INTERCELLULAR ADHESIVE SELECTIVITY

II. Properties of Embryonic Chick Liver Cell-Cell Adhesion

EDWARD J. McGUIRE

From the Division of Molecular and Cellular Biology,
National Jewish Hospital and Research Center, Denver, Colorado 80206

ABSTRACT

Studies directed at understanding the molecular basis of liver cell homotypic adhesion are presented. An assay which measures the rate of adhesion of isotopically labeled ($^{32}PO_4$) embryonic chick liver cells to liver cell aggregates, described in a companion paper, has been used to investigate the problem of intercellular adhesive selectivity.

Cation requirements, the effects of various inhibitors of metabolism and protein synthesis, of chelators (EDTA and EGTA), and the effects of temperature on liver cell adhesion are reported.

Two mechanisms of inhibition of liver intercellular adhesion are suggested. One involves destruction of cell-surface adhesion receptors (sensitivity to proteases); the other is an energy-dependent step which may involve alterations in plasma membrane conformation and/or membrane fluidity. Finally, a model is suggested for liver cell-cell adhesion that incorporates the early tissue selectivity of intercellular adhesion previously reported, followed by a multistep process which leads to histogenic aggregation.

The development of an improved assay for the measurement of the rate of tissue-selective cell-cell adhesion opened the way for investigating the molecular basis of intercellular adhesive selectivity (15, 22-24). (Previously, conclusions were drawn concerning the fundamental properties of tissue-specific adhesion, based on qualitative or semiquantitative observations [17-19, 25].) Many basic questions, such as the need for protein synthesis (11), the requirements for metabolic energy (21, 22, 24), and the temporal nature of tissue selectivity of intercellular adhesion, still remain unsettled.

We reinvestigated these problems by using embryonic chick liver cell-liver aggregate adhesion for studying the homotypic adhesive process. The results of these studies are presented along with a model for liver cell-cell adhesion.

MATERIALS AND METHODS

Materials

Crystalline trypsin (T), egg-white trypsin inhibitor (TI), bovine serum albumin (BSA), ethylenediamine tetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), colchicine, vinblastine sulfate, carbonyl cyanide, m-chlorophenylhydrazone (CCCP), N,N'-dicyclohexylcarbodiimide (DCCD), cycloheximide and puromycin dihydrochloride, and 2-deoxy-D-glucose were obtained from Sigma Chemical Co., St. Louis, Mo.: papain was purchased from Worthington Biochemical Corp., Freehold, N.J.; actinomycin D from Calbiochem, La Jolla, Calif.; and cytochalasin B, from Aldrich Chemical Co., Milwaukee, Wis. Valinomycin, monactin, nigericin, gramicidin, and A23987 were generous gifts of Dr. Franklin M. Harold, National Jewish Hospital and Research Center, Denver,

Colo. All other materials are described in a companion paper.

Methods

The procedures employed to assay the rate of intercellular adhesion of embryonic chick liver cells are described in detail in the companion publication. 8–9-day old embryonic chick livers were used in all adhesion experiments.

The following media were used: Hanks' balanced salts, glucose (1 g/liter) with 0.01 M HEPES buffer adjusted to pH 7.2–7.4 (H); Hanks' lacking calcium and magnesium salts (CMF); dissociation medium for aggregate preparation, 0.1% 1:250 trypsin, 0.1% collagenase, and 10% heated chicken serum (vol/vol) in CMF; dissociation medium for labeled cells, 0.01% crystalline trypsin with 10% heated chicken serum in CMF (T); medium for arresting action of trypsin, 0.01% egg-white trypsin inhibitor with 10% heated chicken serum (vol/vol) in CMF (TI); nutrient medium, medium 199 lacking NaHCO$_3$, with 0.015 M HEPES adjusted to pH 7.2–7.4 (N); collecting or assay medium, Hanks' (H), 25%, with medium 199 (N), 60% with heated chicken serum (CS), 15%; all vol/vol with DNase 1 μg/ml (HNCS). Labeled cells (^{32}PO$_4$) and collecting aggregates were prepared as described in the accompanying paper. The details for each experiment are reported in the legends to the figures or in the footnotes to the tables. All water-insoluble agents were predissolved in 95% ethanol and added to the appropriate medium. Control cells were suspended in medium containing an equivalent ethanol concentration.

RESULTS

Cation Requirements for Liver Adhesion

The adhesion of labeled embryonic chick liver cells to chick liver cell aggregates was dependent on the presence of the divalent cation, calcium (Ca^{++}) (Table I). The rate of adhesion was reduced to 6% of the control value if either Ca^{++} and Mg^{++}, or Ca^{++} alone, was eliminated from the medium during the experiment. The rate of adhesion was identical to the control value if only Mg^{++} was eliminated. The reintroduction of Ca^{++} ions to preincubated cells in Ca^{++}-free medium led to a return to the control rate of adhesion. This demonstrated that the Ca^{++}-free medium is not deleterious to the cells, and that they are capable of adhesion upon readdition of calcium salts. Sodium (Na^{+}) ion could also be replaced by potassium ion (K^{+}) without adversely affecting the adhesion of liver cells to liver cell aggregates.

