Surface tensions of embryonic tissues predict their mutual envelopment behavior

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SUMMARY

During embryonic development, certain tissues stream to their destinations by liquidlike spreading movements. According to the ‘differential adhesion hypothesis’, these movements are guided by cell-adhesion-generated tissue surface tensions (σ), operating in the same manner as surface tensions do in the mutual spreading behavior of immiscible liquids, among which the liquid of lower surface tension is always the one that spreads over its partner. In order to conduct a direct physical test of the ‘differential adhesion hypothesis’, we have measured the σs of aggregates of five chick embryonic tissues, using a parallel plate compression apparatus specifically designed for this purpose, and compared the measured values with these tissues’ mutual spreading behaviors. We show that aggregates of each of these tissues behave for a time as elastico-viscous liquids with characteristic surface tension values. Chick embryonic limb bud mesoderm (σ = 20.1 dyne/cm) is enveloped by pigmented epithelium (σ = 12.6 dyne/cm) which, in turn, is enveloped by heart (σ = 8.5 dyne/cm) which, in turn, is enveloped by liver (σ = 4.6 dyne/cm) which, in turn, is enveloped by neural retina (σ = 1.6 dyne/cm). Thus, as predicted, the tissues’ surface tension values fall in the precise sequence required to account for their mutual envelopment behavior.

Key words: surface tension, tissue spreading, cell sorting, cell adhesion, morphogenesis, morphogenetic movement, tissue affinities, self-assembly

INTRODUCTION

The spreading of one cell population over the surface of another is a common means of embryonic morphogenesis. The causal analysis of tissue spreading movements has been greatly advanced by the finding that such movements are commonly spontaneously initiated when two different embryonic tissue types are brought into mutual contact. This takes place whether or not those tissues actually encounter one another in the course of normal development. In each case it is a specific one of the two tissues that spreads over the surface of the other (Trinkaus and Groves, 1955; Steinberg, 1962c, 1963a,b, 1970).

An important insight into the cause of this preferential spreading was provided by the discovery that the very same final tissue configuration could be arrived at by an entirely different pathway: the sorting-out of the two tissues’ cells after they have been randomly intermixed (Steinberg, 1962c, 1963a, 1970). This finding, together with others relating to the process and geometry of cell sorting (Steinberg, 1962a,b), produced the realization that all of these cell rearrangements closely imitate the behavior of immiscible liquids. The latter are well known to sort out when codispersed and to spread, one over the surface of another, when apposed, to adopt a specific configuration. Indeed, the commonly observed rounding-up of irregular fragments of embryonic tissues into a spheroidal shape is itself a manifestation of liquid behavior.

The determinants of the above behavior in liquids are their surface and interfacial tensions, which are global reflections of the intensities of cohesion and adhesion between their component subunits (Rowlinson and Widom, 1989). Two liquids are immiscible when the tension at their common interface has a positive value, in which case the liquid of lower surface tension will spread to a specific degree over its partner. This means that if one has a series of mutually immiscible liquids and combines them in pairs, their mutual spreading behavior reveals a transitive relationship; a hierarchy of mutual spreading preferences. If liquid a is spread upon by liquid b, and b is spread upon by c, then a will be spread upon by c if those two liquids are mutually adhesive. While it is not easy to find among ordinary liquids a large number all of which are mutually immiscible, this is readily accomplished with vertebrate embryonic tissues. The prediction of transitivity in embryonic tissues’ mutual spreading preferences was made the basis of a test of the ‘differential adhesion hypothesis’ (DAH; Steinberg, 1963a,b, 1970), attributing the liquidlike behavior of cell populations to tissue surface tensions postulated to arise from the adhesive and cohesive interactions of their component cells. Perfect transitivity was demonstrated in the mutual spreading behavior of six chick embryonic tissues (Steinberg, 1963b, 1970).

