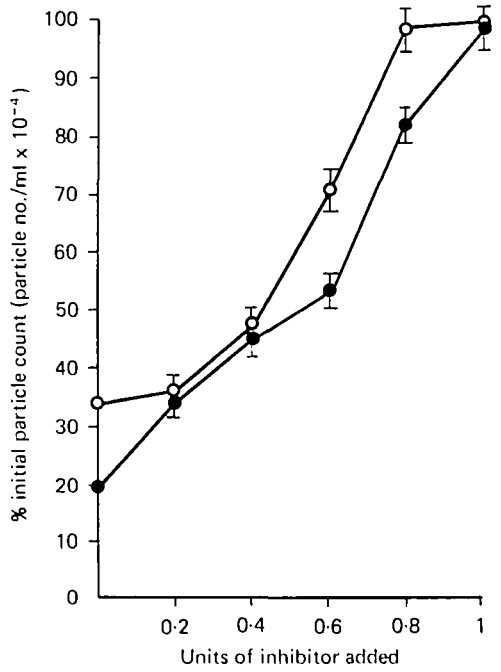

Fig. 3. Effect of increasing inhibitor concentration on cohesion of log cells (●) and log-stationary cell mixtures (○). The points indicate the equilibrium particle numbers obtained after 30 min of cohesion in phosphate buffer with the appropriate amount of inhibitor.

Effect of low-molecular-weight inhibitor from stationary phase medium on cohesion of cell mixtures

The effect of the inhibitor on the cohesion of cell mixtures was studied by assessing the equilibrium particle number obtained after 30 min of shaking in the presence of increasing concentrations of inhibitor.

The results obtained with log cells alone, and 1:1 log:stationary mixtures are shown in Fig. 3. In both cases, the equilibrium particle number increased approximately linearly with increasing inhibitor concentration, and reached a maximum value corresponding to 100% inhibition of cohesion.

The results of similar experiments with aggregation-competent cells and 1:1 stationary phase-aggregation competent mixture are shown in Fig. 4. With aggregation-competent cells alone, less than 50% inhibition of cohesion was achieved, even in

the presence of 1·4 units of inhibitor. In the case of stationary phase-aggregation-competent mixtures, the equilibrium particle number was about 45% of the initial count in the absence of inhibitor, and rose to about 90% at high inhibitor concentrations. However, 100% inhibition was never achieved.

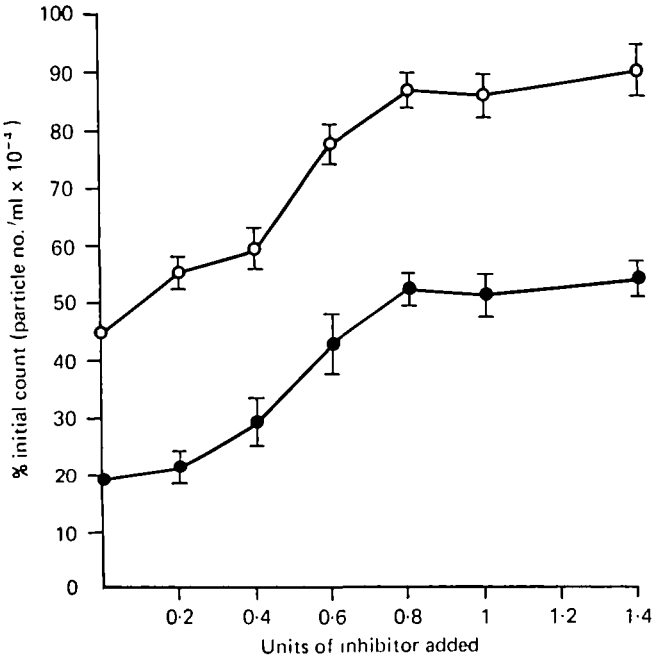

Fig. 4. Effect of increasing inhibitor concentration on cohesion of aggregation-competent cells (●) and aggregation-competent-stationary cell mixtures (○). Equilibrium particle numbers are plotted as in Fig. 3.

Evidence that the inhibitor acts against contact sites B

In the previous section we showed that the cohesion of log phase cells was completely inhibited by our low-molecular-weight factor, whereas with aggregation-competent cells the maximum obtainable inhibition was less than 50%. Thus there would seem to be a parallel between the effect of the inhibitor and the effect of EDTA on slime mould cell cohesion.

