

## INTERCELLULAR ADHESIVE SELECTIVITY

### I. An Improved Assay for the Measurement of Embryonic Chick Intercellular Adhesion (Liver and Other Tissues)

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#### ABSTRACT

An improved assay for measuring intercellular adhesive selectivity of embryonic chick liver cells is described. Three major improvements over earlier procedures are noted: (a) enhanced reproducibility of liver cell-liver cell aggregate adhesion (homotypic adhesion) was achieved; (b) 25%–70% of the input cells adhered to the collecting aggregates during the course of routine experiments as compared to the 0.25% in earlier assays. This increase in cellular adhesion suggests that the observed cell pick-up is a characteristic of the majority of the dissociated liver cell population; (c) the rate of intercellular adhesion was increased 1,000-fold.

The main feature of the assay is that it measures the tissue adhesive selectivities of the dissociated cell population. Studies were undertaken on three embryonic chick tissues (liver, neural retina, and mesencephalon) to determine the tissue selectivity of intercellular adhesion of these dissociated cell types. Some general properties of liver cell homotypic adhesion have been studied and are reported.

Intercellular adhesive selectivity is thought to be of importance in many morphogenetic phenomena (8, 9, 15, 17). Alterations in the adhesive selectivity of cells may also play a role in the loss of growth regulation and metastasis. These important phenomena have been investigated in many laboratories by different methods. Roth and Weston (13) devised an assay involving the collection of  $^3\text{T}$ -labeled cells by unlabeled cell aggregates. The number of cells adhering to the cell aggregates was estimated by autoradiographic procedures. Homotypic cell adhesion was shown to be greater than heterotypic cell adhesion; however, the procedure was slow and difficult to quantitate. Roth et al. (14) improved this method by labeling the cells with  $^{32}\text{PO}_4$  which could be quantitated by standard liquid scintillation techniques. This faster assay

was used primarily to study embryonic chick neural retina cell-neural retina aggregate interactions (homotypic adhesion), with neural retina cell-liver aggregate interactions (heterotypic adhesion) serving as a control for the tissue selectivity of the observed intercellular adhesion. Preliminary results of the reciprocal experiments were described. The above assay procedure measures the adhesive affinities of the dissociated cells for different tissues; however, it is semiquantitative and only determines the rate of adhesion of a small percentage of added cells. An assay developed by Orr and Roseman (10) uses an electronic particle counter to determine adhesion rates. This procedure is rapid and reproducible but cannot distinguish between homo- and heterotypic cell adhesion. Walther et al. (18) introduced an assay which

determines the rate of adhesion of labeled cells to monolayers of cells growing in tissue culture. This procedure is fast and reproducible but requires the collecting cells to grow on a substratum. A large percentage of the labeled cells adhere to the monolayer; however, the tissue selectivity (homotypic vs. heterotypic) of adhesion is lower than that seen with the collecting aggregate assay of Roth et al. (14).

The main advantage of the collecting aggregate assay, as described here, is that it can be used to measure the adhesive affinities of cells for aggregates obtained from the same (homotypic) or different (heterotypic) tissues. Emphasis was placed on the intercellular adhesive properties of liver cells obtained from 8–9-day old chick embryos. The guiding theme of this investigation was to improve the extent and reproducibility of cell-aggregate intercellular adhesion. Heterotypic combinations of cells and aggregates were employed as controls for the tissue selectivity of the procedures under study. The demonstration that a heterotypic tissue aggregate does not collect liver cells is not sufficient to cite tissue selectivity, since we have determined that damaged, dead, or glutaraldehyde-fixed cells behave similarly (data not shown). The reciprocal experiment must be carried out (e.g., labeled neural retina cell collection by unlabeled neural retina aggregates) to demonstrate that the cells and aggregates are capable of cellular adhesion. The results and conditions for these control experiments are given.

## MATERIALS AND METHODS

### Materials

Fertile White Leghorn chicken eggs, obtained from a local hatchery, were incubated in a Humidaire Incubator in this laboratory. The following materials were obtained from the indicated sources: medium 199, chicken, calf, and fetal calf sera, antibiotic-antimycotic, MEM essential, and nonessential amino acid stocks (100×), MEM vitamins (100×), trypan blue vital stain, Grand Island Biological Co., Grand Island, N.Y.; trypsin 1:250, Difco Laboratories Inc., Detroit, Mich.; collagenase and hyaluronidase, Worthington Biochemical Corp., Freehold, N.J.; HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), DNase, catalase, crystalline trypsin, egg-white trypsin inhibitor, and bovine serum albumin, Sigma Chemical Co., St. Louis, Mo.; carrier-free [<sup>32</sup>P]phosphoric acid in 0.01 N HCl, New England Nuclear, Boston, Mass.; New Brunswick G76 gyratory shaking water bath with a 6.25-mm stroke radius, New Brunswick Scientific, New Brunswick, N.J.; Ficoll, Pharmacia, Piscataway, N.J.

**BALANCED SALT SOLUTION (H):** Hanks' basic salt solution, glucose (1 g/l) plus 0.01 M HEPES buffer adjusted to pH 7.2–7.4.

**CALCIUM, MAGNESIUM-FREE BALANCED SALT SOLUTION (CMF):** Hanks' (H) minus calcium and magnesium salts.

**SERUM-FREE MEDIA (HN):** Medium 199, lacking NaHCO<sub>3</sub>, with 0.015 M HEPES buffer adjusted to pH 7.2–7.4, 60%; Hanks' basic salt solution with 0.01 M HEPES buffer adjusted to pH 7.2–7.4.

**COLLECTING MEDIA (HNCS):** HN medium plus 25% heated chicken serum ([56°C for 20 min] CS), or, where indicated, other sera, 15%, all (vol/vol); DNase, 1 µg/ml; antibiotic-antimycotic, (optional) 1 × (penicillin, 100 U/ml, streptomycin, 100 µg/ml, fungizone, 0.25 µg/ml).

