

DO MORPHOGENETIC TISSUE REARRANGEMENTS REQUIRE ACTIVE CELL MOVEMENTS?

The Reversible Inhibition of Cell Sorting and Tissue Spreading by Cytochalasin B

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ABSTRACT

Previous studies have indicated that cell sorting and tissue spreading are caused by cell combination-specific differences in intercellular adhesive energies, acting in a system of motile cells. We wished to determine whether these adhesive energies could *drive* cell rearrangements as well as guide them. Accordingly, aggregates of intermixed embryonic cells were cultured in solutions of the drug cytochalasin B (CCB) at a concentration shown to inhibit the locomotion of cells on a solid surface. In addition, spherical aggregates of several kinds were cultured in mutual contact under similar conditions. Both cell sorting and tissue spreading were found to be inhibited. The prompt release of this inhibition upon removal of the CCB showed that the inhibited cells were not merely injured. Moreover, aggregation experiments showed that CCB did not prevent cells of several kinds from initiating mutual adhesions. In fact, heart cell aggregation was enhanced by CCB. We conclude that interfacial forces, originating outside the cell, act together with forces originating inside it in bringing about the morphogenetic movements of cell sorting and tissue spreading. We propose the term "cooperative cell locomotion" to describe translational movements of cells arising from such a combination of intrinsic and extrinsic forces.

INTRODUCTION

When the dispersed cells from two different vertebrate embryonic tissues are randomly intermixed in a common aggregate, sorting out typically occurs (Holtfreter, 1944; Townes and Holtfreter, 1955; Moscona, 1957; Trinkaus and Gross, 1961; Steinberg, 1962 *a, b*, 1970). Most often the resulting configuration is that of a spheroidal mass of one reconstructed tissue embedded, either partially or totally, in a sphere of the other tissue (see Steinberg, 1963, 1964, 1970, for review). Likewise, when intact fragments of two different em-

bryonic tissues are allowed to fuse into a single mass, one tissue fragment usually tends to spread around the other. These *in vitro* behavioral systems can serve as models for the analysis of mechanisms and cell properties that may underlie tissue rearrangements during embryogenesis.

Sorting out and tissue envelopment, according to the differential adhesion hypothesis (Steinberg, 1963, 1964, 1970; Phillips, 1969), can be explained as a consequence of (*a*) tissue-specific differences in the strengths of cell-cell attachments,

and (b) the ability of the cells to move. Actual differences in the relevant parameter of intercellular adhesiveness (specific interfacial free energy) have been demonstrated among a number of embryonic chick tissues (Phillips, 1969, and manuscripts in preparation; Phillips and Steinberg, 1969, and manuscripts in preparation). Cell movement may be "active"; that is, it may occur as a consequence of intrinsic cellular motor activity. On the other hand, the necessary cell movement might arise directly as a consequence of the action of interfacial forces. In this kind of "passive" movement the energy is supplied by forces outside the cell (see Abercrombie, 1967; Carter, 1967 *a, b*; Gordon et al., 1972; Steinberg and Garrod, manuscript in preparation). Our intention in these experiments has been to determine whether tissue rearrangements require active cell movements. We have used as a tool in this investigation the drug cytochalasin B (CCB) (Carter, 1967 *b*), which has been observed to inhibit active cell locomotion (Carter, 1967 *b*; Spooner et al., 1971; Wessells et al., 1971; Gail and Boone, 1971; Allison et al., 1971; Zigmond and Hirsch, 1971; Sanger and Holtzer, 1972; Armstrong and Parenti, 1972).

MATERIALS AND METHODS

Tissue Dissociation

Organ primordia were excised from White Leghorn chick embryos in cold Hanks' balanced salt solution. The tissues used were heart ventricles and livers from 5-day embryos, forelimb buds from 4-day embryos, and neural retinas from 7-day embryos. Limb buds, after excision, were first incubated in 1.0% crude trypsin (1:250, Difco Laboratories, Detroit, Mich.) in Hanks' solution at 4°C for 1 hr to loosen the epidermis, which was removed and discarded.