Effect of EDTA and EGTA

The demonstration that calcium ions are necessary for the adhesion of liver cells was supported by the finding that EDTA and EGTA, chelators of divalent cations, also inhibited liver cell-cell adhesion. However, it was found that the removal of the chelators and the reintroduction of calcium and magnesium salts did not restore the ability of the liver cells to adhere to liver cell aggregates. This suggests a more complex mechanism for the action of EDTA and EGTA.

Fig. 1 shows the inhibition of the rate of adhesion by various concentrations of EDTA. The liver cells were pretreated with the indicated concentrations of EDTA in CMF (37°C for 10 min). The cells were centrifuged (150 g for 2 min) and resuspended in HNCS. The rate of liver cell adhesion was then determined by the collecting aggregate assay. Fig. 2 shows the rate of adhesion of liver cells pretreated with 7.5 × 10^{-5} M EDTA or EGTA. The liver cells pretreated in CMF with the indicated chelators were unable to adhere to collecting aggregates or to aggregate (visibly) during this time period. Liver cells pretreated with EDTA in HNCS were capable of adhesion. There is sufficient Ca^{++} and Mg^{++} in HNCS to saturate the chelating capacity of this concentration of EDTA (1.26 mM Ca^{++} and 0.9 mM Mg^{++}). (This demonstrated that the effect of EDTA is not a nonspecific toxicity but is related to the chelation of divalent cations on or in the cells.)

TABLE I

Cation Requirement for Liver Adhesion

Condition*	Adhesion rate percent of control
Complete	100
−Ca^{++}, Mg^{++}	6
−Mg^{++}	100
−Ca^{++}	6
−Na^{+}	94
−K^{+}	§
−Ca^{++}; Ca^{++} added at 30 min.‡	(−) 8; (+) 112

* Collecting aggregate assay was carried out in Hanks' medium lacking the indicated cations. The ionic strength was maintained by adding sodium or potassium ions. Chicken serum (15% vol/vol) was included which had previously been dialyzed against the appropriate Hanks' solution to deplete the indicated cation.

‡ Collecting aggregate experiment was run for 30 min minus Ca^{++} (−). 1.26 mM Ca^{++} was then added and experiment continued for a further 30 min (+).
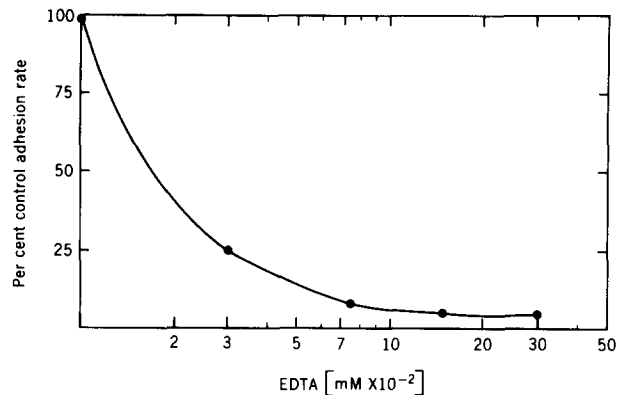
§ Condition was lethal for cells.

E. J. McGuire *Intercellular Adhesive Selectivity. II.*

FIGURE 1 Inhibition of ³²PO₄-labeled embryonic chick liver cell adhesion to liver aggregates by pretreatment of the cells (1.2 × 10⁶/ml) with the indicated concentrations of EDTA in CMF at 37°C for 10 min. After EDTA treatment, cells were centrifuged and resuspended in HNCS medium. The collecting aggregate assay was then carried out for 15 min. The cell concentration was 2.9 × 10⁴ cells/ml (1.7 cpm/cell). The counts per minute/cell was corrected for ³²PO₄ leaked into the medium for each condition.
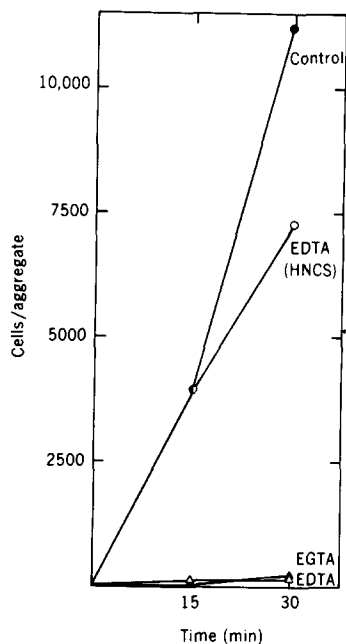

FIGURE 2 The rate of adhesion of ³²PO₄-labeled liver cells to liver aggregates after pretreatment of the cells (1.2 × 10⁶ cells/ml) with 7.5 × 10⁻⁵ M EDTA (Δ) or EGTA (▲) in CMF medium at 37°C for 10 min. Control cells were preincubated in HNCS medium (●) or HNCS medium plus 7.5 × 10⁻⁵ M EDTA (O). After the pre-incubation, the cells were centrifuged and resuspended in HNCS medium. The collecting aggregate assay was carried out with a cell concentration of 2.4 × 10⁴ cells/ml (1.4 cpm/cell) with three aggregates/flask and three flasks/time point.

