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Is Cell Sorting Caused by Differences in the Work of Intercellular Adhesion? A Critique of the Steinberg Hypothesis

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The differential adhesion hypothesis, developed by Malcolm Steinberg, proposes that the histotypic sorting out behavior of aggregated cells is mechanistically equivalent to certain aspects of liquid surface tension, specifically the spontaneous separation of immiscible liquids of differing surface tension. According to Steinberg's hypothesis, the adhesive forces between aggregated cells play essentially the same role in cell sorting as are played by intermolecular attractive forces in liquid surface tension.

In this paper I discuss a number of crucial distinctions between intermolecular attraction (in liquids) and intercellular adhesion (in aggregates). First, liquid drops are closed systems thermodynamically whereas aggregates of living cells can generate an indeterminate amount of metabolic energy capable of altering cell positions and adhesions. Secondly, intercellular adhesions are more than just close range attractions since cells can be held together by forces in addition to those which originally pulled them together. Third, the breakage of intercellular adhesions need not be simply the reverse, thermodynamically, of the formation of those adhesions. And fourthly, because intercellular adhesion is generally concentrated at relatively small foci such as desmosomes, a maximization of intercellular adhesion does not necessarily require a maximization of intercellular contact area, or vice versa.

In addition, several alternative hypotheses are proposed, each of which is theoretically capable of explaining cell sorting and the other surface tension-like aspects of cell aggregate behavior which Steinberg has sought to explain as consequences of differential adhesion. In particular, a differential surface contraction hypothesis is proposed, according to which cell sorting and related phenomena are the results of cell surface contractions induced to occur over those portions of the cell surface which are exposed to the surrounding culture medium. Because of the evidence that various invagination type movements of embryonic epithelia are caused by cell surface contractions, it is suggested that differential surface contraction is the most likely explanation of histotypic cell sorting. A number of experiments are suggested by which these various hypotheses might be tested.

1. Introduction

Various metazoan cells have the remarkable ability to sort out from one another, even after having been dissociated and reaggregated randomly into a jumbled mass. This phenomenon was first encountered by Wilson (1907) in his studies on sponges, and Townes & Holtfreter (1955) have subsequently shown that dissociated cells of early amphibian embryos can sort out from one another according to their germ layer or organ rudiment of origin. Likewise, a similar capacity of embryonic bird and mammal cells to sort out according to histological tissue type was observed by Moscona (1952, 1957), and by Trinkaus & Groves (1955). The patterns of histospecific sorting achieved by dissociated cells of higher animals have been most completely described by Steinberg (1963, 1970 review). In most cases, when cells from two embryonic tissues are dissociated and recombined, the cells of one tissue type form a layer surrounding those of the other tissue type. For example, when heart cells and precartilage cells sort out from one another, the heart cells form a continuous exterior layer surrounding an internal mass of cartilage cells. In such a case, the heart cells are said to have sorted out (or segregated) external to cartilage, and conversely, the cartilage cells are described as having sorted out (or segregated) internally with respect to the heart cells.

Cell sorting has now become one of the most important *in vitro* model systems for the study and interpretation of the morphogenetic cell rearrangements of early metazoan development. Consequently, cell sorting has been the subject of a great deal of theoretical speculation [see Trinkaus (1965, 1970) for reviews], and a variety of hypotheses have been proposed regarding which cellular properties might cause sorting out and determine whether a given cell type will sort out internally or externally to another. For example, Townes & Holtfreter (1955) attributed the sorting out of their amphibian cells to a hypothetical selective intercellular adhesion combined with a tendency of some cell types to undergo directed locomotion away from the aggregate surface, thus segregating internally.

2. The Differential Adhesion Hypothesis

Certainly the most successful and widely accepted theoretical explanation of cell sorting is the "differential adhesion hypothesis" proposed by Steinberg (1963, 1970). According to this hypothesis, histotypic cell sorting, as well as several other aspects of cell behavior (to be described in part 3 below), are mechanistically equivalent to the surface tension behavior of liquids.

Surface tension is the force which tends to minimize the surface area of liquids, causing individual liquid drops to round up and also causes the molecules of two immiscible liquids to sort out from one another when mixed (Adam, 1941). Liquid surface tension is a well understood physical phenomenon and is known to result from the intermolecular attractive forces which pull the individual liquid molecules toward one another. The surface area of liquids tends to shrink because these molecules which lie at the surface are subject to unequal attraction by their neighbors. Surface molecules are attracted by the molecules on their sides and by those "below" them, in the interior of the liquid, but they are not attracted from outside the liquid where there are no other liquid molecules. As a consequence, surface molecules are subject to a net inward force, pulling them into the liquid interior and tending to shrink the surface. This inward force is proportional to the strength of the intermolecular attraction in a given liquid. The resultant force parallel to the surface is called the surface tension.

The effect of this force may of course be summed per unit area of surface and expressed as a surface free energy, equal to the reversible work required to expand the surface area by a unit amount. This surface free energy corresponds to the increased free energy of molecules at the surface relative to those in the interior, due (primarily) to the reduced energy of intermolecular attraction of surface molecules, as compared with molecules in the liquid interior.