**LABELING MEDIA; <sup>32</sup>PO<sub>4</sub> MEDIUM (ME-PO<sub>4</sub>):** Minimum Eagle's medium (ME) without phosphate salts and NaHCO<sub>3</sub>, containing 0.01 M HEPES adjusted to pH 7.2–7.4, heated chicken serum (10% vol/vol), 1 × antibiotic-antimycotic stock plus catalase 1.25 µg/ml (16 U/ml), and 0.5 mCi <sup>32</sup>PO<sub>4</sub>.

**DISSOCIATING MEDIUM, FOR AGGREGATES (CTC):** 1:250 Difco trypsin (0.1%), collagenase (0.1%) in CMF (wt/vol) with heated chicken serum (10% vol/vol). Freezing and thawing reduced the toxicity of CTC.

**DISSOCIATING MEDIUM FOR LABELED CELLS (T):** Crystalline trypsin (0.01%, wt/vol), in CMF solution with 10% heated chicken serum (vol/vol); egg-white trypsin inhibitor (TI): (0.01%, wt/vol) in CMF with 10% heated chicken serum (vol/vol). All media were filter-sterilized before use.

### Methods

The principle of the assay system remains the same as that previously described (16). However, many modifications were introduced which, while seemingly minor, led to large increases in the rate and extent of adhesion and the accuracy of the assay. <sup>32</sup>PO<sub>4</sub>-labeled cells were shaken with unlabeled tissue aggregates. At intervals the aggregates were washed to remove non adherent cells and the radioactivity of the aggregates was measured. The radioactivity is a measure of the number of cells that adhered to the aggregates.

**LABELING OF CELLS:** Four livers from 8-day embryonic chicks were aseptically dissected into H medium and teased into small pieces. The fragments were rinsed with labeling medium (ME-PO<sub>4</sub>) and placed in a 5-cm tissue culture plate with 3–4 ml of ME-PO<sub>4</sub> containing 0.5 mCi <sup>32</sup>PO<sub>4</sub>. The inclusion of catalase improved the viability of the cells, presumably by destroying peroxides generated by the carrier-free <sup>32</sup>PO<sub>4</sub>. The chunks of liver were shaken gently at 37°C in CMF. The CMF was aspirated, and 3 ml of 0.01% crystalline trypsin (T) were added. The fragments were incubated for another 15 min at 37°C and were gently dissociated by repeated pipetting through a 9-in Pasteur pipette (10 min). After dissociation, 3 ml of 0.01% trypsin inhibitor (TI) and 3 ml of

HNCS were added to stop the action of the trypsin, and the cells were collected by centrifugation for 2 min at 150 g. The cells were not washed further since we found that while the amount of  $^{32}\text{PO}_4$  in the supernate could be reduced by washing, the cells were progressively damaged by each subsequent centrifugation. This damage was reflected in a greatly reduced ability to adhere to liver aggregates. The cells were resuspended in 3 ml of HNCS and their number was counted in a hemocytometer. The radioactivity of cells and supernates was also determined at this time. The cells were then diluted with HNCS to obtain an appropriate concentration for each experiment. Other tissues were labeled and dissociated by essentially the same protocol except that CTC was used instead of 0.01% trypsin (T) to dissociate the labeled tissue fragments. The time of incubation in CTC was the same as that used for aggregate preparations.

**AGGREGATE PREPARATION (LIVER):** 25–30 embryonic chick livers (8-day) were dissected aseptically into H medium and teased into small pieces, and the pieces were rinsed with CMF. They were incubated at 37°C for 10 min in CMF. The CMF was removed, and 5–6 ml CTC were added, and the incubation was continued for 30 min. The tissue was dissociated by pipetting with a 9-in Pasteur pipette (1–2 min). 3 ml of HNCS were added to halt proteolysis. The dissociated cells appeared predominantly as single cells, with some groups of two, three, etc. Better aggregates were obtained if the liver was not totally dissociated to a single cell population. If large chunks still existed, they were allowed to settle and were removed. The cells were centrifuged at 150 g for 2 min, allowing recovery of  $5-9 \times 10^7$  viable cells. Viability of the cells was determined by trypan blue dye exclusion. They were then resuspended at a density of approximately  $5 \times 10^6$  cells/ml in HNCS, and 3-ml portions were pipetted into 25-ml DeLong flasks. The flasks were shaken at 66 rpm on a gyratory shaker at 37°C for 1 h. Aggregates of many sizes were obtained. Approximately 75–100 usable aggregates of 1-mm diameter were selected for use as collecting aggregates. Each of them contained approximately  $1 \times 10^6$  cells.

**AGGREGATE PREPARATION; (NEURAL RETINA):** Six 8-day neural retinas were dissected and washed with CMF. They were dissociated in 3 ml of CTC at 37°C for 5 min. Dissociation was stopped by the addition of 3 ml of HNCS. The cells were centrifuged at 150 g for 2.5 min, resuspended in 9 ml of HNCS, and 3-ml portions were pipetted into 25-ml DeLong flasks. Approximately  $6 \times 10^7$  cells were obtained by this procedure. The flasks were shaken at 64–66 rpm for 1 h. Approximately 30–40 aggregates of 1 mm diameter were obtained and selected for the collecting assay. Each aggregate of this size was composed of approximately  $2 \times 10^6$  cells.

