SUMMARY

Using intuitive arguments, several investigators have proposed that the relative strengths of adhesion of cell to cell and of cell to substratum could determine whether or not monolayering — and specifically contact inhibition of cell overlapping — will occur. In the present communication, these ‘strengths of adhesion’ are given precise physical definitions, and the adhesive relationships which would promote spontaneous cell monolayering are rigorously derived, using the thermodynamic approach embodied in the differential adhesion hypothesis. This analysis verifies that contact inhibition of overlapping could, in principle, be a result solely of differential adhesion. In addition, it is demonstrated that for homogeneous populations of uniform cells cultured on a solid, uniform substratum, eleven distinct equilibrium configurations (cell population morphologies) could be generated merely by varying the relative values of cell-to-cell and cell-to-substratum adhesiveness. Most of these configurations have been observed previously in actual cell cultures.

INTRODUCTION

In this report we consider possible relationships between contact inhibition of cell overlapping (monolayering) and the ‘strength of adhesion’ of cells to each other and to the substratum. The general idea of explaining in some way certain aspects of the behaviour and morphology of cells in monolayered cultures on the basis of cell adhesiveness has often been suggested (Holtfreter, 1939; Abercrombie, 1960, 1961, 1964; Carter, 1965, 1967a, b, 1968; Rubin, 1966; Weston & Roth, 1969). Recently, two of us proposed a more explicit adhesion-based mechanism for contact inhibition of cell overlapping (Martz & Steinberg, 1973). Indirect experimental support for this mechanism has been obtained in 2 recent studies (D. R. Garrod & M. S. Steinberg, in preparation; Martz & Steinberg, 1974). The purpose of the present communication is to provide a more rigorous formulation of this mechanism than has been previously attempted. This formulation will be made in the following five steps:

(i) We shall review the operational definition of contact inhibition of overlapping, and distinguish this type of contact inhibition from others which are not treated in the present communication.
(ii) We shall explain the concept that adhesive energies can cause cell populations to rearrange themselves into increasingly stable configurations, and use it to develop an explanation for contact inhibition of overlapping. In order to do this, the intuitively familiar but physically vague concept of ‘strengths of adhesion’ will be formulated in physically unambiguous terms as ‘reversible works of adhesion’.

(iii) A simple model will be presented which allows us to demonstrate how reversible works of adhesion can be related to such processes as cell aggregation, cell attachment to a substratum and cell monolayering on the substratum.

(iv) We shall then derive the intuitively reasonable prediction that monolayering, and hence contact inhibition of overlapping, will tend to occur spontaneously when cell-substratum adherence (the reversible work of attachment) exceeds cell-cell adherence (the reversible work of aggregation).

(v) Finally, we shall illustrate the remarkable variety of configurations of cell populations which could, in principle, arise from variations in simple ‘strengths of adhesion’. Many of these configurations have, in fact, been observed to be characteristic of various cell cultures.

CONTACT INHIBITION: PHENOMENA, DEFINITIONS, AND PROPOSED MECHANISMS

We have recently reviewed the various contact inhibitions of cell movement in detail (Martz & Steinberg, 1973), and so shall here provide an introduction restricted to certain pertinent aspects of the subject. The term ‘contact inhibition’ was introduced by Abercrombie & Heaysman (1954) to describe a phenomenon which they had observed in cultures of chick embryo heart fibroblasts attached to a solid substratum. In the original usage, contact inhibition consists of a contact-mediated ‘inhibition of the locomotion of a cell in a direction that would take it across the surface of another cell’ (cf. Abercrombie, 1967). Since contact inhibition, thus defined, has never been demonstrated by direct observation of a statistically meaningful number of cell-to-cell collisions, it is actually an hypothesis, suggested by a limited number of direct observations, and invoked to account for certain aspects of the movement of cell populations; e.g. the tendency to monolayer, the circularity of the sheet of cells migrating out from an explant on a substratum, and the cessation of net movement in the zone of collision between 2 such sheets of cells which are opposed (Abercrombie & Heaysman, 1954; Abercrombie, 1961). The essential element of ‘contact inhibition’ necessary to account for these secondary population phenomena is a strong tendency of cells to avoid moving over, under, or upon other cells, a point made clearly in the original definition (Abercrombie & Heaysman, 1954).

On occasion, however, other elements have been added to the definition of contact inhibition, or at least to its characterization. For example, Abercrombie & Ambrose (1958, pp. 342–343) seem to associate the term with membrane ruffling when they write that ‘cessation of ruffling in a leading membrane, either through contact with another cell (contact inhibition), or more rarely “spontaneously”, stops cell locomotion in that direction’, adding that ‘Contact inhibition seems then to consist of the abolition or reduction of the power of the leading membrane to direct the general cell move-
Cell adhesion and contact inhibition

And again (Abercrombie, 1961, p. 190), ‘...contact inhibition selectively inhibits...the life of an RM’ (ruffled membrane). Abercrombie later writes (1970, p. 128, referring to Abercrombie & Ambrose, 1958), ‘Not until later were details of the reaction of the individual cells added, making it possible to define contact inhibition in a more elaborate and specific way, as a process involving adhesion, paralysis, and contraction’. He goes on directly to caution that ‘On the whole, however, it seems most useful to continue to define the term in its original broader sense as the directional restriction of displacement on contact, regardless of the way restriction is brought about’, adding further that ‘the detailed reactions of fibroblasts may not be typical of all cells that undergo such contact inhibition’.