Tissue fragments, after three rinses in calcium- and magnesium-free (CMF) Hanks', were finely minced with microscalpels and transferred to 15-ml, screw-capped culture tubes containing 10 ml of 0.1% crude trypsin in CMF Hanks'. After incubation for 5 min in a 37°C water bath, the tubes were placed in a 37°C test tube rotator (at an angle approximately 10° from the horizontal) for 15 min at 60 rpm. The tubes were then centrifuged at 400 *g* for 4 min, a thick pellet of partially dissociated tissue fragments being formed. The supernatant was discarded and 3-4 ml of standard medium (Eagle's minimum essential medium with Hanks' or Earle's salts as required, supplemented with 10% horse serum, 100 units of penicillin, and 100 μ g of streptomycin/ml) was forced through a Millipore filter (Millipore Corp., Bedford, Mass.)

into the tube. The contents of the tube were sheared on a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.) for 15 sec.

After another centrifugation at 250 *g* for 3 min and the discarding of the supernatant, another 3-4 ml of medium was added and the cells were once again Vortex-mixed for 15 sec. A final centrifugation at 40 *g* brought about the separation of tissue fragments and undissociated cell clumps from the dissociated cells. The upper layer containing the cell suspension underwent further treatment according to the particular experiment (see below).

Locomotion of Cells on a Plastic Surface

Small pieces of liver, less than 1 mm in diameter, were explanted to 35-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) containing the appropriate test solution. These explants were maintained at 37°C and the subsequent outgrowth of cells was observed with an inverted phase-contrast microscope. The coordinates of selected cells were recorded at intervals for 24 hr. When solutions were changed, the dish was rinsed three times with the new solution.

Sorting Out

Suspensions of heart and liver cells, after separate incubation on the test tube rotator for 30 min, were mixed in a 1:1 ratio by cell number, approximately $2-3 \times 10^6$ total cells being added to a 15-ml, screw-capped culture tube. The mixed suspension was centrifuged at 400 *g* for 4 min to form a thick pellet of intermixed cells. This pellet was incubated an additional 3 hr in a 37°C water bath, allowing it to become firm enough to handle. It was then carefully removed to a glass Petri dish and cut with microscalpels into small rectangular pieces which, upon culture, would yield spherical reaggregates 0.25-0.5 mm in diameter (several tens of thousands of cells in each). Individual pieces of the pellet were transferred to a drop of test solution on the underside of the cover of a plastic Petri dish, and the cover was inverted over the bottom of the dish (with medium in it to retard evaporation). These hanging-drop cultures were incubated at 37°C in a 5% CO₂, water-saturated atmosphere. Whenever a tissue mass was transferred to a new solution, it was first rinsed in that solution three times.

Tissues were fixed at various times up to 6 days in Zenker's, embedded, sectioned at 5 μ , and stained with hematoxylin and eosin. The sections were examined microscopically for evidence of sorting out.

Fusions

Pellets of cells were made (as above, although composed of cells from one tissue type only) and

incubated for 5 hr in a 37°C water bath. The pellets were cut up and the pieces were allowed to round up overnight on the gyratory shaker at 140 rpm. Two spheres (each approximately 0.25–0.5 mm in diameter) of tissue to be fused were transferred to a hanging drop of the appropriate test solution, whereupon they fell to the deepest point in the drop and lay in contact with one another at the air-medium interface. These hanging-drop cultures were maintained at 37°C in a 5% CO₂, water-saturated atmosphere, and the course of fusion was observed and photographed.

Cell Aggregation

Before the final low-speed centrifugation used in the preparation of the cell suspension (as above), test solutions were added to the tubes. 2 ml of cell suspension, adjusted to a concentration of 0.5×10^6 cells/ml, were transferred to each of several 10-ml Erlenmeyer culture flasks. The flasks were placed on a gyratory water bath shaker (model G-77, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 70 rpm in a water-saturated atmosphere of 5% CO₂. With an inverted microscope, cell aggregation was observed at various time intervals and photographed on Kodak High Contrast Copy Film directly through the bottoms of the culture flasks.

Cytochalasin B and Dimethyl Sulfoxide

Cytochalasin B (CCB) dissolved in dimethyl sulfoxide (DMSO) (see Carter, 1967 *b*) was added to standard medium at a final concentration of 1 or 10 µg/ml, with DMSO at 1.5% or less. A 50 µg/ml stock solution of CCB in saline solution with DMSO at 7.5% was stored at –80°C. Control cultures were kept in standard medium with and without 1.5% DMSO.

