ENZYMATIC DISSECTION OF EMBRYONIC CELL ADHESIVE MECHANISMS

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ABSTRACT

In this paper we describe a kinetic assay for cell adhesion which measures the formation of cell clusters. Cluster formation is dependent on both calcium and protein synthesis, two parameters essential for the formation of histotypic aggregates.

We also describe modifications of the standard method for trypsinization of tissues which result in populations of single cells that appear to bear intact and functional cell surface adhesive systems. These modifications involve the use of chymotrypsin and the inclusion of calcium during enzyme digestion of tissues with trypsin and chymotrypsin.

Using the cluster formation assay and the modified tissue dissociation techniques, we demonstrate the presence of two functionally distinct adhesive systems operating among embryonic chick neural retina cells. These two systems differ in proteolytic sensitivity, protection by calcium against proteolysis, dependence on calcium for function and morphogenetic potential. Cells possessing one of these intact adhesive systems are capable of extensive morphogenetic interactions in the absence of protein synthesis.

Since the classic studies of Townes and Holtfreter (31), the reaggregation of cells derived from embryonic tissues has served as a model system for analysis of morphogenetic cell interactions (see references 13, 19, and 32 for reviews). Two problems are encountered in such studies: the need for quantitative assays that measure adhesions relevant to the formation of histotypic aggregates and the inability to dissociate most embryonic avian or mammalian tissues into single cells without the use of proteolytic enzymes.

The magnitude of the first problem is reflected in the many assays that have been developed to measure intercellular adhesion (reviewed in references 13 and 14). Two discrepant facts emerge when the reaggregation of trypsin-dispersed single cells is monitored. Whereas the formation of histotypic aggregates is dependent on protein synthesis (20) and the presence of divalent cations (18), neither of these dependencies is usually reflected in assays that measure the kinetics of intercellular adhesion (reference 12 and this paper). Furthermore, Roth has shown that the initial adhesions between trypsin-dispersed cells are nonspecific (22). Thus, the relevance of these initial adhesions is questionable with respect to mechanisms of tissue reconstruction and, presumably, morphogenesis.

Most efforts directed at avoiding the use of proteolytic enzymes have relied on the use of chelating agents combined with mechanical shear (5, 11) or a recovery period after trypsinization (3, 22). The first approach generally results in low cell yields and, as Weiss (35) has pointed out, uncer-
tainty with respect to the cleavage planes between cells. The second approach has proved useful in a variety of studies (3, 16, 22) but does not really confront the problem of cell surface modification by enzymatic digestion.

An alternative approach to mechanical dissociation or repair in culture would be the selective digestion of cell surface components leaving intercellular adhesive components intact. Takeichi (28) has taken such an approach with Chinese hamster V79 cells and shown that cells trypsinized in the presence of calcium have an intact calcium-dependent adhesive system, whereas cells trypsinized in the absence of EDTA are nonadhesive. Cells harvested in the presence of EDTA appear to have the calcium-dependent system as well as a calcium-independent adhesive system.

More recently, Takeichi et al. (29) and Uruishihara et al. (34) have extended their studies to include embryonic chick neural retina cells. These cells were also shown to possess both calcium-dependent and independent adhesive systems, both of which are trypsin sensitive. The calcium-dependent system, as with V79 cells, is protected by calcium during trypsinization. Among both V79 and neural retina cells, digestion with low concentrations of trypsin in the absence of calcium destroyed the calcium-dependent, but not the calcium-independent, adhesive system. It was demonstrated that among V79 and neural retina cells, those possessing one adhesive system preferentially interact with cells bearing the same adhesive system. The independence of the two systems was further suggested by the fact that antibody prepared against EDTA-treated V79 cells inhibits adhesion of V79 cells bearing the calcium-independent system, but does not affect calcium-dependent adhesion of these cells nor does it have any effect on neural retina cells.

In the studies reported here, we confirm the observation of Takeichi that embryonic chick neural retina cells possess two functionally distinct adhesive systems, one dependent on and one independent of calcium. We further demonstrate that the two systems are separable by qualitatively different tissue digestion procedures using trypsin and chymotrypsin. The calcium-dependent adhesive system is shown to be sufficient for the assembly of single cells into large histotypic arrays in the absence of protein synthesis. In addition, the retention of an intact calcium-dependent adhesive system after trypsinization is shown to depend on the maintenance of a particular cell surface configuration.