Effect of Inhibitors of Protein Synthesis

The kinetics of adhesion were linear and showed no apparent lag period before the initiation of cell-aggregate adhesion. This observation suggested that, with our tissue dissociation procedures, no subsequent macromolecular synthesis was necessary for homotypic intercellular adhesion. This was confirmed, as shown in Fig. 3. The addition of inhibitors of protein synthesis (puromycin or cycloheximide) did not inhibit the initial rates of adhesion. The concentrations employed were shown to inhibit the incorporation of [³H]leucine into trichloroacetic acid-precipitable proteins (cycloheximide, 96%; puromycin, 95%). The tissue-dissociation procedures used in this study apparently did not destroy the cell-surface adhesion receptors or binding sites. Thus, no resynthesis of cell surface materials was necessary for adhesion to occur.

Several procedures introduced a requirement for the resynthesis of proteins before cell-cell adhesion occurred. Fig. 4 shows the rate of adhesion of embryonic chick liver cells before and after pretreatment with 7.5 × 10⁻⁵ M EDTA in the presence and absence of 100 μM cycloheximide. As shown in Figs. 1 and 2, pretreatment with EDTA prevented rapid adhesion compared to the control, untreated, liver cells. The adhesive capacity of the cells reappeared (Fig. 4) over a period of 3–6 h. The reappearance of the ability to adhere was inhibited by the presence of cycloheximide.
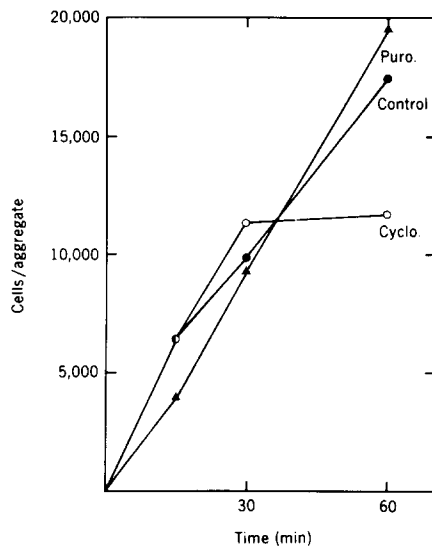
FIGURE 3 The rate of adhesion of $^{32}PO_4$-labeled liver cells to liver aggregates in the presence of inhibitors of protein synthesis. The collecting aggregate assay was run with control cells (●), cells plus 100 μM cycloheximide (O), and cells plus 50 μM puromycin (▲) all in HNCS medium. The cell concentration was 2.9 × 10^4 cells/ml (4.9 cpm/cell) with three aggregates/flask and three flasks/time point.

This suggests that, after pretreatment with divalent cation chelators (EDTA or EGTA), repair is necessary before the cells are capable of cell-cell adhesion. These data suggest that protein(s) are removed, destroyed, or functionally disrupted by pretreatments with EDTA or EGTA. These macromolecules must be resynthesized or reassembled before intercellular adhesion can occur.

Effect of Extensive Proteolysis

Dissociated (T+TI) embryonic chick liver cells treated with cysteine-activated papain (0.37%) for 45 min (room temperature) were unable to adhere to liver cell aggregates. This treatment resulted in viable cells capable of reacquiring the ability to aggregate and adhere to homotypic aggregates. Fig. 5 shows the rate of adhesion of control and papain-treated liver cells. The repair of the papain-treated cells required between 6 and 12 h and was also inhibited by 100 μM cycloheximide. These data suggest that extensive proteolysis removed cell-surface constituents necessary for intercellular adhesion. Cellular protein synthesis was necessary to effect repair. The time for resynthesis was

longer than that seen for EDTA-treated cells. This increased time may reflect the more extensive disruption of the cell surface caused by papain treatment.

Effect of Sodium Periodate Treatment

Pretreatment of the dissociated labeled cells with 1 mM NaIO$_4$ for 15 min (37°C) drastically reduced their ability to adhere to liver collecting aggregates (Fig. 6). The cells were treated in H (-glucose) for the indicated time with 1 mM NaIO$_4$. The NaIO$_4$ was destroyed with 1 mM glycerol. The cells were centrifuged (150 g for 2 min) and resuspended in HNCS. The cells were still viable as demonstrated by the trypan dye exclusion test. Control cells were treated with 1 mM glycerol + 1 mM periodate (preconsumed NaIO$_4$). The treated liver cells showed no indication of repair up to 7 h after initiation of the experiment. Periodate treatment is thought to destroy cell-surface carbohydrates. The observed inhibition of adhesion of liver cells to liver aggregates by periodate treatment may be a result of the destruction of cell surface glycoproteins or glycolipids. Further studies are in progress to determine if periodate-treated cells can regain the ability to demonstrate liver-specific cell adhesive capabilities.

Effect of Metabolic Inhibitors

Fig. 7 shows the inhibition of liver cell-liver aggregate adhesion by a number of known inhibitors of oxidative phosphorylation or of glycolysis. It can be seen that CCCP (carbonyl-cyanide m-chlorophenylhydrazone) completely inhibited the adhesion of liver cells to liver aggregates. Sodium cyanide (NaCN) was not as efficient an inhibitor while sodium arsenate (NaAsO$_4$) and sodium azide (NaN$_3$) did not inhibit significantly.