**AGGREGATE PREPARATION; (MESENCEPHALON [OPTIC LOBES]):** Nine 8-day mesencephala were dissected and washed with CMF. The tissue pieces were

incubated for 30 min in 2 ml of CTC at 37°C. The mesencephala were dissociated by gentle pipetting with a 9-in Pasteur pipette for 2–3 min. The proteolysis was stopped by the addition of 2 ml of HNCS, and the cells were centrifuged at 150 g for 2.5 min. The supernate was removed, and the cells were resuspended in 9 ml of HNCS;  $9 \times 10^7$  cells were obtained. 3-ml portions were pipetted into 25-ml DeLong flasks, and the flasks were shaken at 64–66 rpm. Aggregates of many sizes formed in 1 h. Approximately 30–40 aggregates of 1 mm diameter ( $2 \times 10^6$  cells/aggregate) were obtained for the collecting assay.

**VISCOSITY DETERMINATIONS:** The viscosities of the various media were determined with a size 50 Cannon-Fenske viscometer at 37°C.

**THE ASSAY:** A series of duplicate 25-ml DeLong flasks containing three to four collecting aggregates of each tissue (homotypic or heterotypic) were prepared. An aliquot of dissociated, labeled cells of known cell density and total radioactivity was added to each flask. The final volume of HNCS medium was adjusted to 3.5 ml. The cell density, counts per minute per cell, and time of incubation are given for each experiment. The flasks were rotated on a gyratory shaker at 60–64 rpm (37°C). The depth of the medium, shape of the flask, and rotational speed were shown to be extremely critical: under optimal conditions, the collecting aggregates and labeled cells gathered in the center of the flask. Final adjustment of the shaker speed was made to ensure that the aggregates in all flasks were centered. Any alteration in this pattern resulted in drastically reduced collection since the aggregates and labeled cells rotated in separate orbits and did not come into contact.

At the indicated times, the flasks were taken from the shaker and the aggregates removed with a Pasteur pipette and placed on a sheet of Parafilm in small droplets of HNCS. The nonadhering, labeled cells were washed from the aggregates by transferring the aggregates serially to three fresh droplets of HNCS. The washed aggregates were dried on strips of Whatman 3-mm paper, and the  $^{32}\text{PO}_4$  of each aggregate was counted in toluene scintillation fluid containing 4 g/liter 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) with a Beckman LS 100C liquid scintillation counter. After removal of the aggregates, the remaining contents were centrifuged at 150 g for 2 min to remove cells. A portion of the cell-free supernatant fluid from several flasks for each experimental condition was then counted. The counts per minute per cell for any given time point and for each experimental condition was determined by using the following formula:

$$[\text{cpm/cell}] = \frac{[\text{total cpm/flask}] - [\text{total cpm of cell-free supernate}]}{[\text{cells/flask}]}$$

and the number of cells adhering to a given aggregate

calculated. Approximately 40% of the  $^{32}\text{PO}_4$  was found in the cell-free supernate in experiments of less than 1-h duration. The duplicate counts per minute per cell values for each experimental condition are generally in good agreement ( $\pm 10\%$ ). This method takes into account the fact that the counts per minute per cell changes from one condition to another and during the course of an individual experiment.

In a series of controls the labeled cells were incubated without collecting aggregates for various times, and the cells were removed by centrifugation. The cell-free supernate was then incubated with unlabeled collecting aggregates for various times. The aggregates were washed and counted. They were found to retain 25–50 cpm/aggregate for a 30-min incubation. This control was performed to determine how much of the “leaked”  $^{32}\text{PO}_4$ , in whatever form, was taken up by the collecting aggregates.

**VARIABILITY AND STATISTICAL TREATMENT:** The means and standard deviation were calculated by standard statistical methods. The reproducibility of the assay procedure was improved enough to obtain meaningful quantitative information. Valid comparisons were obtained by performing many duplicates. Each assay consisted of at least two time points, and each time point represented 6–12 aggregates, washed and counted separately. Thus, comparisons were made on a minimum of 24–48 data points. The standard deviation is still quite high (average of  $\pm 37\%$  of the mean), but further improvement in duplication may prove to be very difficult due to the extreme sensitivity of the assay to minor topological variation from flask to flask and from aggregate to aggregate.

## RESULTS

### *Dissociation Methods; (Aggregates)*

The first step in a systematic study of adhesion was to examine various procedures for dissociating the tissue. Difco 1:250 trypsin with chicken serum (CTC), a dissociating medium used previously (16), was employed to provide a base line for comparison. Treatment with crystalline trypsin (T), EDTA, and mechanical dissociation in CMF medium were also tried. The resulting cells were tested for their ability to aggregate as described in Methods. The best aggregates were obtained with the CTC procedure of Roth et al. (14). It appears to be the mildest treatment as judged by viability and by the lack of DNA release during the aspiration of the cells for disruption of the tissue.

### *Dissociation Methods; (Cells)*

Fig. 1 shows the influence of the dissociation procedure upon the rate of adhesion of labeled

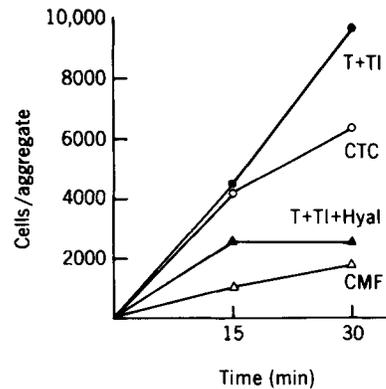


FIGURE 1 Rate of adhesion of  $^{32}\text{PO}_4$ -labeled cells to liver aggregates after dissociation by different methods. Dissociation methods: (●, T+TI) 0.01% crystalline trypsin in CMF at 37°C for 15 min; (○, CTC) 30 min in CTC at 37°C; (▲, T+TI + HYAL) 0.01% trypsin + 0.1% hyaluronidase in CMF at 37°C for 15 min; (△, CMF). Incubation in CMF at 37°C for 15 min, followed by vigorous dissociation with a Pasteur pipette. Assay procedure: the labeled cell concentration was  $1.4 \times 10^4$  cells/ml with three aggregates per flask and three flasks per time point and the temperature was 37°C. The counts per minute per cell were: CTC = 1.56; CMF = 1.27; T+TI = 1.29; T+TI + HYAL = 2.57. The counts per minute per cell were corrected for  $^{32}\text{PO}_4$  leaked from the cells into the medium, for each time point and condition in the experiment.