MATERIALS AND METHODS

Preparation of cells

Neural retinas were obtained from White Leghorn chick embryos by dissection in acidified Tyrode solution. Unless otherwise stated, all manipulations were carried out on ice, using 10-d embryos, at a ratio of one retina to 1 ml of solution. Tissues were then treated by one of four methods:

(a) To produce trypsin-dispersed cells, tissues were washed two times in Ca2+-, Mg2+-free Tyrode solution (CMF) and incubated for 10 min at 37°C on a rotating platform at 40 rpm. The tissues were then washed once in HEPES, 0.01 M, pH 7.4, 0.15 M NaCl, 1 mg/ml glucose (HBSG), and incubated for 30 min at 37°C, 40 rpm in HBSG with 4,000 National Formulary Units/ml trypsin (Miles Laboratories, Inc., Elkhart, Ind., three times crystallized). After gently washing three times in Tyrode solution supplemented with 50 μg/ml DNase1 (Calbiochem-Behring, Corp., American Hoechst Corp., San Diego, Calif., 110,000 Dornase units/ml) and 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo.). The covalent serine protease inhibitor PMSF (6) was added from a 200-mM stock solution in isopropanol. After examination of the cell suspension to ensure complete tissue dispersal, the cells were then pelleted at 200 g for 10 min, washed, and resuspended in HBSG supplemented with 1 mM MgCl2 and 50 μg/ml DNase. Cell counts were determined on a model B Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

(b) Chymotrypsin-dispersed cells were prepared by substituting chymotrypsin (Worthington Biochemical Corp., Freehold, N. J., three times crystallized, 50 U/mg) for trypsin. The activity of the chymotrypsin solution was measured by digestion of azoalbumin (Sigma Chemical Co.).

(c) Calcium-trypsin-dispersed (CaT) and (d) calcium-chymotrypsin-dispersed cells were prepared by substituting Tyrode solution for CMF and including 1 mM CaCl2 in the HBSG before and during digestion.

Culture Conditions

In all cases cells were cultured in HBSG supplemented with 1 mM CaCl2, 3 mM KCl, and 0.5% gentamycin (10 mg/ml Schering Corp., Kenilworth, N. J.). These are referred to as control conditions. Cell suspensions were aliquoted in the cold at 3 ml/35-mm bacteriological dish (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). When all dishes in an experiment were ready, they were transferred to a 72 rpm rotary shaker platform and maintained at 37°C in an atmosphere of moist air.

Assays for Cell Adhesion

Aggregate size and morphology were determined by exami-
ing cultures of 20 × 10^6 cells after 24 h of rotation culture (at low power on an inverted microscope).

Initial cell adhesions were followed in cultures containing 5 × 10^6 cells/dish by counting the number of single cells remaining at various times. The contents of each culture dish was diluted 10-fold and counted on a Coulter counter to record single cells.

Cell cluster formation was measured in cultures of 20 × 10^6 cells/dish by diluting the contents of each dish to 10 ml and counting the number of clusters of 100-150 cells. The Coulter counter settings for single cells were determined empirically. Cell clusters were counted at a setting equivalent to particles with an average of 125 cells (range 100-150) except where stated otherwise. The Coulter counter was calibrated with standardized latex beads (Sigma Chemical Co.). The number of cells per cluster was determined by dividing the average cluster volume by the average cell volume.

Histology
Samples were fixed by the addition of an equal volume of double strength fixative to aggregation culture dishes. The final concentration of fixative was 1.25% glutaraldehyde and 2% paraformaldehyde. Samples were then postfixed in 4% OsO₄, dehydrated, and embedded in Epon-Araldite. Sections were made on a Porter-Blum MT2 ultramicrotome (DuPont Instruments Sorvall, DuPont Co., Newtown, Conn.) and stained with toluidine blue.