Table II summarizes the effectiveness of the various metabolic inhibitors. The indicated concentrations gave a 50% inhibition of adhesion while higher concentrations completely inhibited cell-cell adhesion. The cells remained viable, as measured by trypan blue dye exclusion, and in most instances the inhibition was reversed by washing the cells. The effects of DCCD and high concentrations of DNP were not reversible. The action of DCCD is mediated by the formation of a covalent linkage (1), an irreversible process, whereas the lipophilic DNP is not readily washed from the lipid bilayer.
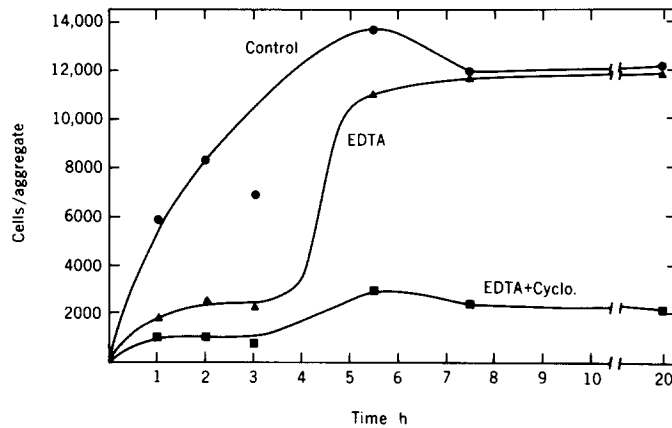
FIGURE 4   An extended time-course for the adhesion of $^{32}PO_4$-labeled liver cells to liver aggregates. Two-thirds of the cells (1.2 × 10^6 cells/ml) were pretreated with 7.5 × 10^{-5} M EDTA in CMF medium at 37°C for 10 min. Control cells were preincubated in HNCS medium. The treated and control cells were centrifuged and resuspended in HNCS medium containing 1% ethanol. The collecting aggregate assay was performed using control (●), EDTA (▲), and EDTA in the presence of 100 μM cycloheximide (■) cells. The cell concentration was 2.9 × 10^4 cells/ml (0.5 cpm/cell) with three aggregates/flask and three flasks/time point.
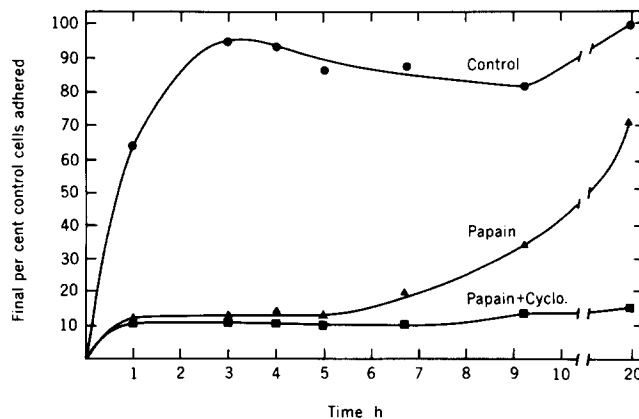

FIGURE 5   An extended time-course for the adhesion of $^{32}PO_4$-labeled liver cells to liver aggregates after papain proteolysis of the dissociated cells. Two-thirds of the dissociated, labeled cells were treated with 0.37% papain in H medium containing 5 mM cysteine and 30 μM dithiothreitol at 25°C for 45 min (8 × 10^6 cells/ml). One-third of the cells were incubated in the same medium lacking papain. After preincubation, the cells were washed with HNCS and the collecting aggregate assay was carried out using control (●), papain-treated (▲), and papain-treated plus 100 μM cycloheximide (■) cells in HNCS medium containing 1% ethanol. The cell concentration was 2.9 × 10^4 cells/ml (0.95 cpm/papain-treated cell; 0.6 cpm/control cell) with three aggregates/flask and three flasks/time point.

All of the above adhesion-inhibiting agents interfere with the oxidative energy metabolism of the liver cells. The intercellular adhesive process appears to reflect the "energy state" of the cells and is probably coupled to an energy-dependent step.

Effect of Ionophores

Ionophores are lipid-soluble ion conductors capable of interfering with or breaking down the ion gradients across biological membranes (8). An
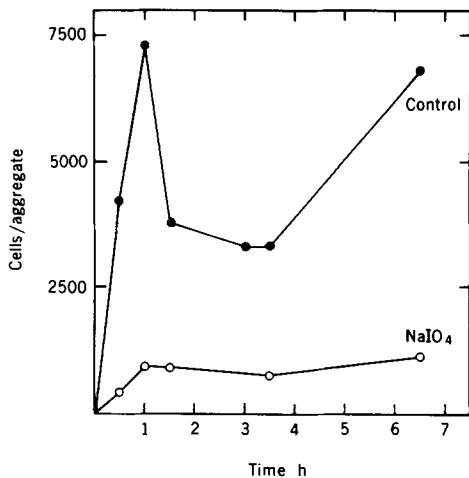
FIGURE 6 The adhesion of control (●) and NaIO₄-treated (O) dissociated ³²PO₄-labeled liver cells to liver aggregates. Periodate-treated cells were incubated at 37°C for 15 min in the dark with 1 mM NaIO₄ in H medium lacking glucose (2 × 10⁶ cells/ml). The oxidation was halted by the addition of 1 mM glycerol. Control cells were incubated in H medium plus preconsumed periodate (NaIO₄ + glycerol). The cells were washed with HNCS medium and the collecting aggregate assay was carried out. The cell concentration was 2 × 10⁴ cells/ml (2.6 cpm/cell) with three aggregates/flask and three flasks/time point.