These same forces of intermolecular attraction can also cause immiscible liquids to "sort out" from one another. When two such liquids are mixed, the molecules having the stronger attraction for one another are drawn together by their mutual attraction to form a central mass, surrounded by the liquid whose molecules attract one another less strongly. By this rearrangement of liquid molecules relative to one another, total intermolecular attraction is maximized, and so the total free energy of the system is minimized.

As Steinberg (1963, 1970) has pointed out, this "sorting out" of immiscible liquids is strongly reminiscent of histotypic cell sorting. Accordingly, the differential adhesion hypothesis seeks to explain cell sorting as a result of a tendency of cells to move so as to maximize their intercellular adhesion just as liquid molecules move so as to maximize their intermolecular attraction. In other words, the differential adhesion hypothesis treats the cells of an aggregate as being equivalent to the molecules of a liquid drop, and likewise regards the forces of adhesion between cells as being equivalent to the forces of attraction between liquid molecules.

For example, the internal segregation of precartilage cells relative to heart cells would be interpreted as the result of the precartilage cells being more adhesive to one another than are the heart cells. Likewise, this hypothesis

holds that the ability of cells to sort out from one another reflects quantitative differences in their work of intercellular adhesion, that is, the greater the amount of adhesive work done (per area of cell surface) in forming intercellular adhesions, the more internally a given cell type will sort out relative to other cell types. As will be discussed more fully below, this hypothesis has even been extended to propose that the surface tension-like behavior of tissue aggregates can actually be used as a measure of the work of intercellular adhesion. (Phillips & Steinberg, 1969.)

It may be noted parenthetically that although the differential adhesion hypothesis explains cell sorting as the consequence of quantitative differences in intercellular adhesion, it in no way excludes, nor is it contradicted by, the existence of qualitative differences in intercellular adhesion. The theory merely holds that it is the quantitative rather than the qualitative differences which are responsible for histotypic cell sorting.

3. The Experimental Evidence Supporting the Differential Adhesion Hypothesis

In support of this hypothesis, Steinberg and his students have produced three principal bodies of experimental evidence (see Steinberg, 1970, for a more complete summary).

First, they have shown that disaggregated cells of a series of six different tissues sort out from one another according to a transitive hierarchical sequence of internal versus external segregation. Listed according to their relative position of segregation, with the more internally segregating tissues being listed first, these tissues are (1) epidermal epithelium; (2) precartilage; (3) pigmented epithelium; (4) heart; (5) neural tube, and (6) liver.

The crucial observation here is that this hierarchy is transitive, that is, if cell type A sorts out internal to B and if B sorts out internal to C, then cell type A will always sort out internal to C. This was found to be true for all the different combinations of the six tissues listed above (Steinberg, 1970) and is the crucial piece of evidence that cell sorting must be attributable to quantitative (rather than purely qualitative) variations in some cell property or properties. The probability of cell sorting being transitive merely by chance difference in qualitatively specific cell properties would be only one in 45, as Steinberg (1970) has demonstrated mathematically. Unfortunately the existence of such a transitive hierarchy does not prove in itself that the quantitative variable responsible for cell sorting is necessarily intercellular adhesiveness. This transitiveness of the hierarchy proves only that quantitative variation in some property (or properties) is responsible. A number

of other quantitatively variable properties will be discussed below which could in principle produce much the same results.

The second type of evidence supporting the differential adhesion hypothesis is Steinberg's demonstration that cell clumps of different tissue types engulf one another when placed in contact, and that the relative internal and external positions achieved by such engulfment correspond exactly to the hierarchy of cell sorting discussed above. That is, if cell type A sorts out internally to cell type B, then fragments of cell type A will always be engulfed by fragments of cell type B whenever such fragments are cultured in contact with one another. (Steinberg, 1962*c*, 1970.)

This pattern of engulfment is, of course, precisely what is predicted by the differential adhesion hypothesis, and these results parallel closely the behavior of immiscible liquids of different surface tension. Consequently, this pattern of tissue engulfment must be taken as conclusive proof that whatever the quantitative variable is which is responsible for cell sorting, this same variable must also be responsible for tissue fragment engulfment. However, such evidence still does not prove that the responsible variable is necessarily intercellular adhesiveness.

The third category of evidence advanced in support of the differential adhesion hypothesis is that of Phillips & Steinberg (1969) who showed that cell aggregates will round up and resist flattening in a centrifugal field, and that they will resist this flattening to a degree which is tissue specific and which varies quantitatively among different tissues according to the very same hierarchy as that previously observed to govern cell sorting and fragment engulfment. That is, the more internally a cell type segregates, the more its aggregates resist flattening. Thus if cell type A sorts out internally to cell type B, then aggregates of cell type B will become more flattened by a given centrifugal field than will aggregates of cell type A. Although not all of the six cell types were actually studied by centrifugal flattening, nonetheless this work does provide quite conclusive evidence that the cell property which governs the degree to which cell aggregates resist centrigual flattening is the same quantitatively varying property which is also responsible for cell sorting and for fragment engulfment. Using this method Phillips attempted to calculate the "specific interfacial free energy of intercellular adhesion," which he calls "sigma" (Phillips, 1969).

