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FUNCTIONAL CHARACTERIZATION OF AN ADHESIVE COMPONENT FROM THE EMBRYONIC CHICK NEURAL RETINA

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SUMMARY

We have developed a quantitative assay for tissue-specific adhesive components which is based on the agglutination of glutaraldehyde-fixed cells. At least 2 components are required for fixed-cell agglutination: a cell-surface ligand which is obtained from tissue culture-conditioned medium, and a soluble 'agglutinin' which accumulates in conditioned medium from monolayer cultures. Our results suggest that the surface-binding ligand and the agglutinin interact directly, resulting in tissue-specific agglutination of cells. The agglutination reaction exhibits divalent cation, temperature, and pH dependence. Several models of cell adhesion are described; the simplest of these which can account for the data is a multicomponent model in which the 2 adhesive components have structural roles.

INTRODUCTION

The reaggregation of embryonic chick neural retina cells can be promoted by media which have been conditioned by monolayers (Lilien, 1968; Garber & Moscona, 1972) or tissue cultures of cells of homologous tissue type (Balsamo & Lilien, 1974*a* and unpublished observations). The aggregation-promoting materials of these media interact specifically with homotypic cells and can be adsorbed from the media by the cells (Lilien, 1968; Balsamo & Lilien, 1974*a*). Furthermore, glutaraldehyde can affix adsorbed activity from tissue culture-conditioned medium (hereafter referred to as 'ligand')† to the cell surface (Balsamo & Lilien, 1974*b*). Glutaraldehyde-fixed cells which have ligand bound to them are incorporated into aggregates of living retinal cells. Cells which do not have bound ligand are not incorporated. Fixed ligand-bound cells also agglutinate when they are suspended over a monolayer of living retinal cells, but do not agglutinate in the absence of the monolayer or when placed over a heterotypic monolayer. Cells which have been fixed without ligand do not agglutinate in any of these cases.

These data suggest that homotypic surface-associated ligand is a necessary component for the formation of tissue-specific adhesive bonds, but that alone it is not sufficient to allow such bonds to form. Another component, supplied in the above

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† Ligand has previously been referred to as 'retinal-aggregation-promoting material' (RAPM) (Balsamo & Lilien, 1974*a*, *b*, 1975).

experiments by homotypic living cells, is also necessary. We refer to this component as 'agglutinin',* and have briefly reported on a quantitative assay for its activity (Lilien & Rutz, 1977). The assay is derived from technical improvements in the procedure reported by Balsamo & Lilien (1974*b*), and involves the harvesting of monolayer-conditioned medium (MCM) followed by the addition of a protease inhibitor to the medium in order to preserve the activity of the labile material. As one result of this improvement, the possibility of living cells detaching from the monolayer and then becoming involved in cell agglutination can be excluded.

The use of fixed cells is advantageous, for it allows cell adhesions to be investigated without the interference of endogenous protein synthesis, and without the (possible) stimulatory effects of media components on the *de novo* synthesis of adhesive molecules or on other cell processes. Moreover, since the single cells which are prepared by trypsinization are not immediately agglutinable, it is possible to reconstruct the specific adhesive bond step-by-step, and to observe the effects of various treatments on the acquisition of adhesive capacity by freshly prepared single cells. Experiments which define the parameters of the assay and which address these points are described below. Several models for the specific adhesive mechanism of the embryonic chick neural retina are discussed, in which agglutinin and ligand are assigned roles in light of the experimental results.

Some of these data have previously been reported in preliminary form (Lilien & Rutz, 1977).

MATERIALS AND METHODS

Preparation of conditioned media

Tissue culture-conditioned medium (TCM). Two neural retinae from 10-day-old white leghorn chick embryos were placed in a 60-mm diameter plastic Petri dish (Falcon) in 3·5 ml of complete medium (the equivalent of 0·5 retinae/ml). This medium consisted of Eagle's basal medium (Gibco), to which were added 2% (v/v) non-essential amino acids (100X, Gibco), 1% glutamine (200 mM, Gibco), 0·5% gentamycin (10 mg/ml, Schering), and 2 mg/ml glucose. The cultures were maintained at 37 °C under a moist atmosphere of 10% CO_2:90% air for 48 h on a gyratory shaker at 40 rev/min. Medium was harvested at 24 and 48 h, centrifuged 30 min at 7700 *g*, and pooled. Cerebral lobe TCM was prepared in a like manner, using twelve 10-day cerebral lobe halves per dish in place of the retinae. Conditioned media prepared in this manner contain tissue-specific components (ligands) which bind at the cell surface (Lilien, 1968; Balsamo & Lilien, 1974*a*) and inhibit lectin-induced capping among freshly trypsinized cells (McDonough & Lilien, 1975*b*). Activity (10 μg of protein/ml for half-maximal binding to 1×10^6 cells) and protein concentration ($21·4 \pm 1·3$ μg/ml; ± S.E.M., $n = 7$) show little variation from preparation to preparation.

Concentrated TCM was prepared by ethanol precipitation. To pooled TCM (10 ml), 0·21 ml of 0·2 M phosphate buffer, pH 7·6, 0·005 ml of 1 M $MgCl_2$, and 7 ml of 95% ethanol were added. After 24 h at −20 °C the precipitate was collected by centrifugation at 7700 *g* for 30 min and redissolved in 0·1 M ethylenediaminetetra-acetic acid (EDTA), pH 6·0, in one-tenth of its original volume. Insoluble material was removed by centrifugation, and the redissolved material was dialysed against Tyrode's solution, pH 7·0, to remove the EDTA. The protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

* Agglutinin has previously been referred to as 'ligator' (Balsamo & Lilien, 1974*b*).

Enzyme-digested TCMs were prepared in the following manner. In one preparation, immobilized protease (Enzite Agarose; Miles) was used at 0·1 u./ml of TCM and incubated at 37 °C, pH 7·0, for 3 h. The enzyme was removed by centrifugation; no residual protease activity was detected. For removal of the terminal sugar from the ligand's oligosaccharide moiety (see Balsamo & Lilien, 1975), purified β-*N*-acetyl-hexosaminidase (*Turbo cornutus*; Miles) was dissolved in 50 mM sodium citrate, pH 4·5, and combined with TCM in a 1:1 ratio (pH was readjusted to 4·5 with HCl). The final concentration of enzyme was 0·10 u./ml of digestion mixture. The mixture was incubated at 30 °C for 30 min, after which the pH was readjusted to 7·2 with NaOH, inactivating the enzyme (Muramatsu, 1968).