RESULTS

Locomotion of Cells on a Plastic Surface

Small pieces of liver (less than 1 mm in diameter) were explanted to plastic dishes. After a day or two in culture, a halo consisting of individual cells and cell groups was observed on the plastic surface all about the explants in standard medium both with and without DMSO. Very little emigration of cells occurred in the presence of 1.0 µg CCB/ml, although a few cells per explant were seen growing out on the plastic surface. No emigration was seen in the presence of 10.0 µg CCB/ml. Moreover, explants usually failed even to attach to the plastic surface at the latter CCB concentration.

When CCB at 10.0 µg/ml was added to cultures growing in standard medium in which emigration was already under way, continued emigration of cells from the explant was prevented. Those cells whose coordinates had been plotted remained stationary as long as the CCB was present. In such cultures, individual cells that had been attached to the plastic changed, during the course of several hours, from a typically flattened to a more stellate shape, in which long, slender processes protruded from a central, rounded cell body (much as reported by Wessells et al., 1971; Spooner et al., 1971; Allison et al., 1971; and Sanger and Holtzer, 1972). When, after 24 hr, the CCB was again replaced by medium, emigration of cells resumed as before.

Sorting Out

Heart-liver sorting-out experiments were performed twice, using 5–10 intermixed reaggregates per test solution. After 3 days in hanging-drop culture (in standard medium with and without 1.5% DMSO or with 1.0 or 10.0 µg CCB/ml), half the reaggregates in each group were fixed and examined histologically; the other half were transferred to standard medium for an additional 3 days of culture.

At the end of 3 days in standard medium with and without DMSO or with 1.0 µg CCB/ml, extensive sorting out had generally taken place, with liver cells tending to surround an inner group of heart cells. (In many instances, when reaggregates were cultured in DMSO, heart rather than liver cells assumed the external position [see Wiseman et al., 1972].) However, in 10.0 µg CCB/ml, the reaggregated, intermixed pellet pieces failed to round up, and the cells within them failed to sort out (Fig. 1). Small areas of heart cells and liver cells were found interspersed throughout these reaggregates, much the same as in newly formed pellets.

Sorting out was observed in all reaggregates transferred to standard medium for 3 days after experimental treatment, with liver surrounding heart in a majority of reaggregates (Fig. 2). Even those reaggregates in which sorting out had been blocked by higher concentrations of CCB exhibited definite sorting out and rounding up.

Reaggregate Fusions

Fusions were made between the following pairs of spherical reaggregates: heart-heart, liver-liver,