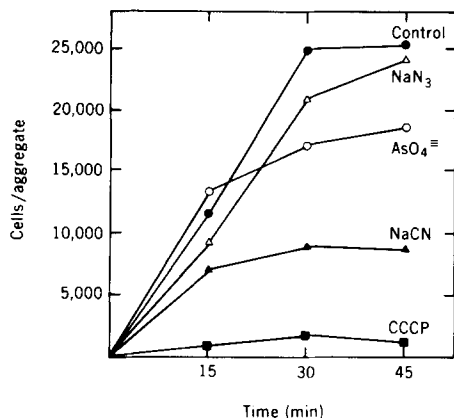FIGURE 7 The effect of various metabolic inhibitors on the rate of adhesion of ³²PO₄-labeled liver cells to liver aggregates. The collecting aggregate assay was carried out in the presence of 100 μM concentration of each of the indicated inhibitors in HNCS medium containing 1.6% ethanol; control (●); NaN₃ (△); AsO₄ (O); NaCN (▲); CCCP (■). The cell concentration was 2.9 × 10⁴ cells/ml (0.7 cpm/cell) with two aggregates/flask and three flasks/time point.

TABLE II

Effect of Metabolic Inhibitors on Liver Cell Adhesion

Metabolic inhibitor	Concentration for 50% inhibition‡ of liver cell:liver aggregate adhesion
	M
CCCP	2×10^{-5}
DCCD*	2×10^{-4}
NaCN	1×10^{-4}
DNP	8×10^{-4}

NaAzide, $(5 \times 10^{-3} \text{ M})$; NaF, (10^{-2} M); Na Arsenate, $(5 \times 10^{-3} \text{ M})$; Ouabain*, (10^{-3} M) did not inhibit at these concentrations.

* Preincubated with labeled liver cells for 15 min at 37°. Control cells, similarly preincubated minus inhibitor.

‡ Collecting aggregate assay run in HNCS medium in the presence or absence of indicated metabolic inhibitors.

investigation of the effect of various ionophores was carried out to further pursue the nature of the energy requirement previously demonstrated for liver cell-liver aggregate adhesion. Table III shows the results of studies with five different ionophores of known molecular mechanisms. Fig. 8 shows the inhibition of adhesion of liver cells by a range of valinomycin concentrations. The ionophores valinomycin and monactin, agents which destroy the K⁺ gradient and the membrane potential of the liver cells, were potent inhibitors of intercellular adhesion. Nigericin and gramicidin break down the ion gradients but act in an electrogenically neutral fashion; therefore, they do not destroy the membrane potential. These ionophores did not inhibit intercellular adhesion at the concentrations tested. The Ca⁺⁺ ionophore, A23187, facilitates the entry of Ca⁺⁺ ions into cells (5, 6). This ionophore had no effect on the rate of liver cell adhesion. These results suggest that liver intercellular adhesion is dependent on the maintenance of ion gradients and the resulting membrane potential across biological membranes.

Studies with metabolic inhibitors and ionophores suggest a strong coupling between cell adhesion and the energetic stage of liver cells. Previously, it was shown that the rate of adhesion of liver cells to liver aggregates was not altered by the exclusion of an energy source, glucose, from the medium. Attempts to starve the liver cells, by preincubation for 30 min (37°C) in nutrient-free

TABLE III

Effect of Ionophores on Liver Cell Adhesion

Ionophore	Mechanism*		Range tested	Action†	Reversibility§
	In	Out			
Valinomycin	K⁺ →		10^{-4}–10^{-6} M	Inhibits	Not reversible
Monactin	K⁺ →		10^{-5}–10^{-7} M	Inhibits	Reversible
Nigericin	K⁺ → ← H⁺		3×10^{-5}–10^{-7} M	No inhibition	—
Gramicidin	K⁺ → ← Na⁺, H⁺		5×10^{-6}–10^{-8} M	No inhibition	—
A 23187	← X⁺ →	Ca⁺⁺	0.1–10 µg/ml	No inhibition	—

* Proposed molecular mechanism. Ionophore facilitates the indicated ion movements across biological membranes. In = Inside the cell (K⁺ concentration high). Out = Outside the cell.

† Collecting aggregate assay run in HNCS medium with indicated concentrations of ionophore. Ionophores dissolved in ethanol and added to medium (ethanol, final concentration 1% vol/vol). Control cells contain equivalent concentration of ethanol lacking ionophore.

§ Cells preincubated with ionophore, centrifuged, and resuspended in ionophore-free medium (HNCS).
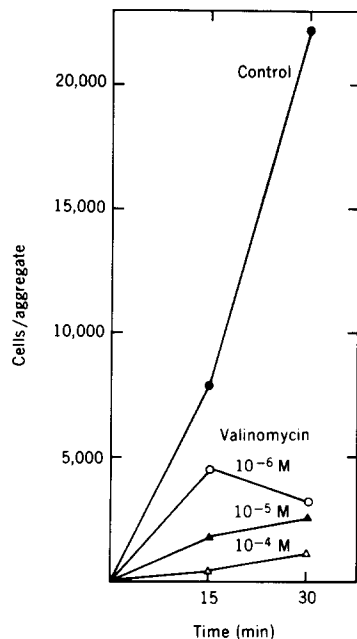

FIGURE 8 The effect of various concentrations of the potassium ionophore, valinomycin, on the rate of adhesion of $^{32}PO_4$-labeled liver cells to liver aggregates. The collecting aggregates assay was carried out in the presence of the indicated concentrations of valinomycin in HNCS medium containing 1% ethanol. Control (●); 1 µM (O); 10 µM (▲); 100 µM (△). The cell concentration was 2.9 × 10⁴ cells/ml (1.9 cpm/flask) with three aggregates/flask and three flasks/time, point.

media, did not decrease that adhesion rate (data not shown).