Heaysman & Pegrum (1973, p. 71) appear to be leaning the other way. They follow Abercrombie (1970) in stating that ‘“Contact inhibition” is a process involving adhesion, paralysis, and contraction’ and go on to state that ‘attempts to reproduce it by causing a cell to collide with a non-living object have failed...The forward movement of the cell may be stopped, that is, it may be unable to move over the surface of the colliding object but the paralysis and contraction so characteristic of contact inhibition is not seen’. Thus, for these authors, the original meaning of contact-mediated, directional inhibition of cell locomotion evidently no longer suffices.

In view of the variations in present usage, the term ‘contact inhibition’ cannot be used unambiguously to denote its original meaning. Hence, we have elsewhere introduced the term contact inhibition of overlapping for this purpose (Martz & Steinberg, 1973). We wish to make clear that the subject of our present analysis, contact inhibition of cell overlapping, is intended to exclude other operationally distinct, and quite possibly mechanistically distinct phenomena such as contact inhibition of speed of cell movement (Abercrombie & Heaysman, 1953; Martz, 1973), inhibition of extension or ruffling of the cell’s leading membrane (Abercrombie & Ambrose, 1958; Abercrombie, 1961; Gustafson & Wolpert, 1967; and Trinkaus, Betchaku & Krulikowski, 1971), induction of leading membrane retraction or contraction (Weiss, 1958; Abercrombie & Ambrose, 1958; Abercrombie, 1970), restrictions on movement leading to parallel alignment of cells (Elsdale, 1969), and so-called ‘contact inhibition of cell division’.

The subject of the following analysis, then, is the original contact inhibition phenomenon; i.e. the contact-mediated inhibition of continued movement in the direction of a cell collision. This contact inhibition of overlapping would result in a suppression of overlapping between cell pairs, which in turn would lead, in a population consisting of many cells, to the formation of a monolayer on the culture sub-

* This last inhibition has also been called density-dependent inhibition of replication (Stoker & Rubin, 1967), cell cycle inhibition (Macieira-Coelho, 1967a), and topoinhibition (Dulbecco, 1970). Direct scrutiny of cells undergoing this inhibition in time-lapse films has failed to reveal any immediate effect of either intercellular contacts or of high local cell density on individual cell generation times (Martz & Steinberg, 1972). Moreover, a connexion between this inhibition of cell division and contact inhibition of speed or of overlapping has not been found when looked for (Macieira-Coelho, 1967b; Njeuma, 1971; Martz, 1973; Gail, 1973). Because this inhibition is correlated with the confluence of a cell culture, we prefer to describe it by the operational term ‘postconfluence inhibition of cell division’ (Martz & Steinberg, 1972).
stratum. Hence, the degree of monolayering has been used to quantitate the intensity of contact inhibition of overlapping in cell cultures (Abercrombie & Heaysman, 1954). However, it is important to realize that while contact inhibition of overlapping would produce a tendency for cells to distribute themselves in a monolayer, it by no means follows that monolayers necessarily originate only as the result of contact inhibition, as Abercrombie has been careful to point out (Abercrombie, 1970). Moreover, before monolayers can be called ‘contact inhibited’, the observed prevention of cell overlaps must first be shown to be contact-mediated (e.g. by experiments like those of Abercrombie & Heaysman, 1954; Abercrombie & Gitlin, 1965). It should be noted that the following analysis applies only to inhibitions of overlapping which are strictly contact-mediated.

The mechanism of contact inhibition of overlapping has not yet been established, nor is it necessarily the same in every case. Proposed explanations for contact-mediated cell monolayering (Abercrombie, 1961) have been based either upon some sort of direct, localized paralysis of cell locomotory systems following cell-cell collisions, or else upon some sort of competition between cell-cell and cell-substratum adhesions. Experimental evidence to date does not rule out either of these alternatives (see Discussion).

In this report, however, our objective is to examine the second alternative, i.e. the possibilities for adhesive control of cell population monolayering and multilayering configurations. Adhesively regulated contact inhibition of cell overlapping is a matter not of suppression of cell movement per se, but simply of confinement of cells – whether moving or not – to a single layer on the culture substratum. Adhesive control mechanisms require that cells be free to change position under the influence of adhesive forces, and hence the explanation we are exploring does not apply to systems of cells immobilized by unbreakable interconnexions or for any other reason. This control would be exerted only upon and during cell-cell contact, but as far as is known, it neither requires nor excludes the concomitant display of any particular form of cell behaviour such as inhibition of ruffling, leading membrane retraction, or the like.

To summarize, then, the following analysis will develop the details of one of two presently tenable mechanisms for contact inhibition of overlapping. By the term ‘contact inhibition of overlapping’, we mean the contact-mediated discouragement of major overlapping between cells on a substratum (regardless of whichever specific contact-mediated mechanism may actually be operating). This term is intended to include ‘contact inhibition’ in the original sense, but to carry no implications of microscopic behaviour often associated with the latter term in current usage.

* The configuration formed by cultured cells when each cell (or, more precisely, when the adhesive part of each cell) has a choice between adhering to the substratum or to other cells cannot be assessed if the cell density is either too high or too low. Unrestrained cell proliferation could cause multilayering simply through overcrowding, and sparse cultures might exhibit little multilayering due to lack of cell collisions. Therefore, the most reliable cell density at which to assess monolayering/multilayering tendencies of cells is that just before the culture becomes confluent.
A THERMODYNAMIC APPROACH: THE DIFFERENTIAL ADHESION HYPOTHESIS

Are the adhesive properties of cells sufficient, in principle, fully to account for monolayering? We will approach this question through methods developed in our laboratory to explain other, but not unrelated phenomena — methods which have been formalized in the differential adhesion hypothesis (Phillips, 1969, and in preparation; Phillips & Steinberg, 1969, and in preparation; Steinberg, 1970). This hypothesis was originally formulated by Steinberg (1962, 1963, 1964, 1970) to account for the rounding-up, segregation (sorting-out), and coalescence (spreading) movements observed in cultured embryonic cell aggregates and tissue masses. According to this hypothesis, cells of different tissues adhere with different strengths; and these cell rearrangements are directed by the tendency of the aggregated cells to maximize adhesive energy evolved by the formation of cell contacts. That is, the cells tend spontaneously to rearrange so as to maximize their adhesive contact area, and so as to exchange weaker for stronger adhesions.