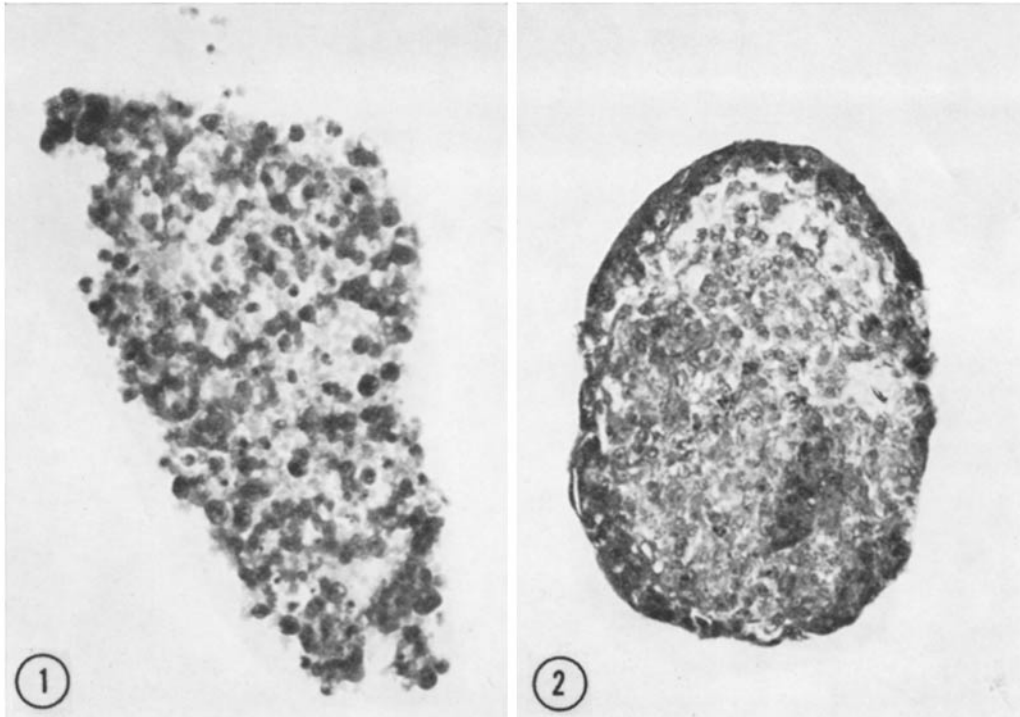


FIGURE 1 CCB inhibition of cell sorting. Mixed aggregate containing heart and liver cells, cultured 3 days in standard medium with cytochalasin B at 10.0 $\mu\text{g}/\text{ml}$. Both rounding up and cell sorting have been inhibited. *Circa* $\times 200$.

FIGURE 2 Recovery from CCB inhibition. Mixed aggregate containing heart and liver cells, cultured 3 days in standard medium with cytochalasin B at 10 $\mu\text{g}/\text{ml}$ and then transferred to standard medium for 3 additional days of culture. An outer rim and an inner island of liver cells have sorted out from the heart cells. *Circa* $\times 200$.

limb bud-limb bud, and liver-limb bud. Each kind of fusion was performed in the same solutions as used in the sorting-out studies above (standard medium with and without 1.5% DMSO, CCB at 1.0 and 10.0 $\mu\text{g}/\text{ml}$). In all cases in which tissues were confronted with one another in standard medium with or without DMSO, tissue spreading occurred to the extent that each pair of tissue spheres rearranged to form a single, spherical mass by the end of 1 day (semispherical masses were formed in the case of liver-limb bud fusions.) However in CCB, tissue spreading was stopped or considerably retarded (Fig. 3).

Tissues did fuse with one another in CCB, as shown by (a) the slight increase, with time, in area of apposition between tissue masses, and (b) the tenacity with which apposed spheres of tissue resisted separation during experimental handling. In CCB at 10.0 $\mu\text{g}/\text{ml}$, essentially no tissue spread-

ing occurred; paired tissues maintained a "dumb-bell" shape for the duration of the culture period (up to 5 days). When the concentration of CCB was 1.0 $\mu\text{g}/\text{ml}$, however, some spreading did occur. In these cases tissues fused together very slowly as compared with controls, tending toward a spherical shape. By 2 days of culture in 1.0 μg CCB/ml, pairs of liver spheres had reorganized to the extent of forming a single, spherical mass. In the other combinations, the area of contact between the two tissue masses had increased considerably, although a modified dumbbell shape was maintained.

These observations were made on hanging-drop cultures in which the solutions were not changed. In liver-liver fusions in which the solution (1.0 μg CCB/ml) was changed five times in the course of 2 days, a single sphere of tissue was not formed, although the area of apposition between the two