Other Reagents
Antipain and chymostatin were the generous gifts of Dr. Philip Cohen of the University of Wisconsin and the U. S.-Japan Cooperative Cancer Research Program through Dr. Walter Troll. Cycloheximide was obtained from Sigma Chemical Co.

RESULTS

Minimal Requirements for Histotypic Aggregate Formation

The minimal requirements that would permit trypsin-dispersed cells to form histotypic aggregates were sought. The criteria used to define histotypic aggregates were large size, smooth edges, stability against trituration, and organization of cells within the aggregate into rosettelike arrays. HBSG supplemented with 1 mM CaCl₂ and 3 mM KCl is sufficient for the formation by trypsin-dispersed cells of large, smooth-edged, stable aggregates (Fig. 1 a) containing rosettelike arrangements of cells (Fig. 7). When either potassium or calcium is left out (Fig. 1 b and c), only small, loose clusters of cells form. Magnesium will not substitute for calcium in aggregate formation. When cycloheximide (5 µg/ml) is included in the culture medium, small, loose cell clusters are formed at 24 h; these clusters are readily dispersed by trituration (Fig. 1 d). Cycloheximide at the concentration used blocks the incorporation of leucine by ~90% (Table I).

Residual Trypsin Remains with Cells

Trypsin-dispersed cells are capable of digesting the surface of gelatin-coated glass plates, whereas mechanically dispersed cells or fragments of intact tissue are not. Treatment of trypsin-dispersed cells with PMSF abolishes this proteolytic activity. PMSF treatment does not affect the organization or appearance of aggregates. Moreover, the data in Table 1 show that PMSF treatment does not affect the incorporation of leucine into TCA-precipitable material.

Development of a Kinetic Assay for Cell Adhesion

Rotation-mediated cell adhesion begins with the adhesion of single cells to one another and proceeds by the gradual accretion of cell clusters of increasing size (1). Because the formation of histotypic aggregates by trypsin-dispersed cells is dependent on both protein synthesis and the presence of calcium, we sought an assay which would measure the first step in the aggregation process (i.e., the smallest cell cluster) which was sensitive to both calcium deprivation and inhibition of protein synthesis.

In our hands, the kinetics of single cell disappearance among trypsin-dispersed cells is not perturbable by cycloheximide or calcium deprivation for at least 2 h and negligibly affected from 2-4 h (Fig. 2).

Cell clusters averaging 30 cells each form in the presence of cycloheximide or absence of calcium (Fig. 3 a); however, only under control conditions do these small clusters proceed to form larger ones. The formation of clusters of 100–150 cells is completely dependent on calcium and protein synthesis (Fig. 3 b). The formation of these large clusters is the basis for the kinetic assay used here; all subsequent reference to cell clusters is to clusters of this size.

Modifications of Tissue Digestion Procedure Produce Adhesion-competent Cells

Although trypsin-dispersed cells are unable to form clusters in cycloheximide, CaT cells are competent to form clusters in the absence of protein synthesis (Fig. 4 A). However, like trypsin-dispersed cells, cluster formation is still calcium de-
dependent. It should also be noted that CaT cells form clusters faster than trypsin-dispersed cells and with little or no lag.

The ability of CaT cells to form clusters in the presence of cycloheximide is dependent upon the concentration of calcium present during trypsinization (Fig. 5). The effect of calcium during trypsinization is very specific, showing a sharp and total loss of activity below 100 μM. Magnesium, even at 1 mM, does not substitute for calcium during either trypsinization or aggregation (Table II).

Cells can also be obtained from tissues by digestion with chymotrypsin with yield and viability equal to that of trypsin-dispersed cells. Chymotrypsin-dispersed cells form large numbers of clusters in the presence of cycloheximide as well as in the absence of calcium (Fig. 4 b). In the absence of cycloheximide, cluster formation is significantly increased, although this increased cluster formation is largely calcium dependent.

Thus, depending on conditions of tissue digestion, cells can be prepared which possess adhesive properties independent of new protein synthesis and either dependent on or independent of the presence of calcium during aggregation.