Work in our laboratory has provided experimental confirmation of a number of predictions arising from the differential adhesion hypothesis. Of these, perhaps the one of most far-reaching significance was the prediction that a given tissue combination would tend to approach the same final configuration from radically different initial configurations (Steinberg, 1962, 1963, 1964, 1970). This demonstration that an equilibrium process is occurring suggested that thermodynamic principles and methods might be used to analyse these morphogenetic rearrangements. (It is important to realize, however, that achievement of equilibrium with respect to the arrangement of cells within a population in no way implies that total thermodynamic equilibrium has been achieved. On the contrary, chemical reactions must continue in order to preserve the constancy of cellular properties upon which the maintenance of an equilibrium configuration depends.)

Thermodynamics analyse events ('reactions') in terms of the energies which drive them, in order to predict both the direction in which changes will occur and their extent (i.e. the point at which a particular reaction will reach equilibrium). We seek to account for contact inhibition of cell overlapping. This phenomenon can be regarded as the outcome of several component 'reactions' (discussed below, illustrated in Figs. 1–3, pp. 407–409). These reactions involve cell movements accompanied by the formation and rupture of cell-cell and cell-substratum contact areas. Hence, our thermodynamic approach to monolayering analyses a change in the arrangement of cells in a culture as a reaction — approaching an equilibrium arrangement and governed by cell-contact energies. We therefore pose 2 questions: (1) Are adhesive relationships alone capable, in principle, of causing cultured cell populations to monolayer; and (2) if so, which particular values of the (reversible) works of adhesion will favour, and which will discourage monolayering?

The 'energy of adhesion' which controls reactions involving changes in contact areas is, in precise physical terms, the reversible work done by the system per unit area of contact formed, called, for simplicity, the work of adhesion (Adam, 1941, p. 8; H. M. Phillips, in preparation). ('Specific work of adhesion' would have been a
more descriptive term, indicating that it signifies work per unit contact area.) It is a measure of the ‘adhesive strength’ of a given kind of contact area, but is not a measure of amount of contact formed. Thus, (reversible) work of adhesion must be distinguished from total adhesive (reversible) work done by a system while forming a particular amount of contact area. Total adhesive work equals work of adhesion multiplied by total contact area formed. It is the total adhesive (reversible) work during an interfacial reaction that determines the course of the reaction.

We shall be guided by the law of thermodynamics which states that any change in the configuration of a system which increases the total reversible work done by the system will tend to occur spontaneously. Thus, monolayering will tend to occur spontaneously if it is accompanied by an increase in the total adhesive work; and the cells will reach an equilibrium arrangement (and thus remain monolayered) if the total adhesive work has been maximized by this process. To answer the questions posed above, then, requires that we determine quantitative relationships between changes in the arrangement of cells in a culture and the accompanying total adhesive works done by the system, a problem to which we now turn.

A SIMPLIFIED DISCRETE-SUBUNIT MODEL FOR DIFFERENTIAL CELL ADHESION

For the purposes of the present model, we shall consider 3-component systems consisting of homogeneous, uniform cells (c) and a uniform, solid substratum (s), both being immersed in a liquid nutrient medium (m). The subunits of such a system can form contact areas of 6 different types: cc, cm, cs, mm, ms, and ss. Since the amount of ss contact area is invariant, we need to consider only the other 5 contact areas.

We wish to inquire whether differences in adhesiveness alone can account for contact inhibition of overlapping. Therefore, for the purposes of the present model, we shall assume that only energies of adhesion contribute to the reversible works associated with the monolayering-multilayering reaction. Other energies, such as those which might be required to change the shapes of individual cells or to increase or decrease the amount of surface membrane per cell in the course of this reaction will be assumed not to contribute to these reversible works. Thus, such other energies are permitted to enter into this model only if they balance out for the initial and final configurations of the reactions of interest.

In particular, to exclude consideration of the non-adhesive works which must be involved in increasing or decreasing the amount of exposed membrane per cell, we shall assume that the surface area per cell is constant. That is to say, the individual cells will not be allowed, in our model, to increase or decrease their surface area by stretching or contracting their membranes or by any other means. This restriction

* The phrase ‘doing reversible work’ is convenient but potentially misleading. ‘Reversible work’ is, of course, a hypothetical concept; in reality, no system undergoes a process reversibly in the thermodynamic sense. The concept has great utility in thermodynamics, however, since ‘reversible work’ can be used to characterize the energy changes that take place during a real process. That reversible work can be measured in cell aggregates during changes in configuration, without any assumption that aggregates are changing reversibly, is discussed in detail by Phillips (1969).
implies that the total cell surface area in the system, \(2a_{cc} + a_{cm} + a_{mc}\) (where \(a\) represents contact area), is constant, and that contact areas can increase in area only by forming new contacts between pre-existing surfaces, equal areas of which must be cleaved from their former contacts in the process. (This assumption also implies that the total medium and substratum surface areas in the system are constant: conservation of the sums \([2a_{mm} + a_{mc} + a_{ms}]\) and \([a_{cc} + a_{mm}]\).)