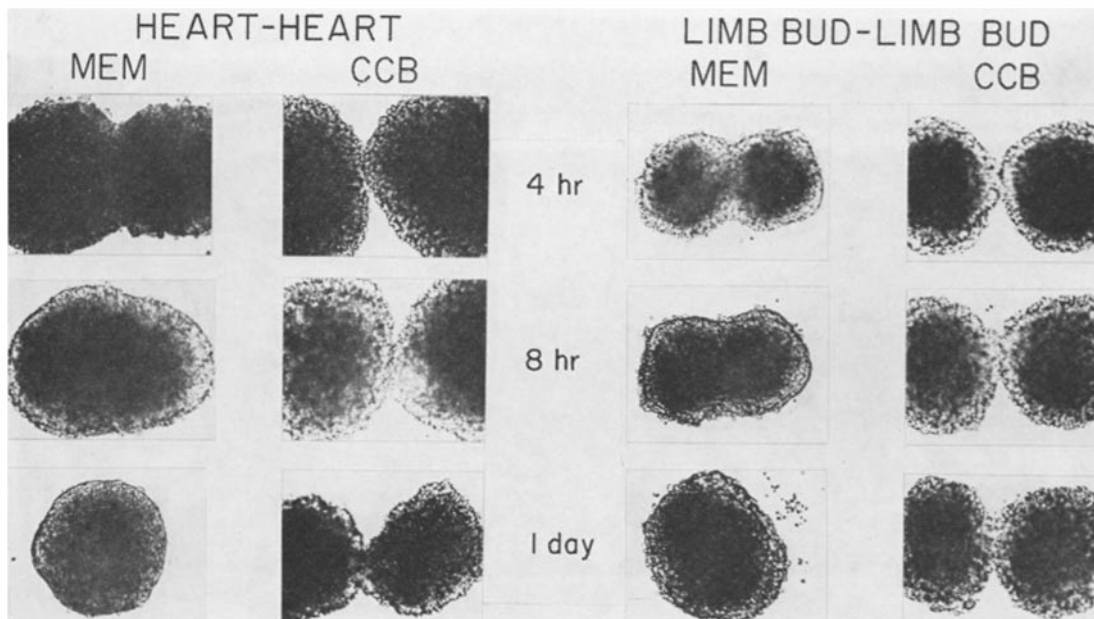


FIGURE 3 Tissue fusions. The time-course of rearrangement of fused heart and limb bud aggregate pairs. Fusions were carried out in standard medium with and without cytochalasin B at $10 \mu\text{g}/\text{ml}$. $\times 50-62$.

apposed spheres did increase. This fact suggests that the CCB is either inactivated or removed in some way. However, a solution of the higher CCB concentration ($10.0 \mu\text{g}/\text{ml}$) which had been used in a hanging-drop culture for 8 days was as active in stopping tissue spreading, when used in a new experiment, as was a fresh CCB solution.

The inhibition of tissue spreading by $10.0 \mu\text{g}$ CCB/ml was readily reversible, as was demonstrated when fusion masses cultured in the higher concentration of CCB were transferred to standard medium (Fig. 4). Pairs of tissue spheres which had not fused into a single, spherical mass during culture for up to 3 days were transferred to medium. They subsequently rounded up into single, spheroidal masses at the same rate and to the same degree as apposed tissue spheres which had been placed directly into medium.

Cell Aggregation

Heart, liver, limb bud, and neural retina cells were all observed to aggregate in standard medium, with and without 1.5% DMSO, and in CCB (plus DMSO) at concentrations of both 1.0 and $10.0 \mu\text{g}/\text{ml}$ of standard medium (Fig. 5). Limb bud cell suspensions reaggregated in the presence of CCB at about the same rate as in

standard medium with or without DMSO. However, reaggregates of both liver and neural retina cells formed in CCB were smaller after 1 day than those formed in either of the control solutions.

The reaggregation of heart cells, on the other hand, was clearly enhanced by the presence of CCB in the medium, definite aggregation being visible after 10-15 min. At the end of 1 day in culture, heart cells in CCB had formed one large, irregularly shaped aggregate in each flask, while cells in medium with or without DMSO had formed a number of small, spherical aggregates. For all tissues, the reaggregates formed in the presence of CCB had fairly irregular contours, while the reaggregates formed in medium with or without DMSO had rounder, smoother profiles.

DISCUSSION

Cell Movement: Active and Passive Components

To move at all, a cell must experience forces. The forces might originate outside the cell or within it, and this is the crux of our distinction between passive and active cell movement. In the latter, cells propel themselves, while in the former they are pulled or pushed by external forces. A