In addition, Phillips & Steinberg (1969) also showed that cell aggregates of a given cell type achieve the same equilibrium degree of flattening in a centrifugal field (of a certain strength) whether these aggregates had initially been flat and had rounded up in the centrifuge, or whether they had initially been spherical and had been flattened in the centrifuge. This proves that the forces which cause aggregates to round up are the same as (or at least equal to) the forces which resist the flattening of aggregates.

According to the differential adhesion hypothesis, the cell property responsible for these various surface tension-like effects is adhesiveness, or more specifically, the reversible work of intercellular adhesion. To quote Steinberg (1970), "the differential adhesion hypothesis attributes sorting out behavior to differences in the strengths of intercellular adhesion. It suggests that cells in mixed population rearrange themselves to minimize their total adhesive free energy." Similarly, the differential adhesion hypothesis explains the tendency of cell aggregates to round up because, by rounding up, "an aggregate minimizes its external surface area thus maximizing internal cell–cell adhesion . . . The driving force for this process arises from the formation of intercellular adhesions." (Phillips & Steinberg, 1969.)

Initially, it does seem quite reasonable to conclude that since liquid surface tension is the result of intermolecular attraction, then the surface tension-like behavior of cell aggregates (sorting out, engulfment, rounding up) must likewise be the result of intercellular attractions (i.e. adhesions). Unfortunately, however, this apparently logical conclusion, upon which the differential adhesion hypothesis is founded, is fallacious. Not only are there a number of alternative ways in which surface tension-like behavior can be explained, other than by attraction between the component parts, but because of several crucial differences between adhesion and attraction, differences in adhesiveness alone would not produce the predicted results.

As Adam (1941) has pointed out in relation to the analysis of liquid surface tension, "any mechanism possessing free energy in the surface will undergo the spontaneous contraction which has led to the idea of surface tension: hence, we can gain practically no idea of the actual nature of the mechanism in the surface from this fact of spontaneous contraction alone." In other words, any circumstances in which the free energy of a system increases with surface area will result in a tendency to minimize this surface area, regardless of the specific reason why the free energy of the system happens to increase with surface area. Thus, the simple fact that a surface area tends to be minimized tells one little about the forces responsible for this tendency, and the same can be said of the other manifestations of interfacial free energy, such as the separation of immiscible liquids.

Thus while the results of Steinberg and his students prove that cell sorting is the result of some microscopic cell property which produces macroscopic effects much like liquid surface tension, these experimental results are not sufficient to prove that the microscopic property responsible for these effects is the reversible work of intercellular adhesion. In the following sections I shall present a number of reasons why intercellular adhesion alone would not produce these effects, as well as several alternative hypotheses which are capable of explaining Steinberg's observations.

4. Crucial Differences Between Liquid Drops and Cell Aggregates

Steinberg's analogy between liquid surface tension and the surface tension behavior of cell aggregates is based upon a number of assumptions about cell adhesion and cell locomotion, and these assumptions need to be analyzed in detail. Comparing the situation of molecules in a liquid to that of cells in an aggregate, Steinberg says, "It is of no substantive consequence that the units happen to be molecules and that their motility happens to be passive rather than active in nature. These properties [i.e. surface tension and its consequences] are independent of the composition of the units [i.e. whether they are molecules or cells], of the cause of their motility, and independent of the nature of the adhesive forces. For example, a liquid drop assumes a spherical shape when subjected to uniform external conditions, because the mobile units of which it is composed attract or adhere to one another until the greatest possible number have the maximum possible contact. Adhesion being nothing more than close range attraction, the same holds true for a population of actively motile uniformly adhesive cells." "The free energy . . . will tend spontaneously to decrease toward a minimum in any population of mobile coherent units. At this minimum the system is in thermodynamic equilibrium." (Steinberg, 1963, material in brackets added.) Thus, the differential adhesion hypothesis holds that cell sorting, fragment engulfment, rounding up, etc., are simply consequences of the cells' tendency to maximize their work of intercellular adhesion, and that they are independent of the chemical causes of intercellular adhesion and of the mechanism of cell motility.

Although there should be no doubt that the cells of an aggregate are as much subject to the laws of physics as are the molecules of a liquid, Steinberg's assertions quoted above can be challenged on at least four grounds.