The intuitive concept of strength of adhesion between cells would seem to connote a quantity that is a function solely of the adhesions formed between cells, independent of the adhesive or attractive properties of the medium surrounding the cells. No such independent quantity can, in fact, be measured, for in reality a liquid medium is always present, and hence \(cm\) contact area (or \(cs\) contact area) is lost (and \(mm\) or \(ms\) area gained) whenever \(cc\) contact area is formed (given our assumption of constant total cell area). To take advantage of the heuristic value of the intuitive concept of 'strength of adhesion', however, one can invoke the hypothetical construct of cells forming adhesions in a vacuum. No \(cm\) or \(cs\) bonds are broken in this imaginary process, nor are \(mm\) or \(ms\) bonds formed. We shall designate this hypothetical work of adhesion as \(W_{cc}^v\), where \(v\) indicates that the imaginary process is carried out in vacuo; analogous symbols will be used for the in vacuo works of adhesion of other contact areas. (\(W^v\)s are similar to the \(\lambda\)'s used by Goel et al. in their computer-assisted model studies of cell sorting (Goel et al. 1970a; Gordon et al. 1972).)

The works of adhesion useful in predicting cellular configurations are net reversible works, per unit contact area formed or lost, done by the system during processes of exchanging certain kinds of contact areas for others. These net works may be defined in terms of the processes of their formation relative to some arbitrary reference state of the system. Here, a suspension of single cells in medium is taken as the reference state (that is, \(a_{cc} = a_{cm} = 0\)).

Consider first the process of cell aggregation from this reference state. \(cc\) contact area will be formed by the process represented schematically in Fig. 1: 2 units of cell-medium area must be given up to form 1 unit of cell-cell area. This also produces one unit of medium-medium area. This process can be represented symbolically by:

\[
2\bar{a}_{cm} \rightarrow \bar{a}_{cc} + \bar{a}_{mm}. \tag{1}
\]

![Fig. 1. Schematic representation of aggregation from the reference state of single cells suspended in medium. Heavy lines accentuate the portions of the cell membranes involved in the exchange of contact areas. C, cell; M, medium; S, substrate.](image-url)
The bars in equation (1) signify unit contact areas of the kinds designated. Since the gain or loss of a unit of contact area (one \( \partial \)) entails the gain or loss of its associated reversible work per unit area (one \( W^r \)), equation (1) leads directly to the net work per unit reaction. The work of aggregation, \( W(\text{agg.}) \), may thus be defined as the sum of the works of adhesion of the products minus the sum of those of the reactants in equation (1):

\[
W(\text{agg.}) = W^r_{\text{ce}} + W^r_{\text{mm}} - 2W^r_{\text{cm}}. 
\]  

(2)

We have called this quantity \( W(\text{agg.}) \), the work of aggregation, because its value specifies whether or not aggregation will occur. If \( W(\text{agg.}) \) is positive, attachment of one cell to another in medium (i.e. aggregation) will tend to occur spontaneously. Conversely, if \( W(\text{agg.}) \) is negative, aggregates of cells in medium will tend to disperse into single cells. If \( W(\text{agg.}) \) is 0, neither aggregation nor disaggregation will be favoured; both single cells and aggregates will be stable, and the equilibrium configuration of the system will be an indeterminate mixture of both.

It is now evident that the 'strength of cell-to-cell adhesion' (in medium) utilized by Martz & Steinberg (1973) is, in fact, \( W(\text{agg.}) \), the net reversible work done during the formation of a unit of cell-to-cell contact area in medium.

In similar fashion, the 'strength of cell-to-substratum adhesion' is defined from the process of a single cell attaching to the substratum in medium, depicted schematically in Fig. 2, and represented symbolically by:

\[
\partial_{\text{cm}} + \partial_{\text{sm}} \rightarrow \partial_{\text{cs}} + \partial_{\text{ss}}.  
\]  

(3)

Fig. 2. Schematic representation of cell attachment to substratum starting from the reference state of single cells suspended in medium. Heavy lines accentuate the portions of the surfaces involved in the exchange of contact areas.

Work of attachment may thus be defined as:

\[
W(\text{att.}) = W^r_{\text{cs}} + W^r_{\text{sm}} - (W^r_{\text{cm}} + W^r_{\text{sm}}).  
\]  

(4)

Finally, we may define a quantity which relates monolayering to reversible works of adhesion by considering the process of monolayering shown in Fig. 3:

\[
\partial_{\text{cs}} + \partial_{\text{sm}} \rightarrow \partial_{\text{cs}} + \partial_{\text{cm}}. 
\]  

(5)

More consistent symbols for \( W(\text{agg.}) \) and \( W(\text{att.}) \) would be \( W^a_{\text{agg.}} \) and \( W^a_{\text{att.}} \), respectively, which designate the work of formation from the reference state (\( \partial_{\text{cs}} = \partial_{\text{sm}} = 0 \)) in the presence of culture medium. The symbols \( W(\text{agg.}) \) and \( W(\text{att.}) \) are used because of their heuristic value.
Fig. 3. Schematic representation of the process of monolayering from a cell multilayer. Heavy lines accentuate the surfaces involved in the exchange of contact areas.

Thus:

$$W(\text{mono.}) = W'_{\text{ce}} + W'_{\text{cm}} - (W'^{\prime}_{\text{cc}} + W'^{\prime}_{\text{en}}).$$

$W(\text{mono.})$ is a measure of the tendency of a culture to display contact inhibition of overlapping on a substratum to which cells adhere. In other words, provided that $W(\text{att.})$ is not negative, if $W(\text{mono.})$ is positive, monolayering will occur spontaneously; if $W(\text{mono.})$ is negative, multilayering will occur spontaneously; if $W(\text{mono.})$ is zero, either a monolayer or a multilayer of cells, as well as intermediate configurations, will be stable (configuration indeterminate). The 3 cases and corresponding predictions made by Martz & Steinberg (1973) are recalled in Table 1, where they are now reformulated in terms of works of adhesion.