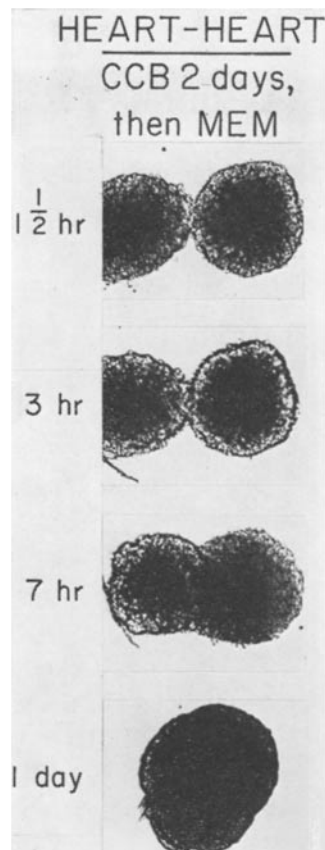


FIGURE 4 Recovery from CCB inhibition. The time-course of rearrangement of a pair of fused heart aggregates in standard medium, after 2 days in $10 \mu\text{g/ml}$ cytochalasin B. $\times 62$.

similar distinction has previously been made by Abercrombie (1967), who has set apart the familiar locomotion of cells on a solid substratum from what he designates as "associative movement": movement, produced by adhesive forces, that "draws the cell towards what it is adhering to." He expresses the "feeling . . . that the rather small cell displacements involved in sorting-out will prove to be of the short-range associative . . . type." Carter (1967 *a*) has proposed that *all* cellular locomotion may be of the latter type, and has coined the term "haptotaxis" to describe the movement of cells propelled by adhesive differentials.

While much evidence supports the view that differentials in adhesive energies *guide* cell sorting and tissue spreading (reviewed in Steinberg 1963, 1964, 1970), there has previously been no experimental test of the possibility that such energies

drive these movements as well. We wish to draw attention here to the fact that passive and active processes, i.e. adhesive differentials and intrinsic cellular motile activity, may potentially contribute in different degrees to the locomotion of cells in various circumstances.

WHOLLY ACTIVE CELL LOCOMOTION: Cell locomotion and cell adhesion are interlinked phenomena; a cell could not locomote on a surface to which it does not adhere. A given cell might translocate by exerting force upon its substratum via contractile machinery in or near its periphery. Here the solid environment provides only a surface to which the cell adheres sufficiently (but not overly) strongly. The cell affords all of the energy for movement. The locomotion of cells in ordinary tissue cultures is regarded by many as belonging to this category.

In a variant of the above case, a cell might extend a filopodium, attach it to a nearby object, and then contract it, pulling the cell toward the object. The cell still provides all of the energy for movement, but here it is especially obvious that certain adhesive relationships must exist for the described movement to occur. The object must be rooted strongly enough, the filopodium must adhere to the object strongly enough and have sufficient tensile strength, and the force must be exerted in such a way that the filopodium neither pulls loose nor snaps and that it is the cell rather than the object that moves. The primary mesenchyme cells of sea urchin gastrulae may fall within this class (Gustafson and Wolpert, 1961; Gustafson, 1963).

Any instance of cell translocation on a substratum necessitates a choice to give up certain adhesions in favor of others, and thus involves a competition among adhesions in which the stronger ones will tend to replace the weaker. Preexisting adhesive anisotropies may provide such a choice; or a large, new area of adhesion may be pitted against a smaller area of "old" adhesion, causing the latter to yield; or the strength of an adhesion may decay with age, causing new adhesions in general to be stronger than older ones, as proposed by Carter (1967 *a*). Thus, interfacial energies unavoidably play a role in even the most active of cell movements. Nevertheless, if environmentally originating heterogeneities play no role in influencing a cell's locomotion, the latter could be regarded for practical purposes as being wholly active.

WHOLLY PASSIVE CELL LOGOMOTION:
 It may be that in certain cases no pseudopodia, filopodia, or the like are extended at all. Because each cell in a solid tissue has some 12 or so neighbors, while each cell in a simple sheet or monolayer has about six, there is in both cases the opportunity for a diversity of preexisting contacts. If sufficient inequalities in adhesive energy exist

among the various regions of contact, the greater energy of the stronger contacts will cause these to "zip up" at the expense of the weaker ones, which will tend to yield. Rearrangements that occur in this way would be propelled exclusively by interfacial forces of adhesion and could be regarded as entirely passive. One could easily imagine the sorting out of cells in a mixed aggregate to pro-