First, cells are alive and molecules are not. This is no vitalistic quibble, but an essential thermodynamic consideration. A liquid drop is a closed system thermodynamically in which we can assume not only that no energy either enters or leaves, but also that no new energy is generated within. In fact, our ability to predict the behavior of the liquid drop on purely thermodynamic grounds depends upon this assumption that "new" energy is not generated within the system. In contrast, when we are considering the behavior of living cells, we have every reason to expect that an indeterminate amount of "new" free energy is being generated within the system. In other words, the system is an open one thermodynamically, and so configurational changes in such an open system cannot so confidently be attributed to the minimization of adhesive free energy. It is much more likely that the cell configurations are the result of steady state pseudoequilibria created and maintained by the

continued expenditure of energy. On the other hand it is not inconceivable that the influence of metabolic energy on cell configuration might turn out to be negligibly small, or might somehow cancel out, but one cannot simply ignore this influence.

A *second* crucial objection can be raised against Steinberg's specific assertion (quoted in full above) that "adhesion is merely close range attraction". Although this is true for some types of intercellular adhesion, it is not true for all. Of course the intermolecular attractive forces which pull the molecules of a liquid together really are the same as the forces which hold them together, whether these forces happen to be electrostatic forces, hydrogen bonds, or van der Waals forces. Thus at the molecular level, the forces of attraction really are the same as the forces of adhesion.

However, in the case of relatively large objects, such as cells, this equivalence between attraction and adhesion does not necessarily hold. For example, certain types of adhesion form only after the adhering objects are already in contact, and in such cases adhesion is much more than just "close range attraction". As examples, we may consider adhesion by covalent bonds linking cells, adhesions by antibody–antigen or enzyme-substrate-like complexes, or certain specialized junctions such as desmosomes which electron microscopic observations suggest form only after cell surfaces are juxtaposed (Overton, 1962). Adhesions of these types do involve attractive forces but the formation of the adhesions would not pull cells together, and that is the crucial distinction. So it should be clear that adhesion can be much more than just close range attraction.

On the other hand, intercellular adhesion by van der Waals forces, hydrogen bonds, or electrostatic attraction would be equivalent to close range attraction; that is, such forces would pull cell surfaces closer together as well as hold them there. The essential point to be made here is that the differential adhesion hypothesis tacitly assumes that intercellular adhesions are all of this latter type, and excludes from consideration those adhesions which form after contact is made and which therefore do not pull cell surfaces together.

A *third* problem with the differential adhesion hypothesis (closely related to the first and second above) is its assumption that the breakage of adhesions between cells is simply the reverse (mechanically and thermodynamically) of the original formation of adhesions. This assumption about the reversibility of adhesions would be valid *only* if intercellular adhesions were entirely the result of short-range attractive forces such as van der Waals forces, electrostatic forces, etc.

When such attractive forces pull cells together, these forces do mechanical work upon the cells' position and this mechanical work of adhesion is thermodynamically reversible. In principle, pulling such cells apart requires

doing the same amount of mechanical work to overcome the attractive forces as was done by these forces in pulling the cells together initially. Thus, with respect to adhesion by attractive forces only, the work of adhesion really is the reverse of the work of de-adhesion, these two works having the same value but opposite sign. This is the "reversible work of adhesion" which, according to the differential adhesion hypothesis, is the parameter responsible for the surface tension-like behavior of cell aggregates. However, when cells are held together by forces different from (or in addition to) those which originally pulled them together, the work of de-adhesion need not be equal to the work of adhesion, so in such cases the work of adhesion is not simply the reverse of the work of de-adhesion.

This difficulty may be clarified by an example from ordinary life. Objects may be stuck to one another using an epoxy or other polymerizing resin. Such objects must first be brought together and the resin then polymerized between them. The only "work of de-adhesion" would be the physical work done in bond breakage and would have no necessary relationship to the "work of adhesion" involved in polymerization.

Specifically, in the case of intercellular adhesion, the work of adhesion would differ from the work of de-adhesion whenever additional intercellular bonds are formed after the cell surfaces are first brought together, whenever the "break plane" of separation differs from the original plane of adhesion, or whenever cells become attached either by covalent chemical bonds or by enzyme-substrate-like complexes (i.e. those depending upon a conformational fit between molecules). In all of these cases the adhering cells would be held together by forces in addition to those which originally brought their surfaces together. So whenever the breakage of intercellular bonds is not simply the reverse of their formation, the work of de-adhesion will be different from the work of adhesion. Consequently the phrase "reversible work of adhesion" really has no meaning when applied to cases in which de-adhesion is not simply the reverse of adhesion.

For example, if we imagine two cell types, both of which exert short range attractive forces of equal strength, but only one of which forms intercellular covalent bonds after contact, how could the differential adhesion hypothesis predict how these cells would sort out from one another? Such questions need to be answered before one can speak of the "reversible work of adhesion" being measurable by the rounding up of aggregates.