Effect of Temperature on Adhesion

Many membrane-mediated physiological events are extremely sensitive to changes in temperature. Sharp alterations in rates are observed, consistent with phase changes within the lipid portion of the membrane, at specific, critical temperatures. Fig. 9 shows that the rate of adhesion of liver cells demonstrated a sharp decrease with the lowering of the temperature. The rate of adhesion of liver cells was reduced when the experiment was carried out at 25°C, and at 4°C intercellular adhesion was eliminated. Preincubation of the cells or aggregates at 4°C did not alter their adhesive rate if the collecting experiment was carried out at 37°C (data not shown). This demonstrated the reversibility of the membrane changes induced by preincubation at lower temperatures.

DISCUSSION

Liver intercellular adhesion was dependent on the presence of calcium ions in the medium. Other agents or treatments which reduced the adhesiveness are shown in Table IV. A suggested mode of action for each condition is given. Fig. 10 gives a model detailing two hypothetical mechanisms for inhibition of adhesion. Liver cells obtained by the
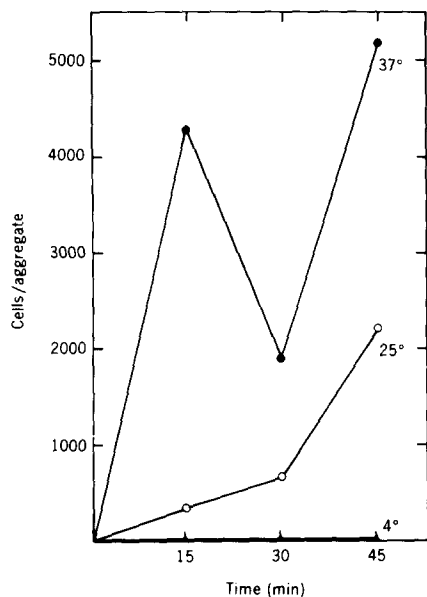
FIGURE 9 The rate of adhesion of ³²PO₄-labeled liver cells to liver aggregates at different temperatures. The collecting aggregate assay was run simultaneously on the same cell preparation at 37°C (●), 25°C (○), and 4°C (▲). The cell concentration was 2.2 × 10⁴ cells/ml (6.8 cpm/cell) with three aggregates/flask and three flasks/time point.

cell adhesion is not inhibited by colchicine, vinblastine sulfate, or cytochalasin B, agents that disrupt cell cytoskeleton function (data not shown). The inhibition of adhesion observed in the presence of valinomycin and monactin may be due to the disruption of the potassium gradients and membrane potential of the plasma membrane. Maintenance of the plasma membrane potential and ion gradients may be a prerequisite for "activation" of the membrane-bound adhesion receptors. Studies are in progress to elucidate the necessity for, and the mechanism of the energy requirement for intercellular adhesion.

The inhibition of intercellular adhesion observed when the liver cells were pretreated with EDTA or EGTA may be due to the removal of the adhesion receptors and/or the deactivation of the plasma membrane. Attempts to detect cell adhesion receptors (proteins removed by EDTA treatment) have thus far been unsuccessful. The mechanism of inhibition by EDTA nevertheless may be by either or both of the suggested paths shown in Fig. 10.

Extensive proteolysis probably removes or destroys the plasma membrane-bound intercellular adhesion receptors. Evidence for this suggestion was obtained by prelabeling liver cells with ¹²⁵I₂ by the technique of Marchalonis (14). Subsequent papain treatment and analysis of the plasma membranes by disc gel electrophoretic techniques showed that most of the labeled plasma membrane

reported dissociation conditions were capable of instantaneous aggregation. No protein synthesis or repair was necessary. The resulting liver cells are postulated to possess adhesion receptors or binding sites which are not destroyed during the dissociation procedure. Further, the receptors are postulated to be bound in the plasma membrane and maintained in an "activated" or correct conformation for intercellular adhesion. Maintenance of the "activated" membrane conformation is postulated to require energy (metabolic or by ion gradients) and calcium ions in the external milieu. Only liver cells possessing adhesion receptors in the correct conformation or in an "activated" state aggregated and demonstrated intercellular adhesion.