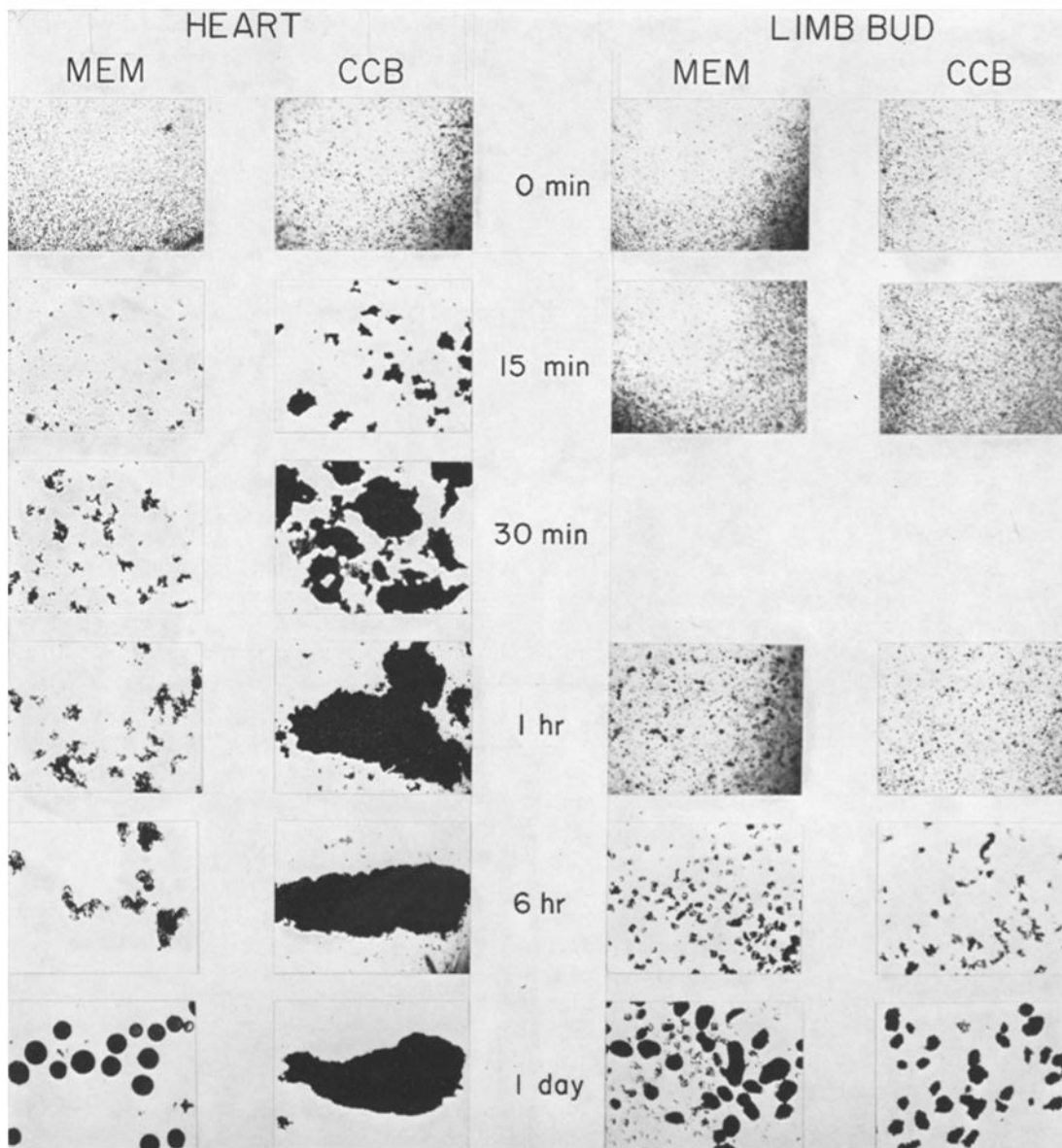


FIGURE 5 Cell aggregation. The time-course of aggregation of heart cells and of limb bud cells in standard medium with and without cytochalasin B at 10 µg/ml. $\times 12$.

ceed in this way, as Abercrombie has already pointed out (see above). The advance of a cell sheet could in principle also be propelled in this way, much as a film of a spreading oil advances over a body of water.