Monolayer culture-conditioned medium (MCM). Single cells (see below) were resuspended in complete medium at a concentration of $4-5 \times 10^6$/ml, and were aliquoted into 60-mm diameter plastic tissue culture dishes (Falcon), 8 ml/dish (the equivalent of 0·05 retina/ml). The monolayers were incubated for 24 h without shaking at 37 °C under an atmosphere of 10 % CO_2 in air. The MCM was harvested and centrifuged 30 min at 7700 *g*. Phenylmethylsulphonylfluoride (PMSF), a serine protease inhibitor which binds irreversibly (Fahrney & Gold, 1963), was dissolved in isopropanol and then added to MCM (final concentration of 2 mM), and the MCM was incubated at 37 °C for 30 min (allowing the PMSF to dissolve). MCM which was prepared in this manner supported agglutination, and was designated 'active MCM'. Activity of these preparations also shows little variation with a protein content of $17·0 \pm 1·6$ μg/ml ($n = 7$). Active MCM could be stored at 4 °C for at least 7 days without loss of activity. Identical MCM preparations which were incubated at 37 °C for an equivalent period without PMSF and then stored at 4 °C without PMSF could not support agglutination, and were designated 'inactive MCM'. (The activity present in MCM will be referred to as a single molecular entity, agglutinin. It should be understood that agglutination activity may reside in more than one molecular species, or that activity may depend on a multimeric complex.) Concentrated MCM was prepared by pressure filtration against an Amicon PM-30 membrane.

Preparation of single cells

Three neural retinae were dissected into warm Tyrode's solution, pH 6·5. The tissues were then transferred to a 10-ml Erlenmeyer flask, washed 3 times with 3 ml of warm calcium- and magnesium-free Tyrode's solution (CMF), pH 7·2, and incubated for 10 min at 37 °C in 3 ml of CMF on a gyratory shaker at 40 rev/min. Following this, the tissues were washed with 3 ml of 0·01 M *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (HEPES)-buffered saline (0·15 M NaCl) with glucose (1 mg/ml) (HBSG), pH 7·2 and incubated for 20 min in 2 ml of HBSG containing 4000 NFU/ml of trypsin (3 times crystallized, Miles) at 37 °C and rotated at 40 rev/min. Subsequently, the tissues were washed 3 times with Tyrode's solution, pH 6·5, after which 2 ml of Eagle's basal medium, containing 50 μg/ml of DNase (NBC, crystalline), were added. The tissues were dissociated by flushing through a Pasteur pipette flamed to an opening of approximately 0·5 mm diameter. The suspension was then centrifuged again. The resulting cell pellet was treated and fixed as described below. Cerebral lobe cells from 10-day embryos were prepared in the same manner, except that the lobes were cut into quarters before the CMF wash, tissue received 2 CMF incubations of 20 min each, and the incubation in trypsin lasted 30 min. The cell yields were 8×10^7 cells/retina and 2×10^7 cells/cerebral lobe.

Test cell populations

Ligand-free cells (fixed freshly-trypsinized cells). Single cell pellets prepared as described above were resuspended in 2 % glutaraldehyde (70 % glutaraldehyde, Ladd) in 0·01 M sodium phosphate-buffered saline (0·12 M NaCl), pH 7·2, at a concentration of $40-80 \times 10^6$ cells/ml and held at 4 °C for 30 min. The cells were then centrifuged (200 *g*, 5 min), resuspended in 2 ml of 0·2 M glycine in water, and incubated for 10 min at room temperature. The cells were then centrifuged for 5 min at 200 *g*, washed once in 0·2 M glycine, and then 4 times in 0·01 M phosphate-buffered saline (0·15 M), pH 7·2, and resuspended in Tyrode's solution, pH 7·0. The cells were counted using a Coulter counter.

Ligand-bound cells. Freshly dispersed single cells were resuspended in TCM, pH 6·5, at a concentration of 20×10^6 cells/ml, and incubated for 10 min at 37 °C. The cells were then centrifuged (200 *g*, 5 min) and fixed as above.

Repaired cells. Single cells were resuspended in complete medium and aliquoted into 60-mm diameter plastic Petri dishes, 40–80 × 10^6 cells/dish, in 4 ml of complete medium, and were incubated for 4 h at 37 °C. The medium usually contained 10 μM UDP (Sigma) (UDP has been shown to inhibit the release of surface-associated ligand (McDonough & Lilien, 1977)). After 4 h, the cultures were chilled by placing them on ice, DNase (25 μg/ml) was added, and the cells were resuspended by flushing gently with a Pasteur pipette; they were then centrifuged and fixed as above.

Agglutination assay

The assay was conducted using 35-mm plastic Petri dishes (Falcon), with 1 ml of 1 % agar (Difco) in Tyrode's solution covering the bottom to prevent any adhesion to the dish. Into each dish was added 2·9 ml of test medium. While the dishes rotated at 70 rev/min on a gyratory shaker, 0·1 ml of Tyrode's solution containing 1 × 10^6 fixed cells was added. The cultures were maintained under a moist atmosphere, buffered by CO_2 to pH 7·2, and incubated for 18 h or more at 37 °C and 70 rev/min. At the end of the incubation the contents of each culture dish were poured into 5 ml of Tyrode's solution, and the dishes rinsed with 2 ml of Tyrode's solution for a total volume of 10 ml each.

The amount of agglutination was determined by counting the number of single cells remaining in suspension, utilizing a Coulter counter. Percent agglutination was computed as follows:

$$\frac{10^6 - \text{no. of cells in experimental}}{10^6} \times 100.$$

RESULTS

Optimization of assay conditions

The agglutination of ligand-bound, glutaraldehyde-fixed neural retinal cells, as originally described by Balsamo & Lilien (1974*b*), required the presence of a monolayer of living retinal cells during the assay. It has since proved possible to dispense

Table 1. *The effect of PMSF and isopropanol on agglutinin activity*

Additions to MCM after collection from monolayers (final concentrations)	Test cell type*	% agglutination†
None	LB	6·4 ± 0·70
None	LF	6·8 ± 0·50
1 % (v/v) isopropanol	LB	7·3 ± 0·82
1 % (v/v) isopropanol	LF	6·6 ± 0·46
2 mM PMSF in 1 % isopropanol	LB	29·1 ± 0·54
2 mM PMSF in 1 % isopropanol	LF	6·3 ± 0·45
2 mM PMSF in 1 % isopropanol added to inactive MCM just prior to assay	LB	6·2 ± 0·96
2 mM PMSF in 1 % isopropanol added to inactive MCM just prior to assay	LF	6·7 ± 0·28

* LB, ligand-bound cells; LF, ligand-free cells (see methods).
† Mean ± S.E.M. (n = 5).

with the monolayer in the assay by substituting monolayer-conditioned medium (MCM) to which has been added the protease inhibitor PMSF (see methods). PMSF protects the agglutination-promoting activity (agglutinin), but does not reactivate MCM to which it has not been added immediately after collection (Table 1). Isopropanol, the solvent for PMSF, has no effect at the final concentration used.