A *fourth* important difference between cells and molecules lies in the localization of (many) intercellular adhesions and the mobility of these adhesions across the cell surface. To a great degree, intercellular adhesion is known to be concentrated in specialized junctions (desmosomes, tight and gap junctions, etc.), and these junctions occupy only a very small fraction

of the total cell surface area (McNutt & Weinstein, 1973). Thus in principle it would be possible for a cell within an aggregate to move from the aggregate interior to the aggregate surface without sacrificing any of these specialized intercellular junctions, assuming that these adhesions could simply "slide" across the cell surface to the area still juxtaposed to other cells. Conversely, surface cells moving to the interior would not necessarily gain new intercellular adhesions in proportion to their increase in total area of contact with other cells. This is contrary to the basic tenet of the differential adhesion hypothesis that cell aggregates tend to minimize their surface area because only in this way can they maximize their area of intercellular adhesion. Consequently (as will be discussed more fully in part 5 below) an aggregate of cells adhering to one another only by small mobile focal adhesions would tend to minimize the aggregate surface area in proportion to the mobility, rather than the strength, of these adhesions. The essential point to be made here is that because adhesiveness is not distributed equally and homogeneously over the cell surface, a maximization of intercellular contact need not involve the maximization of intercellular adhesion (nor is the converse necessarily true).

5. Some Alternatives to the Differential Adhesion Hypothesis

As discussed in part 3 above, the available evidence produced by Steinberg and others is quite sufficient to prove that histospecific cell sorting results from quantitative differences in some cell property, the effects of which on cell aggregates closely resemble the surface tension behavior of liquids. That is, aggregated cells tend to minimize their area of exposure to the surrounding medium, while maximizing their contact with one another, and in mixed aggregates, cells of like histological type tend to maximize their contact with one another while minimizing contact with cells derived from different tissues. In addition, the evidence shows that the strength of these tendencies varies quantitatively by cell type in a known sequence. Thus cell aggregates round up, resist flattening, engulf one another, and their cells sort out. The question remains, however, what property of the individual cells is responsible for these tendencies to maximize intercellular contact, etc. Is it the force of attraction between cell surfaces which produces these effects, as Steinberg contends in his differential adhesion hypothesis, or could other cellular properties be responsible? In the preceding section a number of reasons were advanced for doubting that intercellular attraction can be the cause of these effects, and in this section a series of three alternative hypotheses will be proposed, each of which seeks to account for cell sorting and related phenomena by qualitative variations in a different cell property.

(A) FIRST ALTERNATIVE: A DIFFERENTIAL SURFACE CONTRACTION HYPOTHESIS

This alternative hypothesis is suggested by one of the early theories developed by physicists to account for the surface tension of liquids. Although the idea has long since been discarded, it was at one time believed that liquid surface tension probably resulted from the formation of a contractile surface film of molecules at the air–liquid interface (Adam, 1941). According to this theory, the surface tension of a given liquid would simply reflect the contractility of the molecular film formed at its surface, i.e. the more strongly contractile the film, the greater the surface tension. Actually, this interpretation of surface tension was quite difficult to disprove, for the very simple reason that it could account successfully for all the observed macroscopic phenomena of surface tension, including the rounding up of drops, the separation of immiscible liquids of different surface tension, etc. Eventually, this surface contraction theory of surface tension was supplanted only because it was inconsistent with the known properties of individual liquid molecules, which are not contractile. On the other hand, living cells certainly are contractile, and so this surface contraction hypothesis which turned out to be wrong as an explanation for liquid surface tension could nevertheless be the correct explanation for the surface tension-like behavior of cell aggregates, including sorting out behavior.

Indeed there is a considerable body of ultrastructural evidence showing that the infolding of various epithelial rudiments during embryonic development really is caused by the contraction of cortical layers of actin-like filaments concentrated just within the exposed apical surfaces of the infolding cells (Baker, 1965; Baker & Schroeder, 1967; Karfunkel, 1971; Burnside, 1971; Spooner & Wessels, 1972). While observations of embryonic rudiments might seem at first irrelevant to the behavior of cell aggregates, we should recall that the differential adhesion hypothesis is explicitly intended by its author to explain morphogenetic movements such as invagination as well as events *in vitro* (Steinberg, 1970).

Furthermore, several observations by Townes & Holtfreter (1955) suggest strongly that the sorting out behavior of embryonic amphibian cells, as well as the engulfment of embryonic tissue fragments by one another, must be attributed to the same basic cellular mechanism as that which causes invagination. Specifically, Townes & Holtfreter (1955) found that when neural plate cells were bound together as a sheet, they would penetrate into masses of endoderm by infolding (as in neurulation), but that when these same neural plate cells were dissociated, they would sort out within the endodermal mass as individual cells. Similarly, a solid clump of such neural plate cells was found to be engulfed as a whole by the endodermal cells. Referring

to this almost uncanny ability of the neural plate cells to achieve an internal position within the endoderm either by invagination *or* by engulfment *or* by sorting out, Townes & Holtfreter (1955) remark that "one might wish to find a common mechanism for these three modifications of inward movement". Therefore, since the invagination of the neural plate has subsequently been found to be caused by the differential contraction of the exposed outward cell surfaces (Baker & Schroeder, 1967; Karfunkel, 1971; Burnside, 1971), it seems most likely that such a surface contraction is also the cause of engulfment and of histotypic sorting out.