COOPERATIVE CELL LOCOMOTION: Consider a cell actively moving across an anisotropic surface, to some parts of which it adheres more strongly than to others. The interfacial energies of differing magnitude will exert a directing or guiding influence upon the cell's movements. This influence will contribute a passive component, perhaps only a small one, to the energy causing the translocation of the cell. Even though the cell itself still provides most of the motor energy, its locomotion reflects a synergism of internally and externally originating forces.

In yet another instance, a cell might extend and attach a filopodium to a nearby object, but the filopodium, instead of contracting, might adhere to the object so strongly as to produce a zipping up of the two. Barring the snapping of the filopodium, this would result in the pulling of the cell toward the object, the object toward the cell, or both at once, depending upon the relative firmness of their moorings. In this case, the energy required for movement would clearly be substantially divided between two sources. The extension of the filopodium would presumably require the expenditure of energy by the cell, but the zipping up of the surface of the filopodium against the surface of the object would result from the interfacial forces of adhesion between the two. The immediate "cause" of the resulting movement itself as well as the immediate source of its guidance would lie in these interfacial forces. However, expenditure of energy by the cell to extend filopodia would be required to provide the geometric conditions necessary for these interfacial forces to be brought into play. We propose the term "cooperative cell locomotion" to describe situations in which forces of internal and external origin both contribute to the propulsion of a cell.

Involvement of Active Cell Movements in Cell Sorting and Tissue Spreading

The purpose of the present experiments was to determine whether cell sorting and tissue spreading could occur in the presence of sufficient cytochalasin B to inhibit active cell motility. A positive result would presumably mean that forces of

intercellular adhesion are sufficient not only to *guide* such morphogenetic rearrangements but to *drive* them as well. Cytochalasin B at 10 $\mu\text{g}/\text{ml}$ was found to inhibit the sorting out of chick embryonic heart and liver cells in mixed aggregates and the mutual spreading movements of fused aggregates of several kinds. This concentration of the drug was shown to prevent the locomotion of chick embryonic liver cells on a Falcon plastic surface. That the treated aggregates and cell cultures were not severely harmed by the experimental treatment was shown by their prompt initiation of sorting or spreading activities when the drug was removed.

Because the cell rearrangements studied here depend upon mutual cellular adhesiveness as well as upon cell motility, their prevention by a drug might result from interference with the adhesive capacities of the cells as well as from inhibition of active cell movements. Indeed, Sanger and Holtzer (1972) have concluded that "CCB does interfere with adhesive interactions between various cells." Their conclusion was based upon their observation that pellets of intermixed dissociated cells neither tighten up nor sort out. We have confirmed this observation, but, because of tests reported below, attribute such behavior to the inability of the immobilized cells in the pellet to *move* on one another's surfaces and thus to increase their area of mutual adhesion in the presence of the drug. In order to assess the ability of cytochalasin B to inhibit the *adhesive* capacities of dissociated chick embryonic cells, we performed standard tests of cell aggregation in liquid suspension with and without the drug. In these tests CCB was without effect upon the aggregation rate of limb bud cells, slightly decreased the aggregation rate of liver and neural retina cells, and actually *enhanced* the aggregation rate of heart cells (Fig. 5). The ability of the already-formed aggregates to round up was inhibited (Fig. 1). These findings are in agreement with our observation that spherical aggregates paired in cytochalasin B initiate mutual adhesions strong enough to survive even quite rough manipulation.

We conclude that cytochalasin B inhibits cell sorting and tissue spreading by inhibiting the cells' capacity to engage in active movements. Cell sorting and tissue spreading thus appear to present examples of cooperative cell locomotion, in which forces originating outside the cell act together

with those originating inside it to propel the cell and give direction to its movements.

The decrease in interfacial free energy that accompanies cell sorting or tissue spreading would be greater in some cell combinations than in others. In the particular combinations we have studied, these interfacial forces appear to have been too weak to overcome friction and actually *drive* the rearrangement; assistance from active cell movements was required. Armstrong and Parenti (1972) have now found a cell combination that *does* undergo sorting out in cytochalasin B. Evidently in the combination of neural retina with pigmented retina, interfacial forces between the cells are sufficiently strong to *drive* cell sorting in the absence of active cell movements.

A preliminary report of the present investigation has appeared (Wiseman and Steinberg, 1971).

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