Ligand-bound fixed cells, when assayed over monolayers of living cells, agglutinate above background levels only when they are over monolayers of homologous tissue type (Balsamo & Lilien, 1974*b*). The same specificity can be demonstrated with MCM

Table 2. *Tissue-type specificity of agglutination*

Cell type which produced the MCM	Test cell type*	Test-cell tissue-type	% agglutination†
Neural retina	LF	Retina	6·7 ± 0·34
Neural retina	LB	Retina	29·2 ± 0·33
Neural retina	LF	Cerebrum	6·2 ± 0·45
Neural retina	LB	Cerebrum	7·2 ± 1·02
Cerebral lobe	LF	Retina	6·6 ± 0·65
Cerebral lobe	LB	Retina	6·1 ± 0·78
Cerebral lobe	LF	Cerebrum	7·2 ± 0·30
Cerebral lobe	LB	Cerebrum	25·5 ± 0·60

* LB, ligand-bound cells, prepared using TCM from the *homologous* tissue type. LF, ligand-free cells.
† Mean ± S.E.M. ($n = 5$).

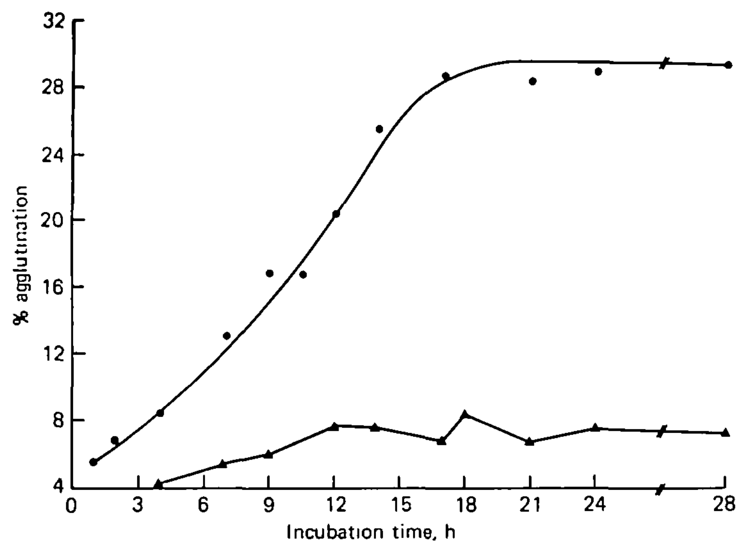

Fig. 1. Time course of agglutination. Ligand-bound cells, ●; ligand-free cells, ▲. Data points are means ($n = 2$).

(Table 2): fixed cells from the neural retina or cerebral lobe agglutinate only when assayed in medium conditioned by cells of homologous tissue type. Heterologous combinations result in agglutination only to background levels, as do heterologous or homologous combinations using ligand-free cells.

Fixed cells agglutinate with a characteristic time course (Fig. 1), maximal agglutination being achieved by 18 h of incubation. Fig. 2 shows the effect of incubation temperature on agglutination. Above 30 °C, a maximal level of agglutination is

attained, with about 28% of the single cells being incorporated into agglutinates. The percentage of agglutinated cells declines steadily with decreasing temperature below 30 °C, reaching background levels below 10 °C. Note that background agglutination with ligand-free cells is largely independent of time (beyond 10 h of incubation, Fig. 1) and temperature (Fig. 2), remaining at 6–8% agglutination throughout.

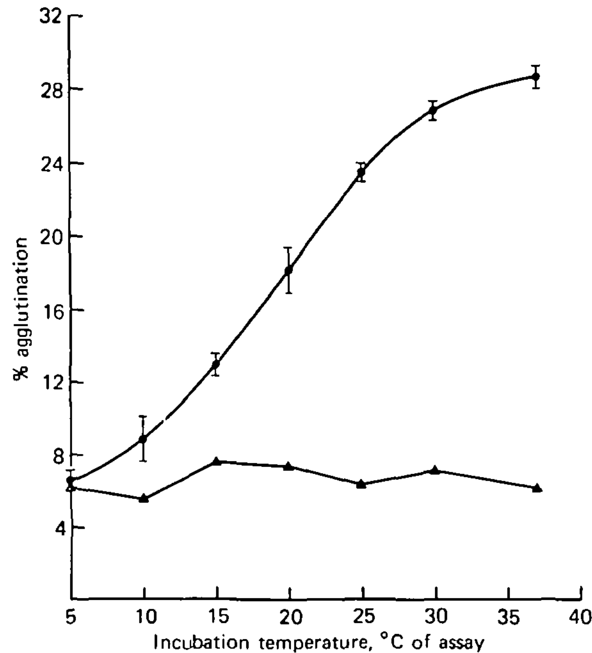

Fig. 2. The effect of assay incubation temperature on agglutination. Ligand-bound cells, ●; ligand-free cells, ▲. Data points are mean values ± S.E.M. ($n = 4$).

Fig. 3 shows that agglutination is affected by pH variations. Agglutination in active MCM increases gradually from pH 6·4 to 7·2; above pH 7·3 agglutination decreases rapidly to background levels. The effect of pH on the stability of MCM was also determined by first dialysing active MCM against buffers ranging in pH from 5 to 9, and then against pH 7·2. The data show that agglutinin is most stable at pH's between 6·0 and 8·0 (Fig. 4).

Salt concentrations in the range from 0·01 to 0·5 M and rotation speeds from 60 to 80 rev/min do not result in any measurable differences in the extent of agglutination; salt concentrations above 0·5 M bring about reductions in agglutination levels (data not shown).

Divalent cations are necessary for agglutination. Approximately half of the activity of MCM is lost after dialysis against buffer lacking divalent cations, and the remaining activity is lost after treatment with chelating agents, either during dialysis or during assay. Most of this loss can be restored by adding back the divalent cations during the assay (Table 3). Chelators do not irreversibly inactivate fixed cells: when fixed cells are treated with chelators prior to assay, but are assayed in standard MCM, they

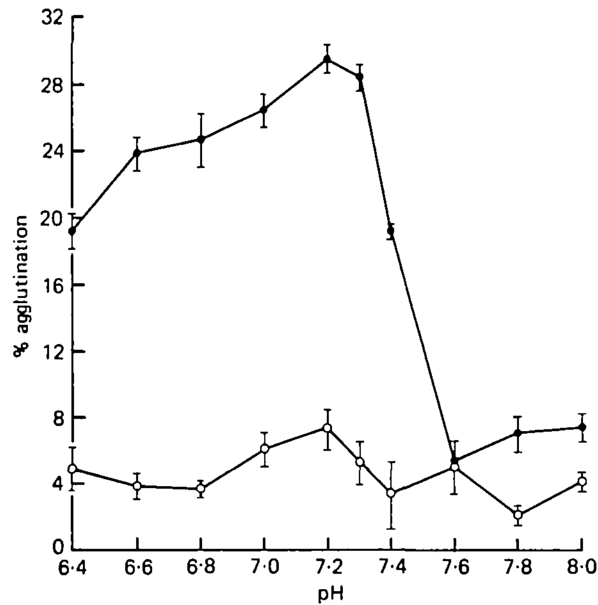

Fig. 3. Agglutination in assay media of different pH. Ligand-bound cells in active (●) and inactive (○) MCM. Means ± S.E.M. (n = 3).