To explain histotypic cell sorting, the rounding up of fragments, engulfment, etc. by a differential cell surface contraction hypothesis, one needs to make two basic postulates. The first postulate is that the cell surface cortex should be differentially contractile, with the force of its contraction becoming greatest where the cell's surface is exposed to the surrounding medium, and being least wherever a cell's surface is juxtaposed to another cell of the same histological type. The cell surface should contract to an intermediate degree wherever it is juxtaposed to a cell of a differing histological type (this is to account for the rounding up of cell masses of one type embedded within cell masses of another type). The second basic postulate is that some histological cell types should be more strongly contractile than other cell types, where they contact the medium. The more strongly contractile a given cell type is over its exposed surface, the more internally it should sort out relative to other, less contractile, cell types. It should be made clear at this point that this hypothesis also presumes a degree of intercellular adhesiveness, and differs from Steinberg's hypothesis not in respect to whether intercellular adhesiveness occurs, but as to whether cell sorting and related phenomena are attributable to quantitative differences in adhesiveness.

How do these two postulates allow us to explain each of the observations upon which the differential adhesion hypothesis rests? The rounding up of aggregates can be explained by the formation of a contractile layer consisting of the exposed portions of all of the cells located at the aggregate surface, all of them together forming what would amount to a single (multicellular) sheet surrounding the aggregate. The contraction of this surface layer would tend to minimize the exposed surface area of the aggregate as a whole, thereby causing it to round up and to resist centrifugal flattening. The more strongly contractile the surface layer, the more the aggregate would resist flattening. Consequently, the parameter "sigma" which Phillips (1969) calculated from his centrifugal flattening data, and which Phillips and Steinberg equate to the free energy of intercellular adhesion, would according to the present hypothesis be equal to the force of contraction of this surface layer (in dynes per cm). Of course, Phillips & Steinberg (1969) also observed

that this tendency of aggregates to reduce their surface area is independent of the total area of the aggregate, so we would have to suppose that as new areas of cell surface come to lie at the aggregate surface, they too become contractile, while conversely areas of cell surface lose contractility if they move inward from the aggregate surface.

It is also not difficult to visualize how such differential contraction of exposed cell surfaces could cause the engulfment of one cell aggregate by another. If aggregates (or fragments) of two different histological cell types are brought into contact, then a contractile surface layer should form consisting of the exposed surfaces of the superficial cells of both aggregates. If the contractile surface layer of one cell type is stronger than that formed by the other cell type, then the more contractile cell aggregate will gradually pull the other aggregate around it. Thus the more contractile cell type would become engulfed by the less contractile cell type. For complete engulfment to occur, it would be necessary for the surface layers to be contractile without limit, so that the exposed surface of the more contractile cell type could continue to shrink until it has disappeared and been covered over by cells of the less contractile type. It is likewise crucial to realize that we are talking about the active contractility of the exposed part of the cell surface relative to the rest of its surface, and are not considering either a generalized contractility or an elasticity.

Although it is somewhat more difficult to visualize how cell sorting would be produced by differential cell surface contraction, the same basic principles would be involved as in the engulfment of one aggregate by another. In a mixed reaggregate, the cells of the type having the strongest induced contraction over their exposed surface would tend to move to interior positions, simply by pulling the cells of the less contractile type over themselves so as to cover these exposed surfaces.

Likewise, within the aggregate, the more internally segregating cell type (i.e. the more strongly contractile) would also contract more strongly over those parts of their surfaces in contact with the other (externally segregating) cell type and so, once in contact, these internally segregating cells would tend to form rounded multicellular masses. Once again the tendency of interior masses to round up would result from the differential contraction of some parts of the cell surfaces (those in contact with cells of differing histological type) relative to other parts (those juxtaposed to cells of the same type).

Thus cell sorting can be explained by a surface contraction hypothesis closely related to the apical contraction hypothesis which is now generally accepted as the explanation for epithelial folding. In this way we can account for the observation that embryonic cells which normally move to an internal

position by invagination, can, if disaggregated, still achieve this internal position by cell sorting. At the cellular level, the basic mechanism would be the same in either case, i.e. a cytoplasmic contraction concentrated at the exposed surface.

Evidence directly supporting this postulated medium induced surface contraction comes from the work of Burnside (1973) on amphibian neural plate cells. When early neural plates were explanted into a saline solution, so that the cells' basal surfaces became exposed to this medium, bundles of microfilaments were induced to form along this newly exposed surface and this basal surface was induced to constrict curling the whole epithelium towards its basal surface. Such induced contractions of the basal surfaces of explanted epithelia are doubtless to blame for the very frustrating tendency of such epithelia to curl during transplantation operations.