Morphology and Stability of 24-h Aggregates

Differences in the morphology of 24-h aggregates are apparent among the three cell preparations. CaT cells form aggregates almost double the size of trypsin-dispersed cells under control conditions (compare Figs. 1 and 6 a). In the presence of cycloheximide, CaT cells form clusters equal in size to those formed in its absence (Fig. 6 b), but these clusters are labile to trituration with a Pasteur pipette. Calcium deprivation prevents the formation of large clusters by CaT cells (Fig. 6 c).

Chymotrypsin-dispersed cells form stable aggregates considerably smaller than trypsin-dispersed cells under control conditions (Fig. 6 d). The small clusters formed in the presence of cycloheximide or absence of calcium (Fig. 6 e and f) are labile to trituration.
Adhesive Properties after Sequential Enzyme Digestions

The adhesive properties of the cells described so far suggest that selective proteolytic treatment of

![Figure 2](image2.png)

**Figure 2** The kinetics of single cell disappearance among trypsin-dispersed cells. Control ( ), without calcium ( ), with 5 μg/ml cycloheximide ( ). Percent single cells remaining were determined at the times indicated. Data are X ± SEM (n = 4).

![Figure 3](image3.png)

**Figure 3** Cluster formation among trypsin-dispersed cells. Control ( ), without calcium ( ), with 5 μg/ml cycloheximide ( ). At the times indicated, the number of 30-cell (A) and 125-cell (B) clusters were determined per culture dish. Data are X ± SEM (n = 3).

### Table 1

**Effect of PMSF on Incorporation of Leucine and Glucosamine**

<table>
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<tr>
<th></th>
<th>Cycloheximide</th>
<th>dpm/μg Protein</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without PMSF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>− 4,027 ± 1,330 (3)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 489 ± 33 (2)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>GlcNH₂</td>
<td>− 801 ± 462 (3)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 460 ± 42 (2)</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td><strong>With PMSF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>− 3,992 ± 1,422 (3)</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 440 ± 37 (2)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>GlcNH₂</td>
<td>− 798 ± 348 (3)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 398 ± 168 (2)</td>
<td>50</td>
<td></td>
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</tbody>
</table>

Trypsinized cells were prepared as described in Materials and Methods with or without PMSF during tissue dissociation. Cells were aggregated for 24 h as described in Materials and Methods, with the addition of 1 μCi of [³H]glucosamine (19 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) or [³H]leucine (53 Ci/mmol, Amersham Corp.) and 5 μg/ml cycloheximide where indicated. Aggregates were collected, washed, lysed in 0.1 N NaOH at 37°C for 30 min, precipitated with an equal volume of 20% TCA on ice for 1 h, washed, and divided into two portions for protein determination and scintillation counting. Data are X ± SEM (n) and are reported as the percent of incorporation for non-PMSF-treated cells aggregated under control conditions.

**Histology of 24-h Aggregates**

Under control conditions, CaT cells form large, densely packed aggregates with distinct areas where cells are arranged into rosette configurations (Fig. 7 a). This is the same organization attained by trypsin-dispersed cells under control conditions (Fig. 7 b) as has been reported previously by several workers (8, 25). Rosette formation has been shown to be an intermediate step in the reconstruction of retina tissue architecture among chorioallantoic membrane grafts of dissociated chick embryo neural retina cells (7).

Significantly, CaT cells incubated in cycloheximide also form large clusters having internal rosettelike organization (Fig. 7 c). Thus, even under conditions of suppressed protein synthesis, CaT cells are able to interact to form organized, tissue-like cellular arrays. The cells are, however, less densely packed than in control aggregates, allowing the rosettes to stand out more clearly than in control aggregates. This loose packing of cells, giving the aggregate a cobblestone appearance in section, correlates with the unstable nature of these large clusters to trituration.
neural retina tissues may reveal more than one type of adhesive system operating between cells.

A series of experiments involving double, sequential enzyme digestions were performed to address the following questions: (a) Does digestion with calcium-trypsin or chymotrypsin endow the cells with their unique properties? (b) Are the calcium-dependent and -independent adhesive systems functionally independent of one another?