While the behavior of mutually confronted embryonic
tissues corresponds closely with that predicted by the DAH, a
direct proof of this explanation would require the demon-
stration that embryonic tissues possess liquidlike surface tensions
whose relative values consistently predict their mutual
spreading tendencies. Using an incubator-centrifuge to apply a
sustained deforming force to aggregates of chick embryonic
limb bud mesoderm, heart ventricle and liver (sessile droplet
method), we established that these three tissues possess liq-
uidlike surface tensions whose values decline in the sequence
cited, consistent with their mutual spreading tendencies
(Phillips and Steinberg, 1969). However, the development of
a density gradient in the serum-containing culture medium
during centrifugation prevented us from obtaining reliable
numerical values of these tissue surface tensions. To avoid this
complication, sustained deforming forces were subsequently
applied to spheroidal aggregates of subsurface amphibian
ectoderm, mesoderm and endoderm by compressing them
between parallel plates, producing the first numerical values of
tissue surface tensions (Davis, 1984). Although the number of
cases was small, those values also fell in the sequence required
to explain these amphibian germ layers’ mutual
spreading tendencies (Davis, 1984; Phillips and Davis, 1978).

Recently we introduced a parallel plate tissue surface ten-
siometer into our experiments, which continuously records
both the force applied to a living cell aggregate and the
aggregate’s profile shape, allowing the approach to shape equi-
lbrium to be constantly monitored in real time (Foty et al.,
1994). Using this device, we obtained numerical surface tension values for chick embryonic heart ventricle and liver
(three aggregates of each) which confirmed the sequence predicted earlier (Phillips and Steinberg, 1969). We report here
an extensive series of such numerical surface tension determini-
ations for those two tissues plus three others, together with the
effects of aggregate volume and culture duration upon the
constancy of aggregate surface tension values. We then
demonstrate that the resulting surface tension hierarchy accu-
trately predicts these tissues’ mutual spreading preferences.

MATERIALS AND METHODS

Preparation of cell aggregates

Fertile White Leghorn chicken eggs (Avian Services Inc., French-
town, NJ) were incubated at 37°C and 95% humidity for 3.5 to 6 days
depending on the tissue required. Limb bud mesoderm (LB) was
obtained from 3.5- to 3.75-day embryos, pigmented epithelium (PE)
from 4.5-day embryos, heart ventricles (H) and livers (L) from 5-day
embryos and neural retinas (NR) from 6-day embryos. Dissections
and dissociations were performed according to previously published
methods (Steinberg, 1970; Moyer and Steinberg, 1976; Heintzelman
et al., 1978; Thomas et al., 1981). Briefly, dissociations of dissected
tissue fragments were conducted in calcium-free Hanks’ balanced salt
solution (CF-HBSS) containing 0.05% trypsin (crystalline, Sigma)
and 0.05% EGTA for 10 minutes at 37°C. PE and LB required pre-
treatment in 1% trypsin at 4°C for 1 hour to remove the choroid coat
or overlying epidermis, respectively. Tissues were centrifuged in a
clinical centrifuge (IEC) at setting 4 for 4 minutes. Pellets were
mechanically dispersed by gentle pipetting in Dulbecco’s Minimal
Essential Medium (DMEM) with Earle’s salts, containing 10% horse
serum (Hyclone), 50 μg/ml DNase I (Boehringer-Mannheim) and the
following antibiotics: 70 μg/ml gentamicin, 50 μg/ml penicillin-
streptomycin-neomycin and 50 μg/ml kanamycin (Gibco-BRL).
Dispersed cells were washed once in the above solution, then cen-
trifuged for 1 minute at setting 1 to pellet clumps. The supernatants
containing single cell suspensions were adjusted to a concentration of
10^6 cells/ml and 3 ml were transferred to 10 ml tissue culture flasks
(Bellco, Vineland, NJ). Flasks were placed in a water bath/shaker at
37°C, 5% CO2 for 2-4 hours at 120 rpm. Upon recovery from
trypsinization, 3-4x10^6 cells per tube were transferred to round
bottom glass tubes 15 mm in diameter, centrifuged at setting 4 for 4
minutes and cultured until they formed a firm, thin pellet (usually 2-
4 hours, depending on tissue type). These thin pellets were then cut
into fragments about 1 mm in diameter and incubated on a gyroratory
shaker at 120 rpm under 5% CO2 at 37°C until fragments rounded up
(1.5 to 2 days, depending on tissue type).