**Table 1. The three adhesive relationships considered by Martz & Steinberg (1973) reformulated with works of adhesion**

<table>
<thead>
<tr>
<th>Original case</th>
<th>Original formulation</th>
<th>Formulation with works of adhesion</th>
<th>Equilibrium configuration</th>
<th>Contact inhibition of overlapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case A</td>
<td>Strength of $cc$ adhesion $&gt;,$ strength of $cs$ adhesion</td>
<td>$W(\text{agg.}) &gt; W(\text{att.})$</td>
<td>Spontaneous multilayering</td>
<td>No</td>
</tr>
<tr>
<td>Case B</td>
<td>Strength of $cc$ adhesion $&lt;,$ strength of $cs$ adhesion</td>
<td>$W(\text{agg.}) &lt; W(\text{att.})$</td>
<td>Spontaneous monolayering</td>
<td>Yes</td>
</tr>
<tr>
<td>Case C</td>
<td>Strength of $cc$ adhesion $\approx,$ strength of $cs$ adhesion</td>
<td>$W(\text{agg.}) \approx W(\text{att.})$</td>
<td>Indeterminate</td>
<td>No†</td>
</tr>
</tbody>
</table>

* $W(\text{agg.})$ and $W(\text{att.})$ were assumed to be positive.
† Contact inhibition will not occur if the cells are motile, since the incidence of nuclear overlaps will approach that expected from a random distribution of nuclei. For further discussion of Case C, see Martz & Steinberg (1973).
DERIVATION OF CONDITIONS FOR MONOLAYERING

Having provided the necessary background and definitions, it is now possible to derive the predictions which were made by Martz & Steinberg (1973) regarding the 3 cases in Table 1. For example, Case B presumed that \( W(\text{agg.}) \) is less than \( W(\text{att.}) \). The implication that monolayering will occur spontaneously in this case (\( W(\text{mono.}) > 0 \)) if \( W(\text{att.}) \) is not negative, is derived as follows. The assumed relationship for Case B is:

\[
W(\text{agg.}) < W(\text{att.}).
\]  

Substituting the definitions of these works from equations (2) and (4) yields:

\[
W^c_{cc} + W^c_{mm} - 2W^c_{cm} < W^c_{es} + W^c_{nm} - (W^e_{cm} + W^e_{sm}).
\]  

By cancelling and rearranging terms, this yields:

\[
W^c_{es} + W^e_{cm} - (W^e_{cc} + W^e_{sm}) > 0.
\]  

The quantity on the left of this inequality is the definition of \( W(\text{mono.}) \) as given in equation (6). Hence, it is shown that under the conditions given by (7), \( W(\text{mono.}) > 0 \), and monolayering will, therefore, be favoured if the cells attach to the substratum.

Analogous derivations confirm the predictions in Cases A and C, where the implications are that \( W(\text{mono.}) < 0 \) and \( W(\text{mono.}) = 0 \), respectively. Hence, the relationship between the work of aggregation and the work of attachment determines whether or not monolayering will occur spontaneously in this model system.

CONFIGURATIONS ARISING FROM THE SIMPLIFIED DIFFERENTIAL ADHESION MODEL

\( W(\text{agg.}) \) may be less than, equal to, or greater than \( W(\text{att.}) \). Moreover, each of these quantities may have positive, zero, or negative values. For a set of adhesive energies in which \( W(\text{agg.}) > W(\text{att.}) \), the corresponding equilibrium configuration when both \( W(\text{agg.}) \) and \( W(\text{att.}) \) are positive will be different from that generated when both are negative, and a still different configuration will be specified when \( W(\text{agg.}) \) is positive and \( W(\text{att.}) \) is negative; and so forth. Detailed consideration of the combinations and permutations here reveals a surprising richness of morphogenetic possibilities.

In Fig. 4 are listed all thirteen possible sets of relative values of \( W(\text{agg.}) \) and \( W(\text{att.}) \) and the corresponding, implied values of \( W(\text{mono.}) \). In each case a schematic representation is given of the configuration of the cell-medium-substratum system favoured by the specified net reversible works of adhesion. Eleven of these configurations are qualitatively different from one another.

It is interesting to note that Case B1 suggests an epithelial-like cell configuration, Case B2 suggests a fibroblast-like cell configuration, Cases A5, B5, and C3 suggest red blood cell configurations, and that cases such as A1 and C1, involving multi-
layering, suggest cell configurations sometimes held to be associated with malignancy. This is not to say, of course, that all of the above-mentioned cell configurations are determined in nature exclusively in this way. But it is worthy of note that several of these configurations have been achieved through manipulations of the adhesive properties of the culture substratum (Carter, 1965; Harris, 1973a).