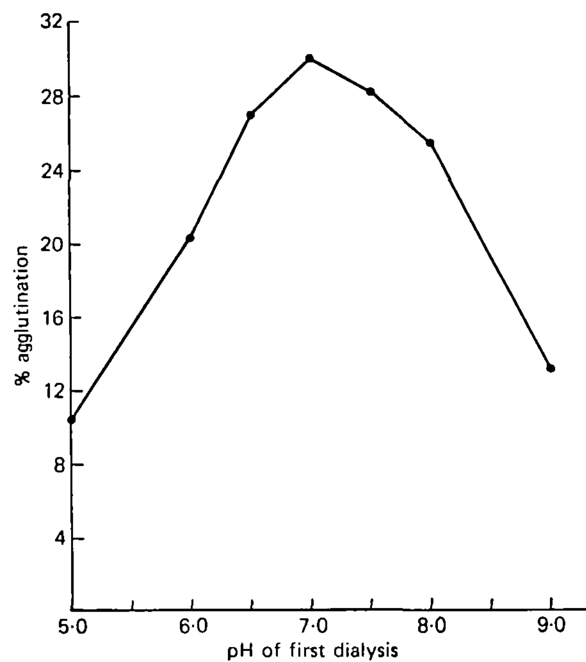

Fig. 4. Stability of agglutinin at various pH. MCM was dialysed against 0·01 M HEPES-buffered saline (0·15 M NaCl) with 1·8 mM $CaCl_2$ and 0·8 mM $MgCl_2$; pH was as indicated. Medium was then dialysed against the same buffer at pH 7·2, and assayed with ligand-bound cells. All dialyses were performed at 4 °C for 12 h. Data points are means (n = 2).

agglutinate at normal levels. A requirement for cations is thus established, although it cannot be determined at this time whether the cations are involved in the interaction between agglutinin and the fixed cells or are necessary for maintaining agglutinin activity.

Table 3. *The effect of chelators on agglutination**

Assay medium		% agglutination of ligand-bound cells‡
Dialysis treatment†	Additions during assay	
Twice *vs.* HBS	No cations	14·8
Same	0·5 mM EGTA	9·9
Same	0·5 mM EDTA	5·0
Same	1·8 mM $CaCl_2$ + 0·8 mM $MgCl_2$	26·2
Same	0·5 mM EGTA + 2·6 mM $CaCl_2$	24·1
Once *vs.* HBS with 0·5 mM EDTA, then twice *vs.* HBS alone	No cations	6·1
Same	0·5 mM EDTA	5·5
Same	1·8 mM $CaCl_2$ + 0·8 mM $MgCl_2$	25·6

* Cells that were treated with 0·5 mM EDTA during phosphate-buffered saline washes following fixation (see methods) gave 27·5 % agglutination.

† The material subjected to dialysis treatment was 100 % active MCM. Each dialysis was conducted at 4 °C for 12 h against the indicated buffers. HBS, 0·01 M HEPES-buffered saline (0·15 M NaCl), pH 7·2.

‡ Mean (n = 2).

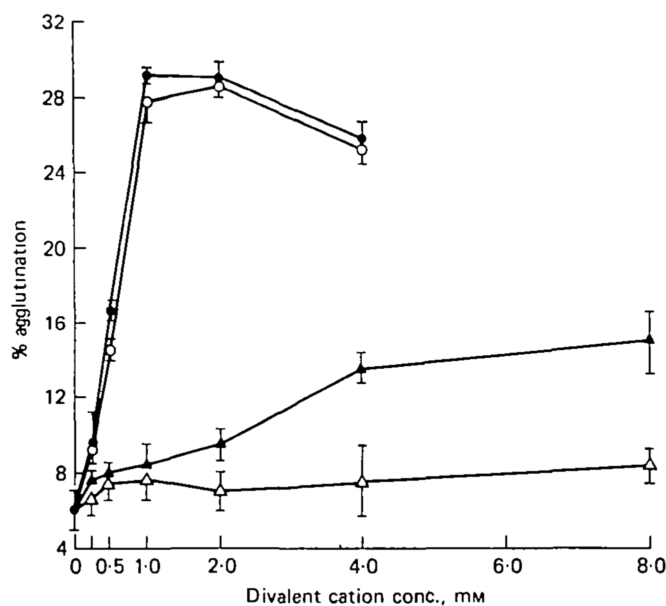

Fig. 5. The effect of divalent cation concentration on agglutination. MCM was dialysed once *vs.* HBS, pH 7·2, without divalent cations but with 0·5 mM EDTA, and then twice *vs.* HBS alone. Divalent cations were added to the MCM at the indicated concentrations prior to assay with ligand-bound cells. Active MCM: $CaCl_2$, ●; $MgCl_2$, ○; $MnCl_2$, ▲. $CaCl_2$ in inactive MCM, △. Means ± S.E.M. (n = 3).

The divalent cation requirement can be met equally well by calcium or magnesium ions in the range of 1–4 mM (Fig. 5). Below this range for either ion, agglutination declines rapidly to background levels. The optimal range includes the concentrations of these ions which are found in Earle's salts (1·8 mM Ca^{2+}, 0·8 mM Mg^{2+}). Manganese ions substitute poorly for calcium or magnesium even at 8 mM, and inactive MCM will not support agglutination above background levels in the presence of any of the tested concentrations of calcium ions.