Measurement of surface tensions

The apparatus and procedures summarized here have been described
in detail (Foty et al., 1994). In brief, cell aggregates produced as
described above were cultured in a shaker flask until they had
approached or attained spherical shape. Aggregates ranging in
diameter from 200 μm to about 600 μm were selected and compressed
between the parallel plates of the tissue surface tensiometer (Fig. 1)
in sterile MEM/HBSS containing 10% horse serum and antibiotics at
37°C. In the case of heart ventricle aggregates, 10^-4 M verapamil, a
blocker of the slow calcium channel, added to the medium prevented
them from beating (Barry and Smith, 1982). Coating of the compres-

![Fig. 1. Parallel plate compression device. The apparatus (not drawn
to scale) contains inner and outer rectangular Plexiglas chambers.
The outer chamber (OC) is connected to a thermostatted circulating
water pump and serves to regulate the temperature of the inner
chamber (IC). The lower assembly (LA) screws into the base of the
inner chamber. The position of its central core (CC), whose tip is the
lower compression plate (LCP), can be adjusted vertically by a screw
thread to set the distance between the two plates. The upper
compression plate (UCP) is a cylinder about 15 mm long suspended
from the arm of a Cahn/Ventron recording electrobalance (B) by a
0.15 mm diameter nickel-chromium wire (NCW). Its position can be
adjusted horizontally to place the UCP directly above the LCP. Both
plates are coated with polyHEMA before each use. During an
experiment, a spheroidal cell aggregate (A) is positioned on the
lower plate and raised until it contacts the upper plate. Compression
of the aggregate reduces the load measured by the balance by an
amount equal to the force acting upon the cell aggregate.
sion plates with poly(2-hydroxyethylmethacrylate) [poly(HEMA)] (Folkman and Moscona, 1978) minimized their adherence to the cell aggregates. The upper compression plate (UCP) was suspended from the arm of a Cahn electrobalance; the recording of its apparent weight being used to establish a precompression zero force baseline.

The balance used was a Cahn/Ventron (Cerritos, CA) Model 2000 recording electrobalance, which operates on the null balance principle. The fulcrum of the balance arm has an armature within a permanent magnetic field. When the balance is operating, it continuously modulates the current passing through the electromagnetic assembly, which in turn maintains the balance arm in the horizontal position. When an object is suspended from the balance arm, the voltage applied by the balance to keep the arm in the horizontal position is proportional to the force acting upon the balance arm (the object’s apparent weight). This voltage is continuously recorded by a strip chart recorder. The balance’s response time was investigated by using the tissue surface tensiometer to apply a force to an incompressible glass chip.

Raising the lower compression plate (LCP) compressed the aggregate between the two plates, the degree of compression being varied through adjustment of the height of the LCP. A strip chart advancing at 0.1 cm/minute continuously recorded changes in the force exerted by the aggregate upon the UCP, equal to the changes in the latter’s apparent weight, while the aggregate’s profile was continuously recorded at 25× magnification with a time lapse video cassette recorder connected to a video camera coupled to a horizontal stereomicroscope. The ultimate leveling-off of the force reading and concomitant cessation of aggregate shape change were taken to denote the attainment of shape equilibrium. After the compressed aggregate had reached shape equilibrium, operationally defined here as its exertion of a constant force upon the UCP for at least 20 minutes, it was released from compression in order to determine the final force it had exerted upon the UCP. When conditions permitted, it was then subjected to a second, greater compression and the entire process was repeated. When possible, a third compression was made. For each set of equilibrium force/shape data, an apparent aggregate surface tension was calculated by the method described below. In order to determine whether aggregate surface tension is influenced by time in culture or aggregate size, the surface tensions of aggregates of different sizes maintained in culture for various periods were determined.