Maintenance of the "activated" plasma membrane conformation is thought to be an energy-dependent process. The effect of metabolic inhibitors and ionophores may be to uncouple the energy supply and thus deactivate the membrane. The uncoupling may simply be the inhibition of ATP production or it may be a more subtle mechanism, such as alterations of the cell cytoskeleton. Liver

TABLE IV

Summary of Conditions that Inhibit Adhesion
(Liver* → Liver)

Condition	Mode of action*
Without calcium ion	(S) or (R)
Without potassium ion	Cell death
EDTA, EGTA treatment	(S) chelate Ca⁺⁺; or (R)
Papain treatment	(R) proteolysis of membrane proteins
CCCP, NaCN, DCCD, DNP, etc.	(S) inhibit energy metabolism; inhibit ATPase
Valinomycin, monactin (ionophores)	(S) uncouple oxidative phosphorylation
Low temperature	(S) plasma membrane "freezes"
Sodium periodate treatment	(R) destruction of cell surface carbohydrate

* (S) = may act via cell membrane conformation change (deactivated). (R) = may act via cell receptor alteration.
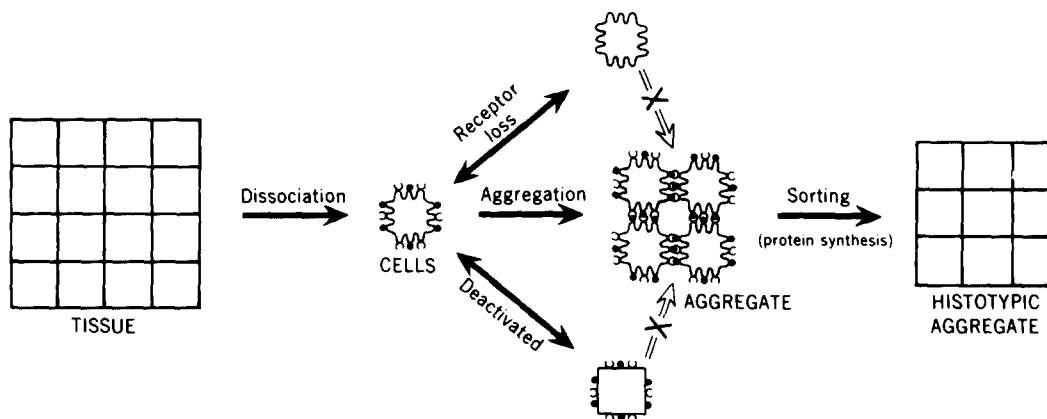
FIGURE 10 A hypothetical model showing the early tissue specificity of intercellular adhesion. The model also suggests at least two ways in which cells can be made aggregation incompetent: (a) adhesion receptor loss (eg: extensive proteolysis); (b) membrane "deactivation"(e.g., no aggregation at low temperature). Tissue-specific aggregation does not require protein synthesis. Aggregation is followed by a multistep process, requiring protein synthesis, which leads to histotypic in vitro tissue formation.

proteins were destroyed. This suggests that most of the external plasma membrane-bound proteins were destroyed by papain. A drastic treatment, such as papain proteolysis, may also lead to extensive membrane alteration. However, the primary mechanism for the inhibition of adhesion is probably the proteolytic cleavage of intercellular adhesion receptors (proteins).

The suggestion that intercellular adhesion is mediated by adhesion receptors is not unique. Evidence has been presented by the groups of Lilien (2, 4, 11), Kuroda (10), and Moscona (7, 9, 20) for the isolation of such tissue-selective adhesion factors. The conformational role or activated plasma membrane concept is more difficult to approach experimentally. We are currently attempting to correlate the intracellular levels of ATP with the adhesion rates of liver cells (with or without metabolic inhibitors). We are also attempting to examine the morphology of the plasma membrane by electron microscope techniques. Alterations in the activated state of the plasma membrane could be mediated by microtubule proteins, actomyosin-like contractile proteins (microfilaments?), changes of the plasma membrane potential by alteration of the ion gradients, or by other unknown mechanisms. The strong inhibition of adhesion observed at low temperatures further suggests that membrane alterations and fluidity are a prerequisite for intercellular adhesion. "Freezing" of the lipid phase of the biological membrane is known to alter its biologi-

cal functions (12, 13). This was confirmed for liver intercellular adhesion in these studies.

The technique of cell aggregation in vitro has been utilized in many studies for analyzing selective cell adhesion and the subsequent sorting out of different cell types (17, 20, 25). Moscona (16, 20) has shown that embryonic cells derived from two different tissues comingled in suspension, coaggregated, and later sorted out into tissue-specific cell groupings. It has been suggested that the early stages of intercellular adhesion are random (demonstrate little tissue selectivity), and that the later sorting-out stage is a tissue specific or recognition stage (20). The results reported in the preceding paper and this report suggest that the earliest stages of intercellular adhesion of embryonic liver, neural retina, and mesencephalon cells manifest a clear tissue selectivity. No repair period or lag time was necessary to demonstrate these early tissue selectivities. Shimada et al. (25) examined neural retina and myocardial cell aggregation by scanning electron microscopy. They also suggested that these cells demonstrated tissue selectivity and recognition during the early stages of aggregation.

The second salient point of the proposed model is that the early stage of intercellular adhesion demonstrates tissue recognition and that the formation of histotypic aggregates is a multistep process. The early tissue selectivity demonstrated by the collecting aggregate assay suggests that tissue-specific adhesion receptors can remain on the cell surface after dissociation of the organ into

a single cell population. The difficulty in reproducibly dissociating embryonic tissues may explain the varying results found in the different laboratories that are studying intercellular adhesion. Second, if histotypic aggregation is a multistep process, as suggested by us and others (25), then it may prove to be difficult to compare the results from laboratories that are studying intercellular adhesion by different assay procedures. The collecting aggregate assay used in this study probably measures earlier events in the complex process of histotypic aggregation than the method of measuring the equilibrium size of aggregates or the sorting out procedure followed by histological examination used by Moscona (17, 20) and Steinberg (26). The future challenge to investigators of intercellular adhesion will be to dissect out and analyze the individual steps leading to histogenesis.

REFERENCES

1. ABRAMS, A., J. B. SMITH, and C. BARON. 1972. Carbodiimide-resistant membrane adenosine triphosphatase in mutants of *Streptococcus faecalis*. I. Studies of the mechanism of resistance. *J. Biol. Chem.* **247**:1484-1488.

2. BALSAMO, J., and J. LILIEN. 1974. Functional identification of three components which mediate tissue-type specific embryonic cell adhesion. *Nature (Lond.)*. **251**:522-524.

3. BALSAMO, J., and J. LILIEN. 1974. Embryonic cell aggregation: kinetics and specificity of binding of enhancing factors. *Proc. Natl. Acad. Sci. U.S.A.* **71**:727-731.

4. BALSAMO, J., and J. LILIEN. 1975. The binding of tissue specific adhesive molecules to the cell surface. A molecular basis for specificity. *Biochemistry.* **14**:167-171.

5. CASE, G. D., J. M. VANDERKOOI, and A. SCARPA. 1974. Physical properties of biological membranes determined by the fluorescence of the calcium ionophore A 23187. *Arch. Biochem. Biophys.* **162**:174-185.

6. CASHWELL, A. H., and B. C. PRESSMAN. 1972. Kinetics of transport of divalent cations across sarcoplasmic reticulum vesicles induced by ionophores. *Biochem. Biophys. Res. Commun.* **49**:292-298.

7. GARBER, B. B., and A. A. MOSCONA. 1972. Reconstruction of brain tissue from cell suspensions II. Specific enhancement of aggregation of embryonic cerebral cells by supernatant from homologous cells. *Dev. Biol.* **27**:235-243.

8. HAROLD, F. M., and K. ALTENDORF. 1974. Current Topics in Membranes and Transport, Vol. 5. F. Bronner and A. Kleinzeller, editors. Academic Press, Inc., New York. 1-50.

9. HAUSMAN, R. E., and A. A. MOSCONA. 1973. Cell surface interactions: differential inhibition by proflavine of embryonic cell aggregation and production of specific cell aggregating factor. *Proc. Natl. Acad. Sci. U.S.A.* **70**:3111-3114.

10. KURODA, Y. 1968. Preparation of an aggregation-promoting supernatant from embryonic chick liver cells. *Exp. Cell Res.* **49**:626-637.

11. LILIEN, J. E. 1968. Specific enhancement of cell aggregation *in vitro. Dev. Biol.* **17**:657-678.

12. LINDEN, C. D., K. WRIGHT, H. M. McCONNEL, and C. F. FOX. 1973. Lateral phase separations in membrane lipids and the mechanism of sugar transport in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **70**:2271-2275.

13. MACHTIGER, N. A., and C. F. FOX. 1973. Biochemistry of bacterial membranes. *Ann. Rev. Biochem.* **42**:575-600.

14. MARCHALONIS, J. J. 1969. An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* **113**:299-305.

15. McGUIRE, E. J., and C. L. BURDICK. 1975. Intercellular adhesive selectivity I. An improved assay for the measurement of embryonic chick intercellular adhesion (liver and other tissues). *J. Cell Biol.* **68**:80-89.

16. MOSCONA, A. A. 1956. Development of heterotypic combinations of dissociated embryonic chick cells. *Proc. Soc. Exp. Biol. Med.* **92**:410-416.

17. MOSCONA, A. A. 1961. Rotation-mediated histogenic aggregation of dissociated cells. A quantifiable approach to cell interactions *in vitro. Exp. Cell Res.* **22**:455-475.

18. MOSCONA, A. A. 1962. Analysis of cell recombinations in experimental synthesis of tissue *in vitro. J. Cell. and Comp. Physiol.* Suppl. 1. **60**:65-80.

19. MOSCONA, A. A. 1965. Cells and tissues in culture. E. N. Willmer, editor. Academic Press, Inc., New York. 1:489-529.

20. MOSCONA, A. A. 1974. The cell suface in development. A. A. Moscona, editor. John Wiley and Sons, New York. 67-99.

21. ORR, C. W., and S. ROSEMAN. 1969. Intercellular adhesion. I. A quantitative assay for measuring the rate of adhesion. *J. Membrane Biol.* **1**:109-124.

22. ROTH, S. 1968. Studies on intercellular adhesive selectivity. *Dev. Biol.* **18**:602-612.

23. ROTH, S., and J. A. WESTON. 1967. The measure-

ment of intercellular adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **58**:974–980.

24. Roth, S., E. J. McGuire, and S. Roseman. 1971. An assay for intercellular adhesive specificity. *J. Cell Biol.* **51**:525–535.

25. Shimada, Y., A. A. Moscona, and D. A. Fischman. 1974. Scanning electron microscopy of cell aggregation: mixed retina-cardiac cell suspensions. *Dev. Biol.* **36**:428–446.

26. Steinberg, M. S. 1963. Reconstruction of tissues by dissociated cells. *Science (Wash. D.C.)* **141**:401–408.