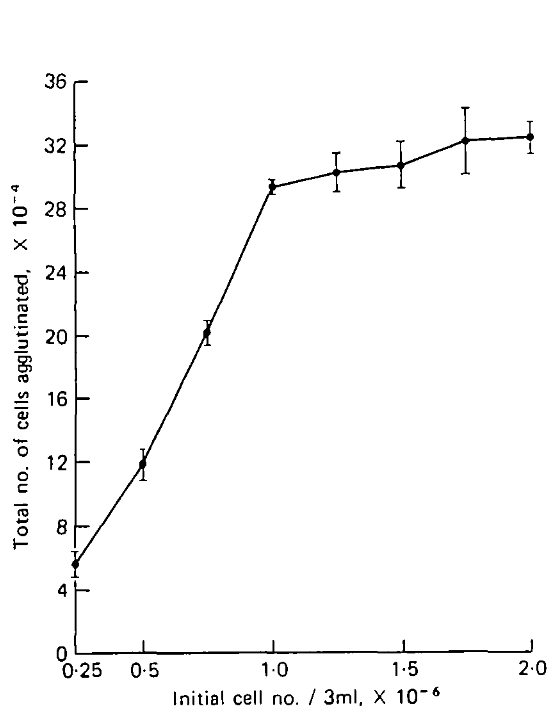

Fig. 6

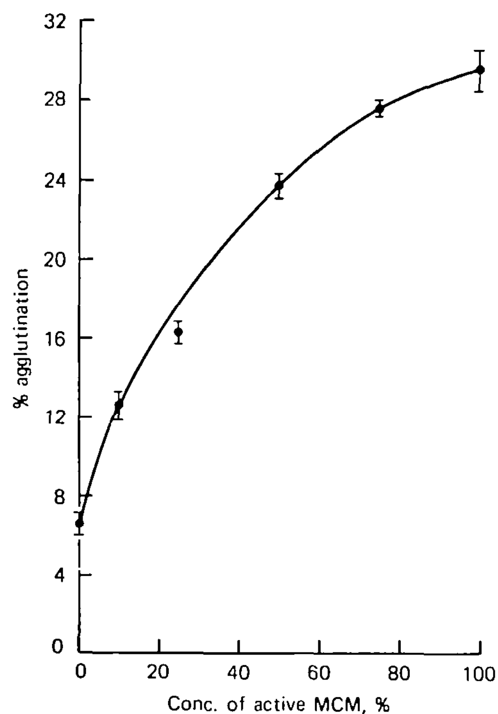

Fig. 7

Fig. 6. Titration of MCM with ligand-bound cells. The indicated number of ligand-bound cells were added to 3 ml of active MCM. Means ± S.E.M. ($n = 6$).

Fig. 7. Quantitative response of agglutination to different initial concentrations of active MCM. Active MCM was combined with inactive MCM (see Methods) in the indicated ratios, and the PMSF concentration was adjusted to 2 mM. The assays were conducted with ligand-bound cells. Means ± S.E.M. ($n = 10$).

Agglutination is dependent in part on the cell concentration in the assay (Fig. 6). Below 1×10^6 cells agglutination decreases while above this number little further agglutination is seen. The extent of agglutination is also a function of the concentration of active MCM (Fig. 7), with the percent agglutination reaching a plateau between 80 and 100% active MCM: concentrations greater than 100% MCM (concentrated MCM; see Materials and methods) yield no additional agglutination. This relationship is consistent from preparation to preparation.

Limits of agglutinability

Two considerations indicate that the upper boundary of approximately 30% agglutination is not due to limiting concentrations of MCM: (1) there is no increase in agglutination at MCM concentrations exceeding 100%, and (2) at cell concentrations below 1×10^6 cells, where MCM is clearly in excess, the number of agglutinated cells decreases.

Table 4. *Unagglutinated cells do not agglutinate when reassayed in fresh MCM**

Test cell type†	No. of times assayed	% agglutination‡
LF	First use	6·5
LB	First use	29·0
LF	Re-use	5·6
LB	Re-use	7·2

* At the end of a 24-h assay period, unagglutinated cells were separated from agglutinated cells by filtering through NITEX P mesh (Tetko, Inc.). The single cells (verified by microscopy) were then reassayed in fresh MCM at 1×10^6 cells/3 ml MCM.

† LF, ligand-free cells; LB, ligand-bound cells.

‡ $n = 1$.

Table 5. *Fixed-cell activity is stable over time in MCM**

Pre-incubation before assay, h	Pre-incubation medium	% agglutination of ligand-bound cells†
None	—	24·6
10·5	Tyrode's solution	14·3
10·5	Complete medium‡	24·8
10·5	Inactive MCM	25·7
24	Tyrode's solution	7·9
24	Complete medium	23·6
24	Inactive MCM	25·2
24	Tyrode's solution + lysine and glycine§	23·7

* Approximately 40×10^6 ligand-bound cells were pre-incubated in 4 different media at 37 °C for the time periods indicated. The cells were then assayed in a 1:1 ratio of active and inactive MCM.

† Mean ($n = 2$).

‡ Eagle's Basal Medium plus additives (see methods).

§ 29·2 μg lysine and 7·5 μg glycine per ml of Tyrode's solution. The amino groups of these amino acids have been shown to react with the free aldehyde groups of glutaraldehyde (see Hopwood, 1972).

The upper limit of agglutination is related to the heterogeneity of the fixed cell population. Cells which have not agglutinated during an assay also do not agglutinate above background levels when they are reassayed in fresh MCM (Table 4). This unagglutinable population is not generated by a loss of cell agglutinability during the

assay (Table 5). When cells are held overnight in PBS or Tyrode's solution, there is a gradual loss of agglutinability. However this loss is not observed when cells are held overnight in inactive MCM, Eagle's basal medium, or Tyrode's solution to which lysine and glycine are added (at concentrations which are found in Earle's salts).

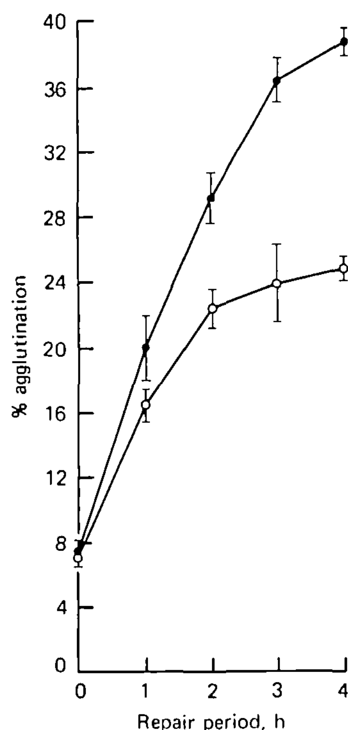

Fig. 8. The effect of UDP on the agglutinability of repaired cells. Cells which were repaired in media containing 10 μM UDP, ●; in media without UDP, ○. Means ± S.E.M. (n = 4).

Fresh, trypsin-dispersed cells which have been allowed to repair trypsin damage for 4 h in culture (see Methods) before fixation agglutinate at somewhat lower levels than do ligand-bound cells (Fig. 8). However, cells which are repaired in the presence of 10 μM UDP (which inhibits the release of a cell-surface ligand that inhibits capping of lectin receptors and mediates agglutination (McDonough & Lilien, 1977)) display a significantly higher percent agglutination than either of the above cell types.

Are there different activities in MCM and TCM?

Differences in the activities detectable in MCM and TCM were further investigated in 2 ways. First, TCM supernantants were treated with PMSF immediately after collection and centrifugation, pooled, and tested in the agglutination assay in place of MCM. TCM does have detectable agglutinin activity (Table 6), but much less than does MCM. Interestingly, TCM which is prepared in the presence of UDP has more agglutinin activity than when UDP is omitted. This suggests that at least some

agglutinin activity is present in TCM, but that it may be masked in TCM produced without UDP. Second, MCM in turn was tested for the presence of ligand by assaying its ability to inhibit lectin-induced capping among freshly dispersed cells as described for TCM by McDonough & Lilien (1975*a*, *b*). By this criterion MCM has less than 20% of the activity that is observed for TCM (data not shown).