liver cells to liver aggregates. Details of the dissociation procedures are given in the legend to Fig. 1. This study showed that the rates of adhesion of CTC- and T+TI-dissociated cells were very similar. However, both mechanical dissociation in CMF and the inclusion of hyaluronidase with the trypsin reduced the rate of adhesion. EDTA-dissociated liver cells did not adhere during the period of the test (data not shown). The trypsin plus trypsin inhibitor procedure (T+TI) was used in subsequent assays to minimize contaminating proteolytic activity. Fig. 2 A shows the effect of trypsin treatment, for varying lengths of time, on the adhesion of labeled liver cells to liver collecting aggregates. It can be seen that prolonged proteolysis reduced the rate of adhesion. Treatments shorter than 15 min were impractical because the tissue was not readily dissociable except by severe mechanical means. Fig. 2 B shows the effect of treatment with varying concentrations of trypsin. There is a marked inverse correlation between the rate of adhesion of liver cells to aggregates and the concentration of trypsin used for dissociation.

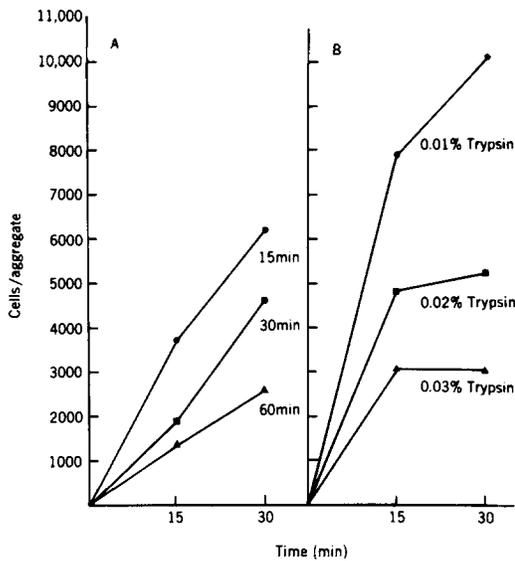


FIGURE 2 (A) The rate of adhesion of  $^{32}\text{PO}_4$ -labeled liver cells to liver aggregates after dissociation in 0.01% T at  $37^\circ\text{C}$  for 15 min (●), 30 min (■), and 60 min (▲). The cell concentration was  $2.9 \times 10^4$  cells/ml (0.6 cpm/cell, ●; 0.5 cpm/cell, ■; 0.6 cpm/cell, ▲) with three aggregates per flask and four flasks per time point. (B) The rate of adhesion of liver cells to liver aggregates after dissociation at  $37^\circ\text{C}$  for 15 min in 0.01% T (●), 0.02% T (■), and 0.03% T (▲). Proteolysis was stopped by the addition of an equivalent concentration of egg-white trypsin inhibitor. The cell concentration was  $1.8 \times 10^4$  cells/ml with three aggregates per flask and four flasks per time point.

#### Studies on Aggregate Preparation; (Collecting Surfaces)

During the course of the investigation, it was found that the surface appearance of the aggregates changed with the time of incubation. After shaking for 5 min, the cells associated to form loose, fragile aggregates. The size of the aggregates did not change appreciably after 15 min. After 1–2 h the aggregates “tightened” and were more amenable to handling. Longer periods of shaking resulted in aggregates with smooth surfaces, which picked up additional cells but not so well as the earlier 1- to 2-h aggregates. 18-h aggregates collected labeled liver cells at 30–60% of the rates of 1- to 2-h aggregates. The 1- to 2-h liver aggregates were routinely used in the assay.

Fig. 3 shows the number of liver cells adhering to liver aggregates of different sizes. This experiment suggests that small variations of aggregate

size will not have large effects on the number of cells collected. Aggregates of 1 mm ( $\pm 0.25$ ) diameter were used routinely. It was also shown that when liver fragments were prepared by teasing the liver with forceps and then allowing the fragments to round up by shaking in HNCS, they were only 4% as efficient in picking up labeled liver cells as were the routinely used, reassociated “collecting aggregates”.

#### Studies on Composition of Media

While most of the studies presented in this and the accompanying paper were conducted with a complex medium (HNCS), it was considered important to determine the nutritional requirements for adhesion. HEPES-buffered Hanks’ medium (H) plus medium 199 (N) with or without 15% (vol/vol) heated chicken serum (HNCS) showed an identical rate of adhesion of liver cells to liver aggregates. This rate was set as 100%. The rate of adhesion with 15% (vol/vol) heated calf serum was 38% of the control rate, while the rate of adhesion when 15% (vol/vol) heated fetal calf serum was employed was 20% of the control value. This suggested that calf and fetal calf sera may contain inhibitors of adhesion. These studies were carried out with only one lot of calf and fetal calf sera, so it is not known whether this is a general property of all calf and fetal calf sera.

Fig. 4 shows the results of a study in which chicken serum was omitted. Liver cells adhered to the liver collecting aggregates even in a simple buffered salt solution containing bovine serum albumin. The replacement of glucose by 2-deoxyglucose, a nonmetabolizable sugar that is actively

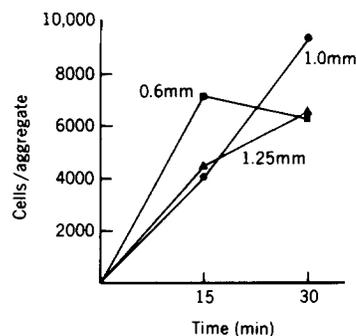


FIGURE 3 The rate of adhesion of liver cells to liver aggregates as a function of aggregate diameter. The cell concentration was  $2.9 \times 10^4$  cells/ml (1.28 cpm/cell) with four aggregates per flask and four flasks per time point.