Testable predictions of this differential surface contraction hypothesis

In general, observations which are confined to the end results of cell sorting will not be sufficient to distinguish between the differential adhesion hypothesis and the alternatives proposed here. This is true simply because all of these hypotheses predict the same end result. The same was true of the physical studies of surface tension where observations of the end results alone were not sufficient to determine the mechanism of the phenomenon. In order to identify correctly a mechanism, it is best to study the process of change by which cell sorting, engulfment, etc. occur, or at least to observe the intermediate stages of these processes. If cell sorting, engulfment, etc., are actually achieved by cell surface contractions, then corresponding cell shape changes should be observable during the process of sorting out and engulfment. Specifically, internally segregating cell types should (be expected to) undergo a progressive narrowing over those parts of their surfaces lying at the aggregate surface, just as is observed in invaginating cells at the blastopore and in the neural plate of embryos (Baker & Schroeder, 1967). Thus for example if the engulfment of one fragment by another were permitted to go part way toward completion, and the partially fused fragments were then fixed and prepared for electron microscopy, one should observe a narrowing of the exposed surfaces of the internally segregating cells and conversely a flattening parallel to the surface of the exposed surfaces of the externally segregating cell types. Furthermore, if fixation were adequate, one would anticipate finding layers of actin-like microfilaments in the cortical cytoplasm of the cells, concentrated at the still exposed surfaces of the internally segregating cell type.

A further testable prediction of this surface contraction hypothesis would be that agents which reduce the contractility of the cell cortex should cause

aggregates to be flattened to a greater degree in a centrifugal field. For example cytochalasin B should have this effect, though its specificity in reducing cortical contractility is open to some doubt (Carter, 1967*a*,*b*; Sanger & Holtzer, 1972). Cytochalasin may alter cell adhesiveness as well as contractility.

(B) SECOND ALTERNATIVE: A DIFFERENTIAL STRENGTH OF ADHESION HYPOTHESIS

Although this alternative differs from Steinberg's hypothesis in only a few respects, these differences are crucial ones and avoid the four objections to the differential adhesion hypothesis discussed in section 4 above. In addition this modified version of the differential adhesion hypothesis is more consistent with recent observations on the role of substratum adhesiveness in determining the direction of fibroblast locomotion in culture (Harris, 1973).

Nearly all tissue cells, including all those known to sort out from one another, are capable of active locomotion when cultured on solid substrata such as glass or plastic. An important aspect of this locomotion is the extension of contractile cellular processes, often in several directions at once. It has been shown that if fibroblasts are cultured on specially prepared substrata, some parts of which are more adhesive than others, the cells will tend to accumulate on the most adhesive areas (Carter, 1965; 1967*a*,*b*).

It was initially suggested by Carter (1965) that the fibroblasts might actually be pulled onto the more adhesive substrata by the work of intercellular adhesion, analogous to the wetting of a solid by a liquid (and also analogous to the differential adhesion hypothesis of cell sorting). However, it has since been shown that this preferential accumulation of fibroblasts on more adhesive substrata does not occur by passive wetting but rather is the result of active cell motility. Net cell movement occurs by the contraction of the cell extensions attached at their tips to the substratum, the direction of movement being determined by which of these extensions adhere most strongly to the substratum. Thus such cells progress by a series of tugs-of-war, moving preferentially onto those areas of the substratum where their extensions form the least breakable adhesions to the substratum. (Harris, 1973.)

It is quite possible that cells within aggregates may move by a similar mechanism, except that the adhesions formed by the cell extensions would be intercellular adhesions rather than cell-substratum adhesions. If a cell within an aggregate were to extend processes outward in several directions, these extensions forming adhesions to nearby cells, then the contraction of these extensions would pull the cell body in the direction in which the strongest, least breakable adhesions were formed. Repeated cycles of such extensions and contractions by the cells of an aggregate would result in

those cells forming the strongest mutual adhesion, being pulled together into a coherent internal mass. Less strongly adhesive cells would be squeezed outward into a peripheral layer. Thus cell sorting could be explained by differences in the breaking strength of the intercellular adhesions formed by different cell types, those with the strongest and least easily broken adhesions segregating internally.

Very much this same possible explanation of cell sorting has been discussed by Steinberg & Wiseman (1972), but it is important to realize that sorting out by this mechanism would be fundamentally quite different from that envisioned in the differential adhesion hypothesis. One essential difference is that cell sorting by this mechanism would be an active rather than a passive process, that is, one driven by the force of cell contractility rather than by the force of attraction between adjacent cell surfaces, as the differential adhesion hypothesis requires. A still more crucial difference is that according to the differential adhesion hypothesis, the quantitatively varying parameter (sigma), determining a given cell type's position in the hierarchy of sorting out, should be the work of intercellular adhesion, i.e. the integral over the distance of attraction of the attractive forces exerted during the formation of an adhesion, per area of cell surface. On the other hand, if cell sorting occurs by the mechanism proposed in this section, then this "sigma" parameter would be a function of the minimum force required to break an intercellular adhesion. To take the simplest case of an adhesion involving only attractive force fields, the work of adhesion would be the area under the curve of attraction *v.* distance, while the strength of adhesion would be the maximum height of this curve. These two parameters are not the same, or even necessarily proportional to one another, especially if covalent bonds form between adhering cells, or if the adhesion is otherwise altered after its formation. So the question remains, which of these two parameters (if either) governs sorting-out, rounding up, etc.