Data in Table III, 1 show that once tissues have been digested with trypsin, which renders them nonadhesive in the presence of cycloheximide, a subsequent digestion with calcium-trypsin or chymotrypsin does not restore the cells to an adhesive state. Thus, the enzyme treatments do not endow the cells with their adhesive properties.

Cross-digestion of either cell preparation (CaT or chymotrypsin dispersed) results in a loss of cycloheximide-insensitive adhesiveness (Table III, 2 A). Data in Table III, 2 A also show that when either cell preparation is redigested with the same enzyme, their adhesive properties are unchanged with respect to calcium dependence or cycloheximide sensitivity. This indicates that the difference between the two cell preparations is qualitative, not quantitative. Digestion conditions that leave one adhesive system intact inactivate the other, indicating the functional independence of the two systems.

Cells obtained by chymotrypsinization of tissues in the presence of calcium behave like CaT cells although they show cluster formation in the absence of calcium (Table III, 2 B). A second digestion of these cells with trypsin in the presence of calcium results in a population with the properties of CaT cells. A second digestion with chymotrypsin results in cells with the properties of chymotrypsin-dispersed cells. These results further indicate the functional independence of the two adhesive systems.
Effect of Calcium on Tryptic Activity during Digestion of Tissues

Calcium does not inhibit tryptic activity in in vitro assays (26) or when used on live cells. When tissues prelabeled for 24 h in culture with [3H]-glucosamine or [3H]leucine are trypsinsized, the time-course and amount of labeled phosphotungstic acid-precipitable material released over a 30-min period in the presence or absence of calcium are identical (data not shown). This suggests that Ca++ does not dramatically alter the amount or character of the cell surface components cleaved by trypsin, but is quite selective in its protective effect.

Effect of a Calcium-free Incubation on CaT Cells

If CaT cells are warmed to 37°C in the absence of calcium, their ability to form clusters in the presence of cycloheximide is lost (Fig. 8). This loss does not occur if calcium is present during the 37°C incubation, or if the cells are kept at 4°C even in the absence of calcium. The protective effect of calcium during this incubation is concentration dependent, with an optimum much below that seen for the effect of calcium during trypsinization. Prevention of the loss of adhesiveness increases gradually from 20 to 75 μM calcium. The effect of calcium during this incubation cannot be substituted for by magnesium (Fig. 8).

Several protease inhibitors were tested for their effects during this 15-min incubation at 37°C in the absence of calcium. Antipain, a specific trypsin inhibitor (33), completely preserves the adhesiveness of CaT cells (Fig. 8). This protection is dose dependent, reaching a plateau at 10 μg/ml. Antipain has the same protective effect even under conditions of calcium chelation (2 mM EGTA). Chymostatin, a specific inhibitor of chymotrypsin (33), has no effect (Fig. 8).

When CaT cells are preincubated in calcium-containing medium at 37°C or calcium-free medium at 4°C, and then redigested with calcium-trypsin, these cells retain their adhesive properties (Fig. 9). However, CaT cells incubated in calcium-free medium with antipain, which retain their adhesive properties (see Fig. 8), are rendered non-
FIGURE 7  Histology of 24-h aggregates. CaT (a and c) and trypsin-dispersed (b) cells were aggregated under control conditions (a and b) or with 5 μg/ml cycloheximide (c). Cultures were fixed and processed for histology as described in Materials and Methods. All pictures are to same scale. Bar, 20 μm. Centers of rosettes are indicated by arrows. x 500.

adhesive by a subsequent calcium-trypsin digestion (Fig. 9). Thus, cell surface components that are initially protected by calcium against trypsinization are no longer protected after a calcium-free incubation at 37°C. This result suggests that cell surface components undergo a rearrangement in the absence of calcium at 37°C, rendering them labile to digestion with trypsin even in the presence of calcium.

All these data taken together suggest that calcium protects a limited number of cell surface-associated substrates against trypsinization. The loss of adhesiveness in the absence of calcium appears to be caused by residual trypsin not accessible to the PMSF used during tissue dissociation. This is supported by our observation that cells trypsinized with fluorescein isothiocyanate (FITC)-labeled trypsin fluoresce throughout their cytoplasm even after extensive washing.