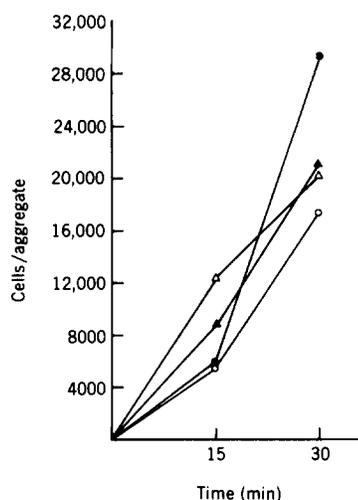


FIGURE 4 The rate of adhesion of liver cells to liver aggregates in different media. The media were: ●, HN medium + 1% bovine serum albumin (BSA); ▲, H medium + glucose (1 g/liter) + 1% BSA; △, H medium + 2-deoxyglucose (1 g/liter) + 1% BSA; ○, H medium minus glucose + 1% BSA. The cell concentration was  $2.9 \times 10^4$  cells/ml (0.4 cpm/cell) with three aggregates per flask and three flasks per time point.

transported into the cells, did not inhibit adhesion. This suggested that the cells have sufficient energy reserves to complete adhesion. If potent metabolic inhibitors were added (e.g.,  $10^{-3}$  M NaCN, see accompanying paper), adhesion was inhibited; but for experiments of less than 1-h duration the liver cells adhered to collecting aggregates in a simple balanced salts medium. Bovine serum albumin can also be eliminated, and the cells will adhere at the control rates (HNCS medium); however, the standard deviation of the resulting data was greatly increased. The increased scatter is caused by the great difficulty in maintaining the aggregates in the center of the flask, using media of lower viscosity than the media containing chicken serum or bovine serum albumin. Collecting aggregate experiments carried in our HN medium plus 15% CS, 0.75% BSA, or 0.1% Ficoll (viscosity = 0.755 centipoise) at 63 rpm all showed identical rates of adhesion of labeled liver cells to liver aggregates. HN medium (viscosity = 0.71 centipoise) will also support adhesion of liver cells to liver aggregates, but the shaker speed must be reduced to approximately 58–60 rpm and the aggregates are more difficult to maintain in the center of the flasks.

Finally, identical rates of adhesion were found in HEPES-buffered medium and in  $\text{CO}_3^{2-}/\text{HCO}_3^-$

buffered medium. This ruled out toxic effects by HEPES on the rate of adhesion.

### Studies on the Geometry of Cell Collection

The major change in the collecting aggregate method from the previously published procedure (14) is that collection of labeled cells by collecting aggregates was carried out with a gyratory shaker instead of a reciprocal shaker. This alteration is consistent with the previously mentioned necessity for "centering" the collecting aggregates into the same "orbits" as the labeled cells. Collection of labeled liver cells by liver aggregates with a reciprocal shaker (60 rpm, 1.5-in stroke) was 5–15% of the value obtained with a gyratory shaker.

Table I shows the effect of the volume of the medium on the rate of adhesion of labeled liver cells to liver aggregates. The dramatic effects on the adhesion rate produced by increasing the volume of the medium from 2.0 to 3.5 ml per flask is probably due to the fact that 3.5 ml of medium provides a sufficient depth so that the aggregates are not washed from the center of the flask by the waves set up by the gyratory shaking. It is impossible to keep the aggregates in the center of the flask with less than 3.0–3.5 ml of medium.

The shape of the bottom of the flask was also found to be important. Each DeLong flask was marked for identification, and experiments were carried out to determine the rate of adhesion of liver cells to liver aggregates and the standard deviation, using four aggregates per flask. Any flask that was found to have a mean value greater than  $\pm 40\%$  of the experimental mean was re-

TABLE I  
The Effect of Media Volume (Depth) on Liver Cell Adhesion to Liver Aggregates

Volume*	Adhesion rate compared to control (3.5 ml)†
	%
2.0	8
2.2	13
2.5	29
3.0	57
3.5	100
4.0	100

\* Milliliters of HNCS (15%) medium per 25-ml DeLong flask.

† The rate of adhesion was measured at 15 min.

checked. Also, any flask that showed exceptional variability of values from one aggregate to another was rechecked (mean of the aggregate values from flask/SD of aggregate values from flask  $> \pm 40\%$ ). If these differences were consistent the flask was discarded. No visible imperfections could be correlated with the variability in these discarded flasks. This precaution served to reduce the variability of the data.

### Studies on the Tissue Selectivity of Adhesion

Three tissues were studied to determine the relative homo- and heterotypic affinities of these dissociated cell types.

**LIVER:** Fig. 5 shows the rate of adhesion of labeled liver cells to liver aggregates, to neural retina aggregates, and to mesencephalon (optic lobe) aggregates. Adhesion of liver cells to liver collecting aggregates (homotypic adhesion) was very rapid. Within 30 min, 37% of the input cells adhered to the liver aggregates. The rate and extent of heterotypic adhesion (liver  $\rightarrow$  neural retina; liver  $\rightarrow$  mesencephalon) was much lower. Under these conditions, there was no lag period before homotypic adhesion occurred. This agrees with the visual observation that aggregation was very rapid, occurring in 5 min or less.