<table>
<thead>
<tr>
<th>ORIGINAL CASE</th>
<th>SUB-CASE</th>
<th>W(agg.)</th>
<th>W(att.)</th>
<th>W(mono.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASE A W(agg.) &gt; W(att.)</td>
<td>A1</td>
<td>+ &gt; +</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>+ &gt; 0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>+ &gt; -</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>0 &gt; -</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A5*</td>
<td>- &gt; -</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

| CASE B W(agg.) < W(att.) | B1 | + < + | + |
| | B2 | 0 < + | + |
| | B3 | - < + | + |
| | B4 | - < 0 | + |
| | B5* | - < - | + |

| CASE C W(agg.) ≡ W(att.) | C1 | + ≡ + | 0 |
| | C2 | 0 ≡ 0 | 0 |
| | C3* | - ≡ - | 0 |

Fig. 4. The eleven distinct equilibrium configurations which would in principle result simply from varying the strength of cell-cell adhesion [reversible work of aggregation $W(agg.)$] and the strength of cell-substratum adhesion [reversible work of attachment $W(att.)$]. Sub-cases A5, B5, and C3 (marked with *) yield identical configurations. The 'original cases' refer (see Table 1) to Martz & Steinberg (1973). Net reversible works are assigned positive (+), negative (−), or zero (0) values. The value of $W(mono.)$ is implied (→) by the relationship ($>$, $<$, ≡) assumed between $W(agg.)$ and $W(att.)$. The actual formation of a monolayer requires not only that $W(agg.) < W(att.)$, but that $W(att.) ≥ 0$ as well.
DISCUSSION

In this communication, we have shown how differential cell adhesion (properly defined in terms of reversible works of adhesion) could, by itself, be responsible for a large constellation of cell population morphologies that are actually observed \textit{in vivo} or in tissue culture, and could account for the phenomenon of contact inhibition of cell overlapping. For the sake of simplicity, we have limited ourselves to modelling the spreading behaviour of homogeneous populations of uniform ('isotropic', see Goel \textit{et al.} 1970a) cells on a uniform solid substratum. In actual situations, heterogeneity in the cell population (Steinberg, 1964; Garrod & Steinberg, 1973) and variations in the adhesive properties of the substratum on the one hand (Weiss & Taylor, 1956; Rosenberg, 1962; Carter, 1965, 1967b; Harris, 1973a) and of the cells on the other (Steinberg, 1964, pp. 355-356; 1970, p. 429; Goel & Leith, 1970b; DiPasquale & Bell, 1972; Middleton, 1973) greatly extend the range of cell population patterns that can be determined by adhesive differentials.

The present demonstration provides another example of the power and utility of what we may term the thermodynamic approach to assembly problems in morphogenesis: that is, the resolution of an overall assembly process into simple, well defined component reactions, followed by the evaluation of the energy changes associated with each such reaction.

In the present model, we have explored the consequences of adhesive differences alone, isolated from the other energies (not so easily incorporated into a simple quantitative model) which might contribute to the final configuration of a cell culture. Many factors besides adhesiveness could, in actuality, contribute to the overall reversible work of adhesion associated with a particular change in the configuration of a cell population, including the reversible works involved in production, absorption, and stretching of cell membrane and in causing changes in cell shape. These energies may not always be trivial.

In addition, there are cases in which a great number of different cell population configurations could equally well maximize the adhesive work done by the system. For example, certain stellate cells adhere strongly to the substratum only at a few points, these being the tips of the cell’s extensions (see Trinkaus \textit{et al.} 1971). Since only these widely separated points on the cell, and not its main body, engage in adhesive exchanges, it does not matter whether a pair of such cells lies parallel or crisscrossed on the substratum; as long as their adhering plaques are not altered in size or quality, the 2 configurations would be equivalent with respect to adhesive energy. Consequently, when the adhering tip of one such cell approaches the non-adhering side of another, crisscrossing or ‘underlapping’ should readily occur, as has been previously pointed out by Weston & Roth (1969), and this has been directly observed (Boyd, Grainger & James, 1969; Abercrombie, 1970; Trinkaus \textit{et al.} 1971; Bell, 1972; Vasiliev & Gelfand, 1973, including discussion of Bell’s observations by Trinkaus, see pp. 330–331 of that volume). In this instance, the adhesive areas of the cells form a monolayer despite the overlapping of the non-adhering cell bodies, and
Cell adhesion and contact inhibition

413

it is the disposition of the former, not the latter, that would be thermodynamically significant in contact area exchanges.

In order to isolate adhesiveness as the sole controlling energy in our simplified model, we have had to assume that the other energies mentioned above do not affect the total reversible work of the reactions in question. Despite this restriction, which may be unrealistic in many cases, it has been illuminating to discover the variety of configurations of cell populations which, in principle, differentials of adhesive energy alone are sufficient to produce. Thus, while energies other than adhesive energies may indeed be involved in generating certain culture morphologies, what we have achieved here is the demonstration that, in principle, they are not necessary components of models that can adequately account for a considerable variety of empirically observed culture morphologies.

Elsewhere, H. M. Phillips (in preparation) shows that the relation for monolayering derived here (equation (7)) can be derived from only 3 underlying assumptions, which may be paraphrased as follows: (i) that cellular configurations of motile cell populations will tend spontaneously to progress towards states of maximal total adhesive reversible work; (ii) that each type of interface has an effectively uniform and constant reversible work of adhesion; and (iii) that the surface area of each cell is constant. The first 2 assumptions, which are probably close to reality for actual motile cell populations, form the basis (Phillips, 1969, and in preparation) for the differential adhesion hypothesis, as originally developed to explain the behaviour of embryonic cells cultured in aggregates. The third assumption is a simplification, invoked to limit the present model to adhesive energies, as discussed above.