Role of Calcium in Cell Adhesion

The ability of calcium to prevent the loss of adhesiveness among CaT cells during a 15-min incubation at 37°C raises the possibility that calcium is required in cell adhesion only to protect

TABLE III

<table>
<thead>
<tr>
<th>Clusters formed at 4 h</th>
<th>1st Digest</th>
<th>2nd Digest</th>
<th>With Ca²⁺</th>
<th>Without Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. CaT 1</td>
<td>100</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaT 2</td>
<td>95</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaT 3</td>
<td>19</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaT 4</td>
<td>100</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaT 5</td>
<td>59</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaT 6</td>
<td>6</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. CaCl 1</td>
<td>100</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaC 2</td>
<td>119</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaC 3</td>
<td>69</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaC 4</td>
<td>59</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaC 5</td>
<td>9</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All cultures contained 5 μg/ml cycloheximide.

* The still intact tissue was digested a second time for 30 min.
† Calcium trypsinization.
§ Standard trypsinization.
¶ Standard chymotrypsinization.
|| Dissociated cells were digested a second time for 5 min.
$$ 25 μg/ml antipain present during second digestion.
†† Calcium chymotrypsinization.
Loss of adhesiveness by CaT cells during a calcium-free incubation at 37°C. CaT cells were incubated for 15 min under various conditions before assay for 125-cell cluster formation in the presence of 5 μg/ml cycloheximide. All incubations were done in HBSG with 5 μg/ml cycloheximide at 37°C (except where indicated). CF, no calcium added; Ca++, 1 mM calcium added; AP, 25 μg/ml antipain added; CS, 25 μg/ml chymostatin added; Mg++, 1 mM magnesium added. After this incubation, the cells were washed with ice-cold HBSG. Data are X ± SEM (n = 2) for clusters formed at 4 h, expressed as percent of control.

This does not appear to be the case; antipain, at a concentration sufficient to preserve CaT cell adhesiveness during a calcium-free incubation, does not substitute for calcium in the formation of adhesive bonds (Table IV). This provides evidence that calcium plays a direct role in intercellular adhesion.

DISCUSSION

The adhesive interactions measured by the cluster formation assay appear to reflect those parameters that are critical to the formation of histotypic aggregates: the presence of divalent cations and protein synthesis. Thus, the cluster formation assay provides a unique tool for the study of cell adhesion.

The tissue dissociation methods employed in these studies demonstrate the feasibility of obtaining cells with intact adhesive systems directly from embryonic tissues, without the need for extensive repair periods or the use of metal ion chelators and mechanical shear. There are two major advantages of using such cells in the analysis of cell adhesion: (a) their properties may be studied under conditions where synthesis is not required, as was done here, reducing the probability that indirect metabolic effects are responsible for the cellular behaviors observed, and (b) they provide an opportunity to dissect the adhesion mechanism as it may exist in situ.

The results of this study indicate that embryonic chick neural retina cells have at least two functionally distinct and separable adhesive systems. These systems differ in their susceptibility to proteolytic digestion, the effect of calcium on that digestion, and the requirement for calcium in the function of the adhesive system itself. CaT cells possess a calcium-dependent adhesive system that is capable of binding cells into large, organized adhesive sites against inactivation by residual trypsin.

**FIGURE 8** Loss of adhesiveness by CaT cells during a calcium-free incubation at 37°C. CaT cells were incubated for 15 min under various conditions before assay for 125-cell cluster formation in the presence of 5 μg/ml cycloheximide. All incubations were done in HBSG with 5 μg/ml cycloheximide at 37°C (except where indicated). CF, no calcium added; Ca++, 1 mM calcium added; AP, 25 μg/ml antipain added; CS, 25 μg/ml chymostatin added; Mg++, 1 mM magnesium added. After this incubation, the cells were washed with ice-cold HBSG. Data are X ± SEM (n = 2) for clusters formed at 4 h, expressed as percent of control.