**NEURAL RETINA:** Fig. 6 shows rates of adhesion of labeled neural retina cells to neural retina, to mesencephalon, and to liver collecting aggregates. Again, as with liver, it was seen that homotypic adhesion proceeded rapidly, 8.5% of

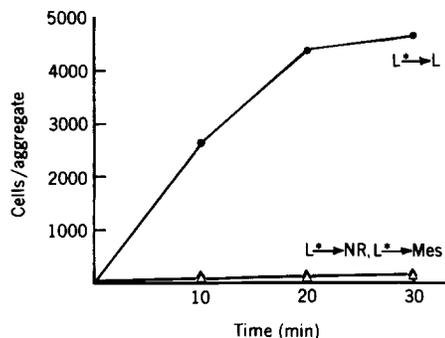


FIGURE 5 The adhesion of liver cells to liver (●), neural retina (▲), and mesencephalon (Δ) aggregates as a function of time. The cell concentration was  $1.4 \times 10^4$  cells/ml (0.5 cpm/cell) with four aggregates per flask and three flasks per time point. Liver aggregate labeling using a cell-free supernate as a source of leaked  $^{32}\text{PO}_4$  was less than 50 cpm/aggregate at 30 min.

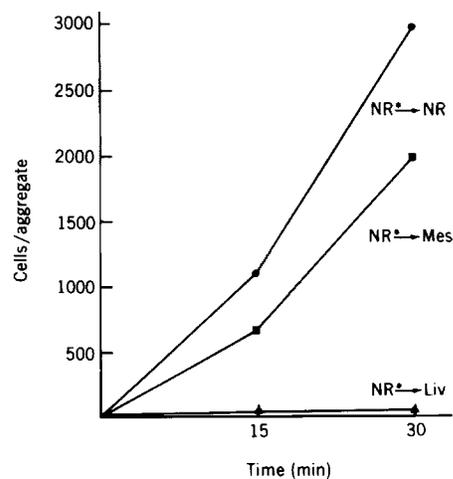


FIGURE 6 The adhesion of neural retina cells to neural retina (●), mesencephalon (■), and liver (▲) aggregates as a function of time. The cell concentration was  $2.9 \times 10^4$  cells/ml (2.5 cpm/cell) with three aggregates per flask and three flasks per time point.

the neural retina cells adhered to neural retina aggregates in 30 min. Heterotypic adhesion to liver aggregates was negligible; however, heterotypic adhesion to mesencephalon aggregates represented about 6% of the input neural retina cells.

**MESENCEPHALON:** Fig. 7 shows the results of the adhesion of labeled mesencephalon cells to mesencephalon, liver, and neural retina aggregates. In this case the homotypic adhesion was greatly favored, with 13.5% of the input mesencephalon cells adhering in 30 min. The mesencephalon cells did not adhere to liver aggregates, and only 0.75% adhered to neural retina aggregates.

Table II shows a summary of the tissue studies. The ratio of homotypic to heterotypic adhesion is a measure of the tissue selectivity reflected by this assay procedure. The ratio for liver was high and reproducible. For other tissues studied, homotypic adhesion was always favored; however, the differences were not so marked as in the case of liver. Further studies are in progress to extend these studies to other embryonic tissues.

### Studies on Effect of Embryonic Age on Liver $\rightarrow$ Liver Adhesion

Other workers (3, 6, 7) have shown that changes in cellular adhesiveness occur during development. It was observed that the size of liver aggregates at equilibrium, for a constant cell density and shaker speed, decreased with the age of the embryo. This

was interpreted as a decrease in cellular adhesiveness with increasing embryonic age. We have studied changes in adhesiveness through development using the adhesion of liver cells to liver collecting aggregates. Table III shows a summary of these studies. All comparisons were based on the rate of adhesion of 8-day liver cells to 8-day liver aggregates. An experiment measuring the rate of adhesion in a four-way reciprocal cross was done for every embryonic age studied.

Far less study has gone into the dissociation and aggregation of livers of ages other than 8-day embryonic liver. Nevertheless, three conclusions were drawn from this study. (a) There was no dramatic change in the rate of adhesion of the liver cells obtained from the embryonic livers of different ages. While the rates given in Table II are not to be considered as absolute values, it is clear that a population of cells capable of adhering to liver aggregates of the same age or to 8-day aggregates was obtained from all stages studied. (b) The livers were increasingly difficult to dissociate with age, and the aggregates from older embryos were inferior (not so compact). (c) The yield of cells from a given weight of liver decreased with the embryonic or posthatching age of the liver. How-

ever, the cells that were obtained could adhere in the collecting aggregate assay. While the interpretation of these data awaits further study, it is clear that cellular adhesion, as measured by the collecting aggregate assay, is not unique to the early embryonic liver.

#### DISCUSSION

The collecting aggregate assay (12-14) was sufficiently improved for its use in probing the molecular character of liver cell-cell adhesion and recognition (the subject of an accompanying paper). The improvements in the assay procedure resulted in a large increase in the rate and extent of liver cell-liver aggregate adhesion. In earlier assays, 0.25% of the input cells adhered in 5 h while the present assay permits 25-70% adhesion in 30 min (14). This increase in the number of cells that adhered to the aggregates eliminates the possibility that the observed adhesion is a property of only a small minority of the dissociated cells (11).