We have chosen to present the above derivation in terms of reversible works of adhesion, basing our arguments on the adhesive interactions between the discrete subunits in the system (individual cells) because of the relative ease with which this approach fits one's intuitive notions. The disadvantage of this approach is that a truly generalized derivation, based solely on assumptions (i) and (ii) above, and without the restricting assumption (iii), becomes exceedingly cumbersome. Therefore, H. M. Phillips (in preparation) has formulated a conventional thermodynamic derivation phrased in terms of the continuous phase properties of the system, namely, surface tensions and specific interfacial free energies. Using this approach, Phillips develops a less-restricted derivation of the conditions for monolayering, and in addition, demonstrates that aggregates of cohesive, multilayering cells in subconfluent cultures should, at configurational equilibrium, adopt spherical-segment shapes. (In vertical profile, small, isolated multilayer colonies would, at configurational equilibrium, form spherical segments (i.e. spheres chopped off by the aggregate-substratum interface) having various (finite) radii, depending upon the relative values of $W(\text{agg.})$ and $W(\text{att.})$. Monolayering is recognized as an extreme case, in which the aggregate-medium interfaces are planar (i.e. spherical segments with infinite radii).)

Weston & Roth (1969) have previously discussed contact inhibition of overlapping and cell adhesiveness. Their conclusions are seemingly different from ours, but this is because of fundamental differences between our approach and theirs. We here explore
the theoretical consequences of pure differential adhesiveness per se, isolated from other phenomena, whereas Weston & Roth's explanation utilized several assumed cell properties not necessarily related to adhesiveness. More importantly, Weston & Roth conceive of the relevant adhesive parameter as one based on rates of aggregation (a kinetic parameter) which they call 'adhesive stability', a parameter which has no theoretically or empirically demonstrated value in predicting morphogenetic behaviour. Our analysis, in contrast, is based on the well documented premise that cell populations can seek increasingly stable configurations, finally reaching an equilibrium morphology controlled by surface free energies (thermodynamic parameters; Steinberg, 1963, 1964, 1970). These differences have been discussed in greater detail elsewhere (Steinberg, 1970; Martz & Steinberg, 1973).

Micromanipulation studies (Harris, 1973a) indicate that, in culture, single cells of many types adhere to a solid substratum mostly in localized peripheral regions. In confluent sheets of monolayered sarcoma-180 cells, each cell remains attached to the substratum (typical case B-i behaviour); but in confluent sheets of monolayered chick fibroblasts, only peripheral cells seem to adhere to the substratum. Moreover, Harris finds that monolayered fibroblasts can be detached from the substratum by pulling on their neighbours, while sarcoma-180 cells cannot. Thus, it appears to Harris that, when fibroblasts collide during monolayer formation, cell-cell adhesions form at the expense of cell-substratum adhesions. From these observations, he concludes that 'while monolayering among sarcoma-180 cells could, in principle, be explained as a result of cell-substratum adhesions competing successfully with intercellular adhesions, this is not a possible explanation in the case of the more typically contact-inhibiting chick fibroblast cells'.

Harris' results suggest that adhesive regions on cell surfaces are confined to discrete 'patches'. It is not surprising that this structural specialization of cell surfaces may (e.g. in fibroblast cultures) but need not (e.g. in sarcoma-180 cultures) lead to the formation of monolayer configurations more complex than those illustrated in Fig. 4 (p. 411). The apparent detachment of fibroblasts from the substratum as they attach to one another may reflect a reorientation of adhesive patches on cell surfaces - from weaker cell-substratum contacts to stronger cell-cell contacts - as Harris himself proposes. Cell surface specializations may thus make the particular adhesive exchanges that lead to monolayering in a given case different from those envisioned for the simple model system analysed above, although the principles governing such exchanges and invoked in our present discussion may nevertheless remain applicable.

Harris' results might be taken to mean that the adhesive relationships of monolayering chick heart fibroblasts are not only not those which we have proposed for monolayered cells in our model system \([W(\text{agg.}) < W(\text{att.})]\), but are in fact exactly the opposite \([W(\text{agg.}) > W(\text{att.})]\). We wish to emphasize that the evidence cited does not support such a conclusion for 3 reasons. First, the process envisioned by Harris does not have any direct implications for the relationship between \(W(\text{agg.})\) and \(W(\text{att.})\). Harris deduced that, upon collision, 2 cells both give up contact with the substratum in exchange for contact with each other. If characterized in terms
of \textit{in vacuo} reversible works of adhesion, this process would be favoured when

\[ W_{cc} + 2W_{mm} - (2W_{cm} + W_{mn}) > 0. \] (10)

By contrast, \( W(\text{agg.}) > W(\text{att.}) \) implies (see Derivation of conditions for monolayering, p. 410) that

\[ W_{cc} + W_{mm} - (W_{cm} + W_{cm}) > 0. \] (11)

Since the last term in (10) is not involved in (11), and vice versa, the relationship implied by Harris' process (10) does not specify any particular relationship between \( W(\text{agg.}) \) and \( W(\text{att.}) \). The second reason why Harris' observations do not support conclusions about works of adhesion is that \textit{reversible} works of adhesion (thermodynamic free energies attained at configurational equilibrium) cannot be measured or compared by measuring or comparing the \textit{forces} required to pull adhering objects apart (kinetic parameters acting during brief periods of rapid configurational change), since these are separate and distinct physical properties (see Steinberg, 1964; Phillips, 1969). Because Harris' technique cannot provide reliable assessments of works of adhesion, it would, of course, be equally fallacious to contend that his results with sarcoma-180 cells, which also exhibit contact inhibition of overlapping on one another, provide definitive evidence confirming the applicability of equation (9) to those cell monolayers. Finally, we wish to caution that the use of 'average' work-of-adhesion parameters for broad areas of cell surfaces whose actual adhesive regions are widely separated as discrete patches can easily lead to incorrect calculation of the cell population's most-stable configurations (M. Burdick, personal communication). Thus, for the 3 reasons given, no conclusions about cellular works of adhesion may be drawn from Harris' experiments.