Table 6. *Agglutinin activity in MCM and TCM*

Assay medium*	Test cell type†	% agglutination‡
MCM – PMSF	LF	5·7
MCM + PMSF	LF	7·2
MCM – PMSF	LB	7·4
MCM + PMSF	LB	29·3
MCM (with UDP) – PMSF	LF	5·8
MCM (with UDP) + PMSF	LF	6·3
MCM (with UDP) – PMSF	LB	7·1
MCM (with UDP) + PMSF	LB	29·7
TCM – PMSF	LF	6·0
TCM + PMSF	LF	7·0
TCM – PMSF	LB	6·8
TCM + PMSF	LB	11·3§
TCM (with UDP) – PMSF	LF	8·0
TCM (with UDP) + PMSF	LF	6·6
TCM (with UDP) – PMSF	LB	6·9
TCM (with UDP) + PMSF	LB	16·0§

* MCM – PMSF, inactive MCM; MCM + PMSF, active MCM. TCM + PMSF was prepared by adding PMSF to each 24-h harvest immediately after collection and centrifugation. MCM and TCM with UDP were prepared by including 10 μM UDP in the culture medium.

† LB, ligand-bound cells; LF, ligand-free cells.

‡ Mean ($n = 2$).

§ 11·3 corresponds to about 8% of the agglutinin activity which is found in MCM; 16·0 corresponds to 20% of the activity of MCM (see Fig. 7).

Mechanism of agglutinin action

Is agglutinin a structural component of the adhesive bonds formed during agglutination? This question was approached by determining whether agglutinin activity is removed from MCM by fixed cells. MCM was preadsorbed for 18 h at both 37 and 4 °C with ligand-bound and ligand-free cells. The experiment summarized in Table 7 indicates that ligand-bound cells do adsorb considerable (roughly half: *cf.* Fig. 7) activity from MCM at 37 °C, but not at 4 °C. Moreover, ligand-free cells do not remove activity from MCM at either temperature.

Do ligand and agglutinin interact directly? If the association of surface-bound ligand and free agglutinin be direct, ligand which is free in solution should be able to compete with surface-bound ligand for the agglutinin. This competition should result in decreasing levels of agglutination as the concentration of TCM protein is increased. Alternatively, if the interaction be through another cell-surface moiety, one would predict that ligand free in solution should have no effect on the functional attachment

Table 7. *Preincubation of MCM with ligand-bound cells removes agglutinin activity**

Temperature of pre-incubation, °C	Absorbing cell type in pre-incubation	% agglutination of ligand-bound cells†	% of activity found in unabsorbed MCM‡
No preincubation	25 % active MCM control	17·6	90
4	Ligand-free	17·9	92
4	Ligand-bound	17·7	91
37	Ligand-free	18·8	100
37	Ligand-bound	13·4†	54

* Active MCM was added to inactive MCM (1:1 ratio) and the mixture was pre-incubated with 1×10^6 cells per 3 ml under the conditions indicated for 24 h. Cells were removed by centrifugation, and the media were diluted 1:1 with inactive medium (for a final concentration of 25 % active medium). The media were then assayed at 37 °C using 1×10^6 ligand-bound cells per 3 ml. Where active MCM was diluted with inactive MCM, the PMSF concentration was adjusted to 2 mM.

† $n = 1$.

‡ See Fig. 7.

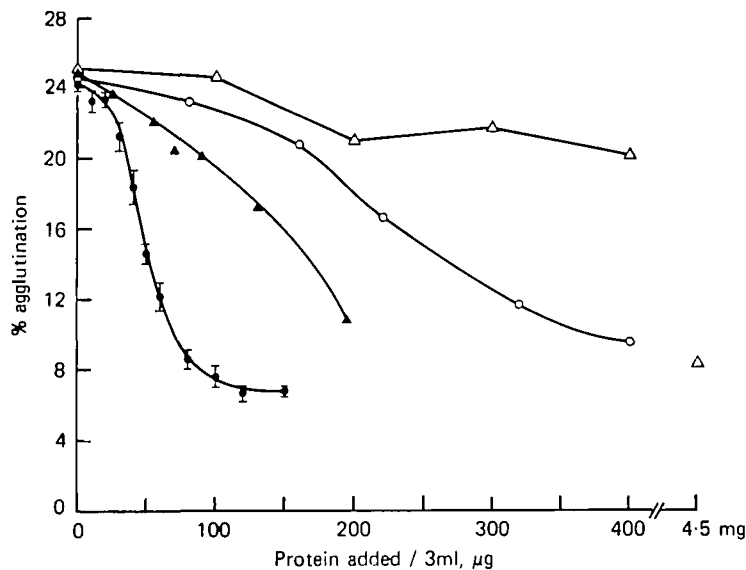

Fig. 9. Competition between free ligand and cell-surface-bound ligand for agglutinin. Assay cultures contained 1·5 ml active MCM, and 1·5 ml Tyrode's solution containing concentrated TCM. PMSF concentrations were adjusted to 2 mM. Test cells were retinal cells with retinal ligand affixed to their surfaces. Assay additions: retinal TCM, ● (means ± S.E.M., $n = 6$); retinal TCM prepared in the presence of 10 μM UDP, ▲ (means, $n = 2$); cerebral lobe TCM, ○ (means, $n = 2$); and bovine serum albumin (fraction V, Sigma), △ ($n = 1$).

of agglutinin to a cell surface which has been previously saturated with bound ligand. TCM does inhibit the agglutination reaction quite effectively (Fig. 9). The active component exhibits these important characteristics in common with ligand: (*a*) When TCM is prepared in the presence of UDP, it contains much reduced levels both of ligand (McDonough & Lilien, 1977) and of the agglutination-inhibiting component (Fig. 9, ▲). (*b*) As in the case of ligand (Balsamo & Lilien, 1974*a*, *b*) the agglutination-inhibiting component of TCM exhibits tissue-type specificity. Cerebral lobe TCM (Fig. 9, ○) is a much less potent inhibitor of the agglutination of neural retinal cells than is neural retinal TCM (Fig. 9, ●). Bovine serum albumin (Fig. 9, △) has no inhibitory effect at concentrations less than several hundred μg/ml. And (*c*), it now appears (see below) that while ligand binds to the cell surface through an oligosaccharide moiety, both bound ligand and the agglutination-inhibiting component in TCM interact with agglutinin through a pronase-labile moiety.