These improvements were achieved by careful evaluation of three basic parameters of the assay procedure. (a) The basic procedures for dissociation and reaggregation were studied in detail. (b) The preparation and the adhesive affinities of collecting aggregates were carefully studied. Moscona (6) has shown that the final (equilibrium) size of an aggregate, at a constant cell density, can be varied depending on the shaker speed. The faster the flasks were shaken, the smaller the final aggregate size. We have prepared collecting aggregates using conditions that favor smaller aggregates (faster shaker speed) than do the standard assay conditions for collection of labeled cells. Cell-aggregate interactions and adhesion may be favored (the equilibrium has been shifted toward larger aggregates) at the slower shaker speed used during labeled cell collection. Also, early aggregates had more irregular surfaces than aggregates that were allowed to shake for a longer time. The earlier, irregular aggregates proved to be better collecting aggregates for labeled cells. After aggregation, secondary events occurred which resulted in the "tightening," "rounding up," and histogenesis of the aggregates. This suggests that adhesion, aggregation, and histogenesis are multi-stage events. (c) The geometry of the collection of the labeled cells by the aggregates was carefully studied. Collection was carried out in a gyratory rather than a reciprocal shaker, to favor cell-aggregate interactions (14). Also, the depth of

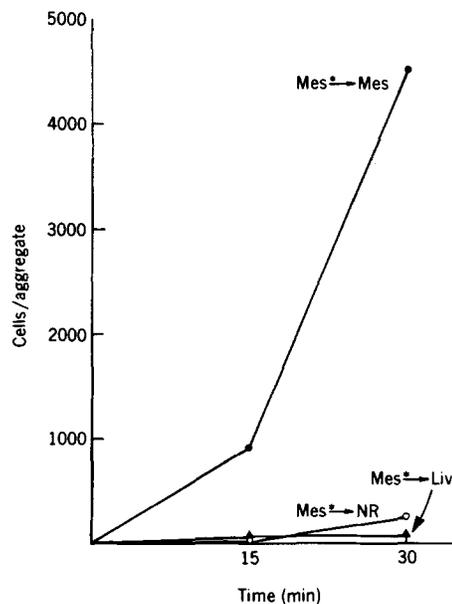


FIGURE 7 The adhesion of mesencephalon cells to mesencephalon (●), neural retina (○), and liver (▲) aggregates. The cell concentration was  $2.9 \times 10^4$  cells/ml ( $1.5$  cpm/cell) with three aggregates per flask and three flasks per time point.

TABLE II  
Tissue Selectivity of Collecting Aggregate Assay

Time	Liver (HOM*/HET)	Time	NR (HOM†/HET)	Time	MES (HOMO§/HET)
<i>min</i>		<i>min</i>		<i>min</i>	
10	$\frac{\text{LIV} \rightarrow \text{LIV}}{\text{LIV} \rightarrow \text{NR}} = 133$ $\frac{\text{LIV} \rightarrow \text{LIV}}{\text{LIV} \rightarrow \text{MES}} = 296$	15	$\frac{\text{NR} \rightarrow \text{NR}}{\text{NR} \rightarrow \text{LIV}} = 13$ $\frac{\text{NR} \rightarrow \text{NR}}{\text{NR} \rightarrow \text{MES}} = 2$	15	$\frac{\text{MES} \rightarrow \text{MES}}{\text{MES} \rightarrow \text{LIV}} = 12$ $\frac{\text{MES} \rightarrow \text{MES}}{\text{MES} \rightarrow \text{NR}} = 21$
20	$\frac{\text{LIV} \rightarrow \text{LIV}}{\text{LIV} \rightarrow \text{NR}} = 183$ $\frac{\text{LIV} \rightarrow \text{LIV}}{\text{LIV} \rightarrow \text{MES}} = 314$				
30	$\frac{\text{LIV} \rightarrow \text{LIV}}{\text{LIV} \rightarrow \text{NR}} = 108$ $\frac{\text{LIV} \rightarrow \text{LIV}}{\text{LIV} \rightarrow \text{MES}} = 188$	30	$\frac{\text{NR} \rightarrow \text{NR}}{\text{NR} \rightarrow \text{LIV}} = 70$ $\frac{\text{NR} \rightarrow \text{NR}}{\text{NR} \rightarrow \text{MES}} = 1.4$	30	$\frac{\text{MES} \rightarrow \text{MES}}{\text{MES} \rightarrow \text{LIV}} = 45$ $\frac{\text{MES} \rightarrow \text{MES}}{\text{MES} \rightarrow \text{NR}} = 16$

\* The ratio of <sup>32</sup>PO<sub>4</sub>-labeled liver cells adhering to liver aggregates (homotypic adhesion) and to neural retina (NR) and mesencephalon (MES) aggregates (heterotypic adhesion) at the indicated times.

† The ratio of <sup>32</sup>PO<sub>4</sub>-labeled neural retina cells adhering to neural retina aggregates (homotypic) and to the indicated heterotypic aggregates.

§ The ratio of <sup>32</sup>PO<sub>4</sub>-labeled mesencephalon cells adhering to homotypic and heterotypic aggregates.

TABLE III  
The Homotypic Adhesive Rate of Embryonic and Chick Liver Cells at Different ages

Age of liver tested	Adhesion rate compared to 8-day liver cell adhesion to 8-day liver aggregates*			
	<sup>32</sup> X → X Agg.	<sup>32</sup> X → 8 Agg.	<sup>32</sup> 8 → X Agg.	<sup>32</sup> 8 → 8 Agg.
	%	%	%	%
12-day embryo	89	113	112	100
15-day embryo	112	133	87	100
19-day embryo	92	108	127	100
2-day chick	33	61	37	100

\* Adhesive Rate of Labeled Cell (<sup>32</sup>X age) to Aggregates → (x Agg.)  
Adhesive Rate of Labeled Cell (<sup>32</sup>8-day) to Aggregates → (8-day Agg.) × 100.

medium, the speed of shaking, and the shape of the bottom of the flask were critical. Small changes in any of these parameters drastically altered collection. The aggregates and the labeled dissociated cells had to be shaken in the center of the flask and in the same orbits. If the cells were in the middle and the aggregates were on the outside, there was no interaction.