**FIGURE 9** Effect of a second calcium-trypsin digestion on 125-cell cluster formation among CaT cells after a 15-min 37°C incubation. Incubations were done in HBSG with 5 μg/ml cycloheximide at 37°C (unless otherwise indicated). Without added calcium (CF), with 1 mM calcium added (Ca++) or with 25 μg/ml antipain added (AP). After the incubation, cells were digested for 5 min with trypsin in the presence of 1 mM Ca++. The digested cells were aggregated with 5 μg/ml cycloheximide. Clusters were counted at 4 h. Data are means of two experiments, expressed as percent of control.

**TABLE IV**

<table>
<thead>
<tr>
<th>Assay medium</th>
<th>Clusters at 4 h</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + cycloheximide*</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ca++-free + cycloheximide</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Ca++-free + cycloheximide + antipain†</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Data are means of two experiments.
* 5 μg/ml cycloheximide.
† 25 μg/ml antipain.
clusters in the absence of protein synthesis. This is a particularly unique situation; we know of no other case where cells derived from embryonic tissues are capable of such a degree of morphogenetic interaction in the absence of protein synthesis. Preliminary data indicate that the CaT cell adhesive system is developmentally regulated; CaT cells prepared from embryos ranging in age from 7 to 18 d of incubation show a continually decreasing capacity to aggregate in the presence of cycloheximide. Thus the properties of CaT cells strongly suggest a morphogenetic role for the CaT cell adhesive system. Cells derived from tissues by chymotrypsinization possess a calcium-independent adhesive system whose morphogenetic potential remains unclear.

The data presented here are largely in agreement with the studies of Takeichi et al. (29) and Urushihara et al. (34), although there are significant differences. These authors state that 10 mM calcium is required to produce CaT cells from neural retina. Although we have routinely used 1 mM calcium, 0.2 mM is sufficient to produce cells with an intact calcium-dependent adhesive system. The reason for this discrepancy is not clear, although it may be because of variations in the conditions of trypsinization. Also, because inhibitors of protein synthesis were not included in the cultures, it is possible that resynthesis of adhesive components during the course of aggregation could have modulated the effect of the particular adhesive system being examined. Because our studies were largely conducted in the presence of cycloheximide, we can unambiguously ascribe the properties of the cells examined, such as the ability of CaT cells to form large histotypic arrays, to the particular adhesive system they possess immediately after their derivation from fresh tissue.

The cleavage of cell surface components on CaT cells during a brief calcium-free incubation appears to reflect the activity of residual trypsin among CaT cells. This is implied by the ability of antipain but not chymostatin to prevent the loss of CaT cell adhesiveness upon calcium-free incubation. The persistence of active trypsin among trypsin-dispersed cells and CaT cells, despite PMSF treatment, suggests that cells may internalize the enzyme and leach it back out. The retention of active trypsin by trypsinized cells has been reported previously (4, 10, 21). The effect of this residual protease on the CaT cell adhesive system during a brief incubation in calcium-free medium suggests that small amounts of residual protease may modulate cellular behavior under certain conditions.

Calcium appears to protect cell surface adhesive components against trypsinization by maintaining them in a configuration in which they are inaccessible to trypsin. This is supported by the effects of calcium and antipain on maintenance of adhesive competence of CaT cells. Despite the fact that CaT cells incubated in the presence of antipain have retained the ability to form clusters in the presence of cycloheximide, they have lost the ability to be protected by calcium during a second round of trypsinization. Because CaT cells incubated in the presence of calcium retain both properties, it is clear that calcium and antipain act differently in protecting CaT cells against the loss of adhesiveness which occurs in calcium-free medium at 37°C. Apparently the trypsin-resistant configuration is not necessary for the function of the CaT cell adhesive system per se because, before redigestion, cells incubated either way are capable of forming equal numbers of clusters in cycloheximide.

The existence of multiple adhesive systems may prove to be a general phenomenon. In addition to the Chinese hamster lung cell line V79 and embryonic chick neural retina, several neuronally derived cell lines have also been suggested to have multiple adhesive systems (24, 27). The relationship of the adhesive systems described here to those characterized previously by our (2, 23) and other (9, 17, 29, 30) laboratories remains to be elucidated. We are at present analyzing CaT and chymotrypsin-dispersed cells serologically and by radiiodination to identify components unique to each of the two adhesive systems.

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