In order to pursue this parallel, we carried out experiments in which cells were allowed to develop aggregation-competence by shaking for 8 h in phosphate buffer. Samples were taken at 2-h intervals, the cells dissociated, and their cohesion assessed in the presence of either 10 mM EDTA, or 1 unit of inhibitor, for 20 min. Controls were conducted in phosphate buffer alone. The results are shown in Fig. 5. It can be seen that there was indeed an excellent correspondence between the effects of both agents, a sharp decrease in inhibition occurring as the cells approached the aggregation stage. This suggests that the inhibitor, like EDTA, acts against contact sites B but not against contact sites A.

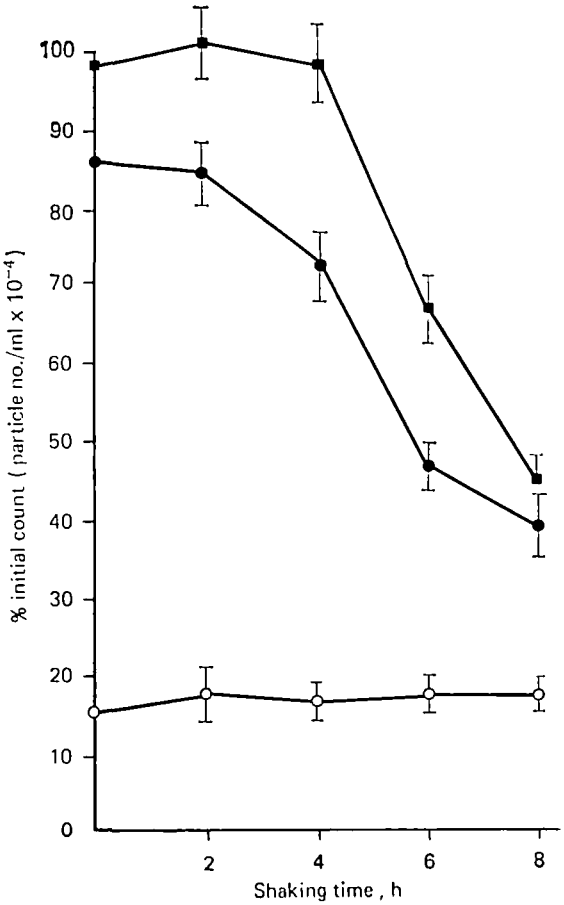

Fig. 5. Comparison of the effects of EDTA and the inhibitor on cell cohesion at different times during the acquisition of aggregation competence. Equilibrium particle numbers obtained after 20 min of shaking in 17 mM phosphate buffer alone (○), in 10 mM EDTA in phosphate buffer (●), and with 1 unit of inhibitor in phosphate buffer (■) are plotted.

Table 2. *Effect of Ca^{2+} on activity of 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+} + 1 u. inhibitor	97 ± 2·0
10 mM Ca^{2+} + 1 u. inhibitor	100 ± 5·0

* In all cases, log phase cells at a final concentration of 1×10^{-6} cells/ml, were shaken at the indicated conditions, according to Methods. Control conditions were as in Table 1; $CaCl_2$ and 1 unit (u) of inhibitor were used in phosphate buffer.

† Obtained as in Table 1. Standard deviations are shown.

These results raise the possibility that the inhibitor might be a chelating agent. In order to investigate this, we attempted to reverse its effect with calcium chloride. As shown in Table 2, complete inhibition of log cell cohesion was obtained with 1 unit of inhibitor, in the presence of 1 mM and 10 mM $CaCl_2$.

DISCUSSION

Although considerable progress has been made in investigating the molecular basis of cohesion of aggregation-competent cells of *Dictyostelium discoideum* (see Müller & Gerisch, 1978), little is known about the cohesion of vegetative cells. Our purpose here has been to analyse the cohesive behaviour of vegetative cells of strain Ax-2 in order to develop a working hypothesis for the study of the molecular mechanism at the earliest phase of the life-cycle.