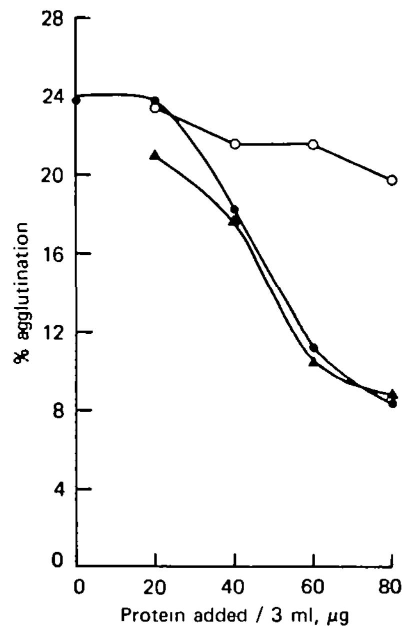

Fig. 10. Competition for agglutinin between enzyme-digested free ligand and cell-surface-bound ligand. Assay conditions were as in Fig. 9. Retinal TCM (undigested control), ●; TCM digested by β-*N*-acetylhexosaminidase (see Methods), ▲; TCM digested by protease (see Methods), ○. Data points are means ($n = 2$).

Experiments using enzyme-digested TCM preparations have helped to clarify the relative roles of protein and oligosaccharide in agglutination. Balsamo & Lilien (1975) reported that β-*N*-acetyl-hexosaminidase treatment of neural retinal TCM both selectively removes terminal β-*N*-acetyl-galactosamine residues and inactivates tissue-type specific binding. On the other hand, binding is not significantly altered by pronase treatment. Fig. 10 suggests that a complementary enzyme-sensitivity pattern is present for the agglutination-inhibiting activity of TCM: β-*N*-acetyl-hexosamini-

dase digestion of TCM does *not* remove agglutination-inhibiting activity (Fig. 10, ▲), whereas pronase digestion does remove this activity (Fig. 10, ○). These 2 kinds of enzyme-digested preparations were then examined for their ability to substitute for untreated TCM as a source of bound ligand in the agglutination reaction: i.e., freshly dispersed cells were subjected to the standard binding procedure with these preparations and were then fixed and assayed for agglutination in MCM (Lilien & Rutz, 1977). The cells are not agglutinable in either case, presumably because the glycosidase-digested preparation contains ligand which cannot bind to the cell surface, while the protease-digested preparation contains ligand which can bind to the cells (Balsamo & Lilien, 1975) but which is unable to interact with agglutinin.

Table 8. *Agglutinin and ligand cannot substitute for each other, but must interact in an ordered fashion*

Test cell types*	Assay medium	% agglutination†
LB (1·0)	Inactive MCM	8·0
AT (1·0)	Inactive MCM	6·6
LB (1·0)	MCM	28·7
AT (1·0)	MCM	9·4
LB (1·0)	TCM	8·8
AT (1·0)	TCM	7·9
LB (0·5) + AT (0·5)	Inactive MCM	8·5
LB (0·5) + AT (0·5)	MCM	10·9‡
LB (0·5) + AT (0·5)	TCM	6·6

* LB, ligand-bound cells; AT, agglutinin-treated cells, prepared by incubation with MCM rather than TCM prior to fixation (all other procedural details were exactly the same as with TCM fixation – see Methods). Figures in parentheses are initial cell numbers ($\times 10^{-6}$) per 3 ml of assay medium.

† $n = 1$.

‡ Equivalent to agglutination of $0{\cdot}5 \times 10^6$ cells (cf. Fig. 6).

Are the assembly steps sequentially ordered? Two kinds of experiments were performed to explore whether the roles of ligand and agglutinin in adhesive bond formation could be reversed. In the first, cells were incubated in MCM, fixed and assayed in TCM: no agglutination of these cells above background is observed (Table 8). Cells fixed in the same manner were also mixed with ligand-bound cells ($0{\cdot}5 \times 10^6$ of each) and assayed in both MCM and TCM. When mixed populations are assayed in MCM, agglutination equivalent to $0{\cdot}5 \times 10^6$ cells is observed (cf. Fig. 7) while equivalent mixtures show no agglutination when assayed in TCM (Table 8). It thus appears that ligand (in TCM) and agglutinin (in MCM) do not detectably substitute for each other; more importantly, the agglutination reaction appears to require that ligand and agglutinin interact in an ordered fashion; ligand must first be functionally bound to the cell surface in order for ligand and agglutinin to interact to bring about agglutination.

DISCUSSION

Agglutination and aggregation

The assay described herein measures the agglutination of glutaraldehyde-fixed cells. Whether the adhesions which are monitored in this assay utilize cellular components which are normally involved in the adhesion of live cells is of the utmost importance. Many of the parameters described for this assay do appear to be similar to those described for the formation of aggregates of live cells. For example, both aggregate formation over a 24-h culture period and agglutination proceed best at 37 °C (Steinberg, 1962; Ball, 1966; Moscona, 1965), the assays both proceed optimally when the pH is in the neighbourhood of 7·0 (Steinberg, 1958), and divalent cations must be present (Steinberg, 1958; Armstrong, 1966; Armstrong & Jones, 1968; McGuire, 1976). Most importantly, both processes exhibit tissue-type specificity. This has previously been shown for cell sorting (Steinberg, 1964; Trinkaus & Lentz, 1964; Garber & Moscona, 1972) and cell adhesion (Roth, 1968; McGuire & Burdick, 1976).

It should also be noted that fixed cells have previously been utilized to probe specific cell adhesive interactions. In the case of sponges, it has been shown (Moscona, 1968) that the aggregation factor is able to agglutinate fixed cells. Oppenheimer & Humphreys (1971) reported that the factor preparation which stimulates teratoma cell aggregation is active in agglutinating fixed teratoma cells. And Balsamo & Lilien (1974*b*) have shown that fixed neural retinal cells (like those utilized in this report) are incorporated into aggregates of living neural retinal cells but not into aggregates of cerebral lobe cells, thereby demonstrating that functional specific adhesive components are present on fixed neural retinal cells.

The similarities between agglutination and aggregation suggest that agglutination does reflect adhesive mechanisms similar to those utilized by live cells. An interesting case in point has to do with the possible correspondence of the agglutinin characterized in this paper and the aggregation-promoting factor which has been characterized by McClay & Moscona (1974), Hausman & Moscona (1975, 1976), and Hausman, Knapp & Moscona (1976). Both components are obtained from monolayer cultures, exhibit tissue-type specificity, and show similar time and temperature profiles of activity. The aggregation-promoter cannot be analogous to our ligand since the ligand requires its oligosaccharide moiety for activity (Balsamo & Lilien, 1975) while the aggregation-promoter does not (Hausman & Moscona, 1975). On the other hand, the one known difference between the aggregation-promoting factor and agglutinin is protease sensitivity. This may not be critical, however, because the stability of the aggregation-promoting factor may be a result of the presence of serum (during the first day of culture) in the monolayers used by Hausman & Moscona (1973, 1975). Furthermore, although the aggregation-promoter is stable in crude medium without added protease inhibitor, it is readily inactivated by trypsin. Conversely, the requirement for protease inactivation by PMSF in MCM may reflect the absence of serum in our monolayer cultures.