The most important aspect of the assay procedure is that it measures tissue selective cell adhesion, which presumably reflects inherent affinities of the dissociated cell population. It should be emphasized that the various alterations in the assay procedure led to large increases in cell adhesion (homo- or heterotypic). No alteration selectively suppressed heterotypic adhesion without affecting homotypic adhesion. We examined three tissues (liver, neural retina, and mesencephalon) by the assay procedure. The homotypic to heterotypic ratio for liver collection (Table I) was high. Previously, the selectivity of the procedure was inferred from the failure of other aggregate types to collect test cells (12-14). The most reliable method for measuring selectivity of collection utilizes reciprocal experiments, which demonstrated the viability and adhesion competence of both the heterotypic cells and aggregates. This was accomplished with two chick tissues other than liver (neural retina and mesencephalon). There was heteroselectivity between mesencephalon and neural retina. Barbera et al. (1) and Gottlieb et al. (2) have also demonstrated neural retina cell adhesion to optic tectum, a constituent of mesencephalon. The neural retina cell adhesion to mesencephalon aggregates which we observed probably represents a normal heterotypic affinity between the cells of the optic tectum and the neurons of the retina that innervate this organ. We are currently extending these studies to other tissues and other species to

examine the adhesive affinities of other cell types.

Other investigators (3, 6, 7) have inferred from the final (equilibrium) size of aggregates obtained that the cell-cell adhesiveness of embryonic tissues decreases with the age of the embryo. We examined this hypothesis and found that a population of adhesion-competent cells was obtained for all ages tested. Livers of older embryos were more difficult to dissociate into single cell populations, which may be due to an increase in the connective tissue present in older livers. There also may be other factors that contribute to this difficulty, such as increasing differentiation of junctional complexes. Any decrease in adhesiveness may reflect the difficulty of dissociating older embryonic or post-natal tissues. It appears that the cellular adhesiveness of embryonic chick liver does not change dramatically through development, and that this adhesiveness is not a unique early embryonic phenomenon.

The kinetics of liver cell-cell adhesion showed that there was tissue selectivity at the earliest times measured. At no time was heterotypic adhesion equal to homotypic adhesion (Table II). This suggests that the earliest stage of liver and neural retina cell aggregation is tissue selective and not random, as has previously been suggested (5, 9). (See Discussion of companion paper.)

We observed no lag time before adhesion began, as has been reported previously (12, 14). This difference is probably best explained by the variation in procedures used by different investigators to dissociate the tissues. In our experiments, extended proteolysis resulted in a lag before cell-aggregate adhesion and introduced a requirement for protein synthesis for adhesion to occur (see accompanying paper). Steinberg et al. (16) have reported that neural retina cells will aggregate without a lag if trypsinization is carried out in the presence of calcium ion or a variety of proteins. Trypsinization in the absence of calcium ion or protein resulted in cells that demonstrated a 30-min lag before aggregation occurred. On the basis of the results of that investigation and our own observations, we suggest that many controversies in the field of intercellular adhesion can be explained by variations in the extent and experimental conditions used during proteolysis of the tissues during dissociation and by the different procedures used to examine the complex, multistep phenomena of intercellular adhesion and histogenesis.

This investigation was supported by Public Health

Service Research Grant no. CA-14431 from the National Cancer Institute.

Received for publication 16 April 1975, and in revised form 21 July 1975.

## REFERENCES

1. BARBERA, A. J., R. B. MARCHASE, and S. ROTH. 1973. Adhesion recognition and retinotectal specificity. *Proc. Natl. Acad. Sci. U.S.A.* **70**:2482-2486.
2. GOTTLIEB, D. I., R. MERRELL, and L. GLASER. 1974. Temporal changes in embryonal cell surface recognition. *Proc. Natl. Acad. Sci. U.S.A.* **71**:1800-1802.
3. KURODA, Y. 1968. Preparation of an aggregation-promoting supernatant from embryonic chick liver cells. *Exp. Cell Res.* **49**:626-637.
4. LILIE, J. E. 1968. Specific enhancement of cell aggregation *in vitro*. *Dev. Biol.* **17**:657-678.
5. MOSCONA, A. A. 1956. Development of heterotypic combinations of dissociated embryonic chick cells. *Proc. Soc. Exp. Biol. Med.* **92**:410-416.
6. 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.
7. MOSCONA, A. A. 1962. Analysis of cell recombinations in experimental synthesis of tissue *in vitro*. *J. Cell Comp. Physiol. Suppl.* **1**, 60:65-80.
8. MOSCONA, A. A. 1965. *Cells and Tissues in Culture*. E. N. Willmer, editor. Academic Press, Inc., New York. 1:489-529.
9. MOSCONA, A. A. 1974. *The Cell Surface in Development*. A. A. Moscona, editor. John Wiley and Sons, New York. 67-99.
10. 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.
11. ROSEMAN, S. 1974. *The Cell Surface in Development*. A. A. Moscona, editor. John Wiley and Sons, New York. 255-271.
12. ROTH, S. 1968. Studies on intercellular adhesive selectivity. *Dev. Biol.* **18**:602-612.
13. ROTH, S., and J. A. WESTON. 1967. The measurement of intercellular adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **58**:974-980.
14. ROTH, S., E. J. MCGUIRE, and S. ROSEMAN. 1971. An assay for intercellular adhesive specificity. *J. Cell Biol.* **51**:525-535.
15. STEINBERG, M. S. 1963. Reconstruction of tissues by dissociated cells. *Science (Wash. D.C.)*. **141**:401-408.
16. STEINBERG, M. S., P. B. ARMSTRONG, and R. E. GRANGER. 1973. On the recovery of adhesiveness by trypsin-dissociated cells. *J. Membr. Biol.* **13**:97-128.
17. TRINKAUS, J. P. 1969. *Cells Into Organs: The Forces that Shape the Embryo*. Prentice-Hall, Inc., Englewood Cliffs, N.J.
18. WALTHER, B. B., R. ÖHMAN, and S. ROSEMAN. 1973. A quantitative assay for intercellular adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **70**:1569-1573.