The main results which we have obtained are as follows: (*a*) Stationary phase cells, although they do not stick to each other, are able to cohere both with log phase and with aggregation-competent cells. (*b*) This cohesiveness and that of log phase cells alone is inhibited by both EDTA and the low-molecular-weight factor from stationary phase medium. (*c*) The effect of the inhibitory factor, like that of EDTA, appears to be specific for the cohesion of vegetative cells. Thus, using the terminology of Beug *et al.* (1970), it seems to act against contact sites B. (*d*) We have previously presented evidence that the inhibitor binds to log phase cells (Swan *et al.* 1977). (*e*) Preliminary analysis of the nature of the inhibitor by thin layer and gas-liquid chromatography suggests that it is a carbohydrate (A. P. Swan, unpublished).

We now propose our working hypothesis which seems to be the simplest possible explanation of these observations.

The EDTA-sensitive cohesion of log phase cells could be dependent on the interaction of 2 different complementary molecules, i.e. a ligand and a receptor. If so, cohesion should be inhibited by inactivation of either of these components.

The fact that stationary phase cells are non-cohesive suggests that the proposed ligand-receptor system becomes inactivated in some way during the transition from log phase. In addition, we have to account for the appearance of the low-molecular-weight inhibitor of log cell cohesion in stationary phase medium. The accumulation of this inhibitor could be a purely fortuitous consequence of cellular metabolism. However, since the inhibitor is a carbohydrate moeity of some description, the simplest interpretation of our results would be that as cells enter stationary phase, the terminal portion of the receptor which normally binds to the ligand on the opposing cell surface in some way becomes detached and is released into the medium. Thus, stationary phase cells would have an incomplete ligand-receptor system and therefore would be unable to stick together. When log and stationary cells are mixed, cohesive interactions would occur between the ligands on the surfaces of stationary cells and the complete receptors of the log cells. The terminal fragment of the receptors released into stationary medium would retain the ability to bind to its complementary ligands. Thus, when added to log phase cells, it would block the

cohesive apparatus by so binding. This terminal fragment of the receptor would be the inhibitor. It is to be expected from this argument, that the inhibitor would block cohesion between log and stationary cells completely, which it does.

The effect of the inhibitor appears to be specific for the cohesive mechanism of log phase cells. There is a developmentally regulated decline in sensitivity to the inhibitor, which parallels the decline in EDTA sensitivity as aggregation-competence is approached. Beug *et al.* (1970), on the basis of their immunological studies, have proposed that EDTA-sensitive cohesion is mediated by contact sites B, and the decline in EDTA sensitivity is due to the acquisition of contact sites A. If our model is correct it would suggest that contact sites B are a ligand-receptor system. The reason why the inhibitor only partially inhibits the cohesion of the aggregation-competent cells could be that they possess 2 cohesive mechanisms only one of which is blocked by the inhibitor. The inhibitor would only partially block their cohesion because it would interact specifically with contact sites B, but would have no effects on contact sites A. Stationary cells could then stick to aggregation-competent cells by interaction between their B-site ligands and the B-receptors of the aggregation-competent cells. The inhibitor would not cause complete inhibition of cohesion in stationary-aggregation-competent mixtures because there would be some residual cohesiveness between aggregation-competent cells due to their contact sites A.

At present there is no direct evidence relating to the chemical nature of contact sites B. However, both systems which have been proposed to account for the cohesion of aggregation-competent cells, i.e. 'discoidin' and contact sites A, have been analysed in some detail. The discoidin system depends upon the interaction of discoidin itself, a carbohydrate-binding protein or lectin, with a receptor which is a glycoprotein (Rosen & Barondes, 1978). Isolation of contact sites A has shown these to be glycoprotein in nature (Heusgen & Gerisch, 1975) and there is some evidence suggesting an interaction between complementary molecules in contact sites-A-mediated cohesion (Gerisch, Beug, Schwarz & von Stein, 1974). Most recently it has been suggested that complementarity may occur between different sites of the same glycoprotein molecule and that cell-to-cell linkage may occur by trans-membrane homodimer formation (Müller & Gerisch, 1978). It is not without precedent, therefore, to propose, as we have done for contact sites B, that slime mould cell cohesion depends upon binding between a ligand and a glycoprotein receptor. We are now attempting to isolate the components of the system and to characterize the inhibitor.

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