Data analysis
The surface tension of a liquid droplet compressed between parallel plates to which it does not adhere is given by equation 1 (Foty et al., 1994),

$$\sigma = \frac{F}{\pi R^2} \left( \frac{1}{R_1} + \frac{1}{R_2} \right)^{-1}$$

where $\sigma$ is the interfacial tension between the droplet and the immersion medium, $F$ is the measured decrease in apparent weight of the UCP in dynes, $R_1$ and $R_2$ are the two principal radii of curvature of the droplet’s surface and $\pi R^2$ is the area of contact between the droplet and either of the parallel compression plates (Fig. 2). Of the three radii, $R_3$ is the most difficult to measure and is easy to overestimate (Yoneda, 1964; Adamson, 1967), since the image is blurred at the angle of contact between aggregate and compression plate. An initially spherical aggregate compressed between parallel plates to which it does not adhere will assume an equilibrium configuration in which the sides of the aggregate approximate semicircles. In that case, $R_2 = H/2$ (Fig. 2) and $R_3$ can be calculated from the relationship $R_3 = R_1 - R_2$, as has been done in a concurrent study of amphibian germ layer tissues (Davis, Phillips and Steinberg, unpublished data). However, when the chick embryonic cell aggregates used in the present study were released from compression, they were sometimes observed to adhere to the UCP despite its poly(HEMA) coating; and $R_2$ usually exceeded $H/2$ in our measurements, as is expected when there is a degree of adhesion between droplet and compression plate. Consequently we calculated $R_3$ as follows.

From the Pythagorean theorem, in equation 2

$$X^2 + \left(\frac{H}{2}\right)^2 = R_2^2$$

where $H$ is the distance separating the UCP from the LCP, equal to the height of the aggregate. It follows that

$$X = \sqrt{(R_2)^2 - \left(\frac{H}{2}\right)^2}$$

Since

$$R_3 = (R_1 - R_2) + X,$$ combining equations (3) and (4) yields

$$R_3 = (R_1 - R_2) + \sqrt{(R_2)^2 - \left(\frac{H}{2}\right)^2}.$$ Because $R_1$, $R_2$ and $H$ can all be directly measured with greater accuracy than $R_3$, the latter parameter was calculated using equation (5).

Videorecorded aggregate profile images, representing their equilibrium shapes, were transferred to a Silicon Graphix Cimron VGX workstation equipped with Videolab imaging software. Images were analyzed using NIH Image software. Circles of adjustable radii were superimposed upon and matched to the images of the aggregate’s sides. A tracing function was used to measure $R_1$, $R_2$ and $H$. $R_3$ was then calculated from eq. 5, taking $R_2$ as the average of the values measured on the left and right sides. The aggregate’s apparent surface tension ($\sigma$) was calculated from eq. 1. This value was taken to approximate its actual surface tension only if successive compressions of the same aggregate yielded similar $\sigma$ values (see Discussion).

Statistical methods
To determine whether the surface tensions measured in successive compressions of the same aggregate differed significantly from one another, $F$ tests were conducted (Zar, 1974). A one-factor analysis of variance (ANOVA) and multiple comparisons tests (Fisher’s PLSD and Scheffé’s $F$ test; Winer, 1971) were performed to resolve statistically significant differences between the mean surface tensions of the various tissues.
**Confirmation of envelopment behavior**

To examine the relationship between surface tension and envelopment behavior, tissues were ranked according to their surface tensions, after which adjacent tissues in the resulting surface tension hierarchy were combined in vitro and cultured in the same medium used for aggregate preparation. The tissue envelopment studies were necessary because the culture medium used in the present experiments differs from the media used in our earlier experiments to demonstrate a hierarchy in chick embryonic tissues’ mutual envelopment tendencies. Prior to aggregate formation, cells from each tissue were stained with either PKH26 Red Fluorescent General Cell Linker or PKH2 Green Fluorescent General Cell Linker (Sigma, St. Louis MO) as recommended by the manufacturer. Cells from each tissue were then either mixed in equal proportions or, in the case of the heart and liver combination, aggregates of the two types were prepared and then fused by overnight culture in a hanging drop. Mixed and fused aggregates were transferred to 10 ml culture flasks (Bellco Inc., Vineland NJ) and incubated in a shaker bath at 120 rpm under tissue culture conditions. When rearrangements appeared to be complete, aggregates were fixed in 2% paraformaldehyde in phosphate-buffered saline and viewed with a BioRad MRC600 scanning laser confocal microscope system attached to a Nikon Optiphot-2 microscope. Optical sections from both green and red channels were collected and a Silicon Graphix Crimson VGX workstation equipped with Vital Images Voxel View Ultra imaging software was used to assign false color to both channels and to merge the images, revealing the anatomical configurations generated.