Opposed to the differential adhesion explanation for contact inhibition of overlapping is an alternative explanation based upon the direct inhibition of the cellular machinery causing forward locomotion. Clearly, a cell might stop short upon collision with another cell either because it is held back by stronger adhesion to the substratum or because its 'motor' is locally shut off or disengaged (Abercrombie & Heaysman, 1954; Abercrombie, 1961, 1967, 1970). Certain observations make it probable that cells, or at least certain kinds of cells, do experience local changes in their contractile systems rapidly following mutual contact. Abercrombie & Ambrose (1958) occasionally observed 'retraction' of the leading membranes of fibroblasts following mutual contact, and Weiss (1958) reported similar retractions in the leading edges of colliding mesenchyme cells. Abercrombie (1970) later reported that 'with higher resolution it (the retraction) seems to be always present'. Most recently, Heaysman & Pegrum (1973; see also Heaysman, 1973) have documented, following the collision of 2 leading lamellae, the rapid local appearance, immediately beneath the unit membranes, of collections of microfilaments which become oriented parallel to the longitudinal axis of collision. The facing microfilamentar tracts of confronted cells then line up as though subjected to tension, concurrently with the appearance of a distortion of the cell surfaces in the region of tract apposition. After further elaboration of this micro-
filamentar system, electron micrographs were obtained, suggesting 'tension parallel to the orientation of the microfilaments in certain regions' as local pulling apart of the cells was observed. The entire process was completed in only a few minutes.

The elegant observations described above, taken together with earlier descriptions of the paralysis of ruffled membranes or leading lamellae upon mutual contact, suggest a contact-mediated, local reorganization of cellular contractile machinery, leading to a contraction that disrupts the contact: a negative feedback mechanism seemingly well suited to account for a failure of cells to superimpose following head-on collision. Heaysman & Pegrum follow Abercrombie (1970) in regarding contact inhibition (of 'locomotion') as a multifactorial 'process involving adhesion, paralysis, and contraction'. However, while a chain of successive local events may indeed follow intercellular collision, it is by no means self-evident that all elements of this chain are essential for or contribute to the failure of overlapping, persisting over many hours or days, which is the subject of our present analysis.

In fact, certain observations provide significant support for the differential adhesion hypothesis for contact inhibition of overlapping. Carter (1965) observed that a cell population on an adhesive gradient formed a monolayer where adhesion to the substratum was high but overlapped where it was low. In the absence of evidence to the contrary, it must be assumed that contact-induced changes in the cellular contractile machinery were similar in both circumstances. Differences in adhesive intensity were evidently sufficient in themselves to determine whether or not cells would monolayer. This point needs further investigation, however, since Harris (1973a) has observed that in some circumstances multilayering on low adhesiveness substrata can be produced by 'retraction clumping', a procedure which might result from changes in properties other than strengths of adhesion, such as the elastic tension in the cell sheet.

Additional evidence has now come from a different quarter. If monolayering results from the inhibition of cell locomotion in the direction of a cell contact, then cells within a confluent monolayer, contacted on all sides by other cells with which they resist overlapping, should be inhibited from moving in all directions; they should be immobilized. D. R. Garrod & M. S. Steinberg (in preparation), studying confluent embryonic chick liver parenchyma monolayers, and Martz & Steinberg (1974), studying confluent monolayers of 3T3 mouse fibroblasts, saw extensive movements of cell nuclei, strongly suggestive of movements of whole cells over several cell diameters, resulting in much shifting of neighbour relationships (see Steinberg, 1973).

It seems implausible that extensive cell movements would be permitted within a confluent monolayer if regional paralysis of a cell's locomotory machinery, sufficient to prevent overlapping over long periods of time, occurs wherever intercellular contact is present. Rarely, however, small gaps were seen briefly to open up in the monolayer (Martz & Steinberg, 1974), and it is conceivable that these were sufficient to permit the apparent movements. On the other hand, extensive cell movements in a confluent monolayer are perfectly consistent with a mechanism based simply upon a more intense adhesion to the substratum. Direct evidence (DiPasquale & Bell, 1972; Middleton, 1973) has now shown that epithelial cells of 2 different kinds do indeed adhere more intensely to culture substrata than to the upper surfaces of already
attached cells of like kind. This differential adhesion mechanism does not, as has been previously pointed out (Abercrombie, 1961, 1970), account for the ultrastructural and contractile events following contact. These require a separate explanation. But these latter events may be irrelevant to the determination of cell overlapping, as seems likely in Carter's (1965) experiments.

It remains for empirical work to evaluate the extent to which differential adhesion does, in fact, control cell culture morphologies. This will ultimately require direct physical measurements of the appropriate cell adhesion parameters, similar to those now being conducted in quantitative testing of the differential adhesion hypothesis as applied to cell behaviour in aggregates of embryonic cells cultured on non-adhesive substrata (Phillips, 1969; Phillips & Steinberg, 1969, and in preparation).

The above-mentioned experimental findings concerning cells on adhesive substrata, when added to the earlier, extensive observations of the behaviour of cell aggregates and tissue fragments on non-adhesive substrata (e.g. rounding up, mutual spreading of cell aggregates, and cell sorting-out) provide a wide spectrum of morphogenetic phenomena for which the differential adhesion hypothesis provides, in principle, a common physical basis.

We thank Dr David Barkley for helpful criticisms of the manuscript. This study was supported in part by grants GB-5759X and GB-40041 from the National Science Foundation, by a special grant for research (P-532) from the New Jersey Division of the American Cancer Society, and by N.I.H. research grant CA13605. E.M. held a Damon Runyon Memorial Fund Postdoctoral Fellowship. The manuscript was completed while M.S.S. was a Visiting Fellow at the Salk Institute.

REFERENCES


Cell adhesion and contact inhibition


(Received 4 March 1974)