Two basic approaches might be suggested as possible ways of testing this hypothesis. Cell sorting might be directly observed within cell masses to determine to what extent the cell movements may resemble those observed on solid substrata (cf. Trinkaus & Lentz, 1964). Alternatively if methods could be developed for the direct measurement of the works of intercellular adhesion, as compared with the breaking strength of these adhesions, one could determine which parameter varies according to the observed hierarchy of cell sorting, i.e. which property is greatest in epidermal cells and smallest in liver cells, etc.

The observation that cytochalasin B (a drug which is known to inhibit cell locomotion and contractility) also inhibits cell sorting within aggregates (Armstrong & Parenti, 1972; Maslow & Mayhew, 1972; Steinberg &

Wiseman, 1972) supports both this hypothesis and the preceding one (differential surface contraction), but this information is by no means conclusive since cytochalasin may also affect cell adhesiveness (Sanger & Holtzer, 1972).

(C) THIRD ALTERNATIVE: A DIFFERENTIAL MOBILITY OF ADHESION HYPOTHESIS

This third hypothesis is included primarily to show just how many factors could (in principle) govern a cell's position within an aggregate. As discussed in part 4 above, intercellular adhesions tend to be concentrated in discrete loci distributed variously over the cell surface. These localized adhesions, such as desmosomes, tight junctions, gap junctions, etc., are often found concentrated in particular areas of the cell surface, indicating that these adhesions are capable of a certain degree of lateral mobility within the plane of the plasma membrane. For example, in epithelial sheets these localized adhesions concentrate between the apical ends of the epithelial cells to form the terminal bar complex of adhesions.

Let us now imagine the case of an aggregate of cells held together largely by such localized adhesions, desmosomes for example, and let us suppose that these desmosomes are completely free to move about over the cell surface. In such a case, cells at the aggregate surface need not have any fewer adhesions than a cell within the interior of the aggregate. A surface cell would indeed have a smaller fraction of its plasma membrane juxtaposed to other cells, but if the actual intercellular adhesions were concentrated at small mobile foci, then a surface cell could have just as many of these focal adhesions as an interior cell. Likewise, an individual cell moving from the aggregate surface to the aggregate interior or vice versa wouldn't necessarily gain or lose any of these local adhesions. Thus the differential adhesion hypothesis takes no account of such freely mobile focal adhesions.

It might be objected, however, that such focal adhesions may not be freely mobile, that they may either be constrained in some way or even propelled about the cell surface by some cytoplasmic force. For example, in our imaginary cell aggregate discussed above, if there were some force tending to keep the desmosomes widely separated, then this force would necessarily tend to resist the movement of cells from the aggregate interior to the surface. Likewise such a force tending to separate the focal cell adhesions would cause the individual cells to maximize their area of contact with one another, since only in this way can they maximize the separation distance between various desmosomes on their surface. The effective "surface tension", sigma, of such an aggregate would thus be a function of the force with which the desmosomes repelled one another. The net result would be an effect closely mimicking the surface tension of liquids yet entirely different in its causation.

6. Conclusion

As discussed in part 3 above, the experimental observations of Steinberg and his students are sufficient to prove that histotypic cell sorting results from quantitative variations in some cell property, and this same cell property also governs the engulfment of cell aggregates by one another and the tendency of such aggregates to round up. In addition Phillips (1969) showed that the cell property responsible for these effects (which he calls "sigma") can actually be measured in cells of different tissues by the degree to which their aggregates resist centrifugal flattening. The question still remains, however, by what property of the individual cells is this sigma parameter determined.

As described in part 4 above, there are a number of reasons for doubting that this parameter can simply be equated to the reversible work of intercellular adhesion, as proposed in Steinberg's differential adhesion hypothesis (Steinberg, 1962*b*, 1970). Consequently, a number of alternative hypotheses are suggested in part 5. In my view the most probable of these alternative explanations is the first, i.e. the differential surface contraction hypothesis, as it is most consistent with what we know about comparable morphogenetic movements in embryonic development. However, we should also not ignore the possibility that the surface tension-like behavior of cell aggregates might be the additive result of several different mechanisms (such as those described in this section), the effect of which add up to give the net "sigma" (apparent surface tension) of a given cell type. The essential point is that quite a number of different mechanisms can, in principle, produce the same macroscopic results and so the "surface tension" of cell aggregates need not have the same cause as the surface tension of liquids. After all, if it had turned out that liquid surface tension really was caused by a contractile molecular surface film, one couldn't necessarily have concluded that the "surface tension" of cell aggregates is also caused by a contractile surface layer.

Nonetheless, even if it should turn out that one of these alternative hypotheses is actually the true explanation of cell sorting, the principal credit for elucidating the mechanics of this phenomenon will still belong to Malcolm Steinberg, because of his fundamental insight that cell sorting closely resembles the separation of immiscible liquids and that a quantitative variation in cell properties can therefore provide a sufficient explanation.

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