**RESULTS**

**Force/time tracings**

Upon application of a compressive force to a cell aggregate, in all cases a significant fraction of the imposed stress was dissipated very rapidly, followed by a slower approach to an equilibrium value (Fig. 3). To determine whether the initial, rapid response to compression is a property of the aggregates or an artifact produced by the measuring device itself, the latter was used to apply a similar force to an incompressible glass chip. The balance’s full reaction appeared instantaneous, the strip chart recording a ‘square’ response when advancing at the usual rate of 0.1 cm/minute. The reaction of the cell aggregates studied here to a very brief compression is that of an elastic solid, aggregates springing back to their original shapes when the compression is quickly released (Fig. 4). Although cell aggregates respond to brief compression as elastic solids, cell rearrangements occurring during prolonged compression can relax compression-induced stresses (Phillips et al., 1977; Phillips and Steinberg, 1978; Phillips and Davis, 1978). This is demonstrated by the failure of the aggregate in Fig. 5 to spring back immediately to its pre-compression shape when the compression is finally released. These results suggest that the rapid initial relaxation of a compressed cell aggregate is due to its short-term elastic properties, whereas the longer-term relaxation reflects cell rearrangements which continue until the compressive force acting to increase the aggregate’s surface area comes into balance with the intercellular adhesive forces tending to decrease it. In the case of the cell aggregates utilized for our surface tension measurements, maximal relaxation took from as little as fifteen minutes (neural retina) to as long as 6 hours (heart ventricle; limb bud mesoderm). When an aggregate was subjected to a second, greater compression, the second equilibrium was often reached more quickly than the first.

**Tissue surface tension as a function of time in culture**

Aggregates of each tissue type were produced in batches and allowed to round up in shaker flasks from which aggregates

![Fig. 3.](image_url) **Fig. 3.** Force exerted upon the upper compression plate by an initially spheroidal liver cell aggregate as a function of time after application of an initial compressive force. Achievement of shape equilibrium by a compressed aggregate is denoted by the leveling off of the force reading and is confirmed by observation of the cessation of aggregate shape change. Relaxation of a liver aggregate, like that of all the cell aggregates studied, was bimodal, a significant fraction of the imposed stress being dissipated within the first few minutes after compression.

![Fig. 4.](image_url) **Fig. 4.** A spherical heart aggregate on the lower compression plate at 37°C (A) before compression, (B) after initiation of compression and (C) released from compression after a few seconds behaves as an elastic solid, springing back to its original, spherical shape.
Tissue envelopment and surface tensions were then periodically removed and transferred to our tissue surface tensiometer for measurement. This procedure yielded apparent surface tension measurements for aggregates maintained in culture for various time periods. As a general rule, the apparent surface tensions of aggregates which were just approaching spheroidal shape in culture were relatively low. These values increased with continued incubation for about a day and then stabilized for a time period which differed from tissue to tissue. Eventually a further increase in apparent surface tension often occurred (Fig. 6). Surface tensions of limb bud mesoderm aggregates remained constant from 1.5 to 2.75 days, those of pigmented epithelium, heart and liver were constant from 2 to 5 days and those of neural retina were constant from 2 to 3.5 days in culture. Except where noted to the contrary, aggregates employed for the measurements described below were cultured for a time period during which surface tension remained constant.

To be utilized for our analysis, aggregate surface tension values must be independent of the applied force, a characteristic of liquid rather than of solid bodies. This means that distinct sets of the quantities $F, R_1, R_2$ and $R_3$ measured on the

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**Fig. 5.** A spherical heart aggregate on the lower compression plate at 37°C (A), compressed for 3.5 hours (B), and then released (C), temporarily retains flat upper and lower surfaces. Behaving as a viscous liquid, it slowly rounds up again (D,E) when incubated for an additional few hours.

**Fig. 6.** Apparent surface tension of limb bud mesoderm (LB), pigmented epithelium (PE), heart ventricle (H), liver (L) and neural retina (NR) aggregates as a function of time in culture. In general, apparent surface tension increased for the first day in culture to a value which held relatively constant for a period and then increased again.
same aggregate at different degrees of compression should yield the same value of $\sigma$. When groups of aggregates were cultured long enough for their apparent surface tensions, measured in a first compression, to have begun their secondary increase and these aggregates were subjected to a second, greater compression, the $\sigma$ values were found to increase as a function of compressive force, rather than remaining constant. Thus these aggregates displayed a property of elastic solid bodies. Similar behavior was observed with a polyacrylamide sphere, a lesser followed by a greater compression yielding apparent surface tension values of 11.4 and 17.0 dyne/cm, respectively. In contrast, cell aggregates cultured long enough to have entered the surface tension plateau but not long enough to have left it yielded $\sigma$ values that were independent of the force applied to measure them. Fisher’s F test (Zar, 1974) confirmed for each such tissue that surface tensions measured in the first compression did not differ significantly from those measured in the second compression. Fisher’s F test was applied to evaluate the statistical significance of the differences between these two sets of means. Since $F_{\text{calc}}$ is in each case smaller than $F_{0.05}$, we must accept the null hypothesis that the mean surface tension values measured in the two sets of compressions are equal.

### Table 1. Statistical comparison of surface tensions measured in two successive compressions

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>$\sigma$ compression 1 (dyne/cm) mean ± s.d.</th>
<th>$\sigma$ compression 2 (dyne/cm) mean ± s.d.</th>
<th>F calc.</th>
<th>F0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limb bud mesoderm</td>
<td>5</td>
<td>19.96±1.56</td>
<td>19.26±2.45</td>
<td>2.47</td>
<td>6.52</td>
</tr>
<tr>
<td>Pigmented epithelium</td>
<td>6</td>
<td>11.67±1.92</td>
<td>12.97±2.40</td>
<td>1.56</td>
<td>5.33</td>
</tr>
<tr>
<td>Heart</td>
<td>4</td>
<td>9.03±0.78</td>
<td>8.98±0.57</td>
<td>1.87</td>
<td>8.79</td>
</tr>
<tr>
<td>Liver</td>
<td>7</td>
<td>4.47±0.34</td>
<td>4.43±0.44</td>
<td>1.67</td>
<td>4.63</td>
</tr>
<tr>
<td>Neural retina</td>
<td>9</td>
<td>1.61±0.59</td>
<td>1.70±0.52</td>
<td>1.29</td>
<td>3.91</td>
</tr>
</tbody>
</table>

Of the 62 aggregates whose surface tension measurements are shown in Table 2, 31 were subjected to two successive compressions. For each aggregate type, the mean surface tension values measured in the first compression are here compared with the mean values measured in the second compression. Fisher’s F test was applied to evaluate the statistical significance of the differences between these two sets of means. Since $F_{\text{calc}}$ is in each case smaller than $F_{0.05}$, we must accept the null hypothesis that the mean surface tension values measured in the two sets of compressions are equal.

### Tissue surface tension as a function of aggregate volume

The surface tension of a true liquid body is independent of its volume. To determine whether the cell aggregates whose surface tensions we have measured possess this property of liquids, aggregates of various sizes were prepared from each of the tissues utilized here and their surface tensions were determined. Aggregate surface tension was found in each case to be independent of aggregate volume, the surface tension of liver aggregates, for example, remaining constant over a 9-fold range of volumes (Fig. 7).

### Tissue surface tensions predict mutual envelopment behavior

Table 2 shows the mean surface tension values calculated for aggregates of each of the five tissues examined during their period of surface tension stability. Altogether, 62 aggregates were subjected to a total of 98 compressions. Limb bud mesoderm is the most cohesive of these tissues, with a surface tension averaging 20.1 dyne/cm (the equivalent of erg/cm²). Next most cohesive is pigmented epithelium, with an average surface tension of 12.6 dyne/cm. Heart ventricle and liver aggregate surface tensions averaged 8.5 and 4.6 dyne/cm, respectively. The least cohesive among these aggregates were those of neural retina, with surface tensions averaging 1.6 dyne/cm. A one-factor analysis of variance (ANOVA) and multiple comparisons tests (Fisher’s PLSD and Scheffé’s F test; Table 3) confirmed statistically significant differences between the mean surface tension values of all of these tissues.

To examine the relationship between tissue surface tensions and envelopment behavior, binary