Limits of agglutinability

Fixed cell agglutination is complete at 18 h with a majority of the cells still present as single cells. This result is not due to any loss of agglutinability during 24 h of incubation in MCM or other appropriate media (see Table 5). Thus, unagglutinable cells are not produced during the assay.

Unagglutinable cells are not readily distinguishable morphologically from agglutinable cells. Moreover, the unagglutinable cells do not constitute a fixed percentage of cell preparations. Cell preparations which are allowed to repair in the presence of UDP have a greater number of agglutinable cells than do preparations in which ligand is bound to the cell surface from TCM and then fixed in place, or in which cells are repaired in the absence of UDP. Since UDP inhibits the release of surface-bound ligand (McDonough & Lilien, 1977), it can be suggested that the degree of agglutination is in large part a function of the amount of active ligand at the cell surface following fixation. The amount of active surface-bound ligand may in turn be limited both by binding kinetics and by inactivation during fixation. (The agglutinability of ligand-bound cells decreases at glutaraldehyde concentrations above 2·5% (data not shown).) The agglutinability of the cells may also be limited by fixation in a second way, i.e. by the restriction of ligand receptor mobility in the plane of the membrane (Inbar *et al.* 1973).

Distinction between the predominant activities present in MCM and TCM

The agglutination assay has functionally identified 2 distinct activities in conditioned media. One is a cell-surface-associated ligand, the other a soluble agglutinin. The assay has also allowed the order of addition of the 2 activities to be determined: ligand must first associate with the cell surface before agglutinin can function. This ordered addition of the 2 components, and their inability to substitute for each other, strongly suggests that the 2 components are qualitatively different.

The observed enrichment of MCM and TCM for different adhesive components may result from differences in cell density (MCM: 5×10^6 cells/ml; TCM: 50×10^6 cells/ml) or in the arrangements of cells in monolayer or tissue culture. For example, these differences may affect to different extents the ability of the 2 components to diffuse away from the cells. Alternatively, there may be differences in the synthesizing capabilities of the cells in the 2 culture systems. Evidence in support of such an hypothesis has been reported by Morris & Moscona (1971). Neural retinal cells are able to synthesize the enzyme glutamine synthetase in response to hydrocortisone when they are in tissues or aggregates of single cells, but not when they are grown in monolayer cultures.

Mechanism of agglutinin activity

The agglutination of retinal cells requires the presence of at least 3 functionally definable activities: a cell-surface receptor, a cell-surface-associating component (ligand) in TCM, and an agglutinin present in MCM. For the purpose of considering mechanistic interaction, each of the 3 activities will be treated as though it were a

single molecular entity. Even if ligand and agglutinin were multiple entities, the models discussed below (and depicted in Fig. 11) would have the same essential features.

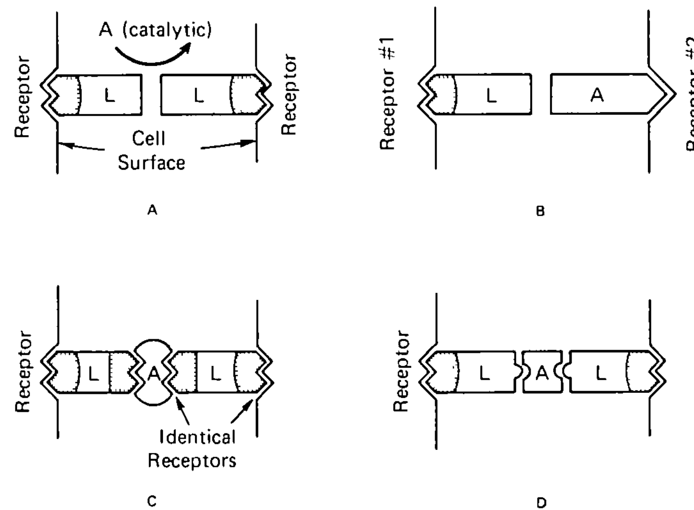

Fig. 11. Four possible models for cell adhesion which incorporate three separable adhesive components (see text). Model A: agglutinin acts enzymically making possible a ligand–ligand interaction. Model B: agglutinin has its own cell-surface receptor. Model C: agglutinin is a soluble form of the cell-surface receptor for the ligand. Model D: agglutinin links 2 surface-associated ligand molecules together. A, agglutinin; L, ligand.

In these models, ligand and agglutinin are depicted as mediating agglutination via a direct interaction. This is suggested by the ability of TCM to compete with surface-bound ligand for agglutinin. While this interpretation is supported by the tissue specificity of TCM competition and by the reduced ability of TCM prepared in the presence of UDP to compete, it is possible that the 2 activities (direct binding of ligand and TCM competition) are separable.

Two features of the agglutination assay, the temperature dependence and time course, might suggest that agglutinin has a catalytic function, and that it affects the ligand (or proligand) so as to allow 2 ligand molecules to interact (Fig. 11, model A). However, evidence has been presented above that agglutinin is removed from MCM by homologous cells. This result is not easily reconciled with a strictly catalytic role for agglutinin: perhaps agglutinin acts in both an enzymic and a structural capacity. Clarification of this question will require studies on the interaction of purified ligand and agglutinin.

In model B, agglutinin is shown to interact both with a cell-surface receptor of its own and with TCM ligand associated with another cell. According to this model, it should be possible to agglutinate cells by first affixing agglutinin to the cell surface and then mixing these cells with ligand-bound cells. Agglutinin-bound cells might also be agglutinable when assayed in TCM. Although cells which have been prepared and assayed as described above have not been observed to agglutinate, this may be due

to inactivation of the agglutinin by fixation. Thus, model B cannot be rigorously excluded at this time.

It can also be suggested that agglutinin is a soluble form of cell surface receptor, which interacts with residues on the ligand which are identical to those involved in the binding of ligand to the cell surface (Fig. 11, model C). This model is inconsistent with the studies employing enzyme-digested TCM: TCM which has been digested with β-*N*-acetyl-hexosaminidase can interact with agglutinin but cannot bind to the cell surface, while protease-digested TCM can bind to the cell but cannot interact with agglutinin.

The simplest model which can account for all of the data presented here is model D. Although agglutinin is represented here in a structural capacity, structural and catalytic roles need not be exclusive of each other. The components also need not interact with each other in a strictly linear array, as is represented in the figure. For example, ligand may contain a 'notch' into which agglutinin fits, completing the interface between 2 ligand molecules. Or alternatively, agglutinin may combine with ligand, altering its overall conformation so as to present a new combining site, in a manner similar to that suggested to occur for the alteration of H-2 antigenicity by viral proteins (Doherty, Blanden & Zinkernagel, 1976; Hale, Witte, Baltimore & Eisen, 1978). Model D, moreover, has been developed for an adhesive system consisting of 3 interacting components. It is possible that other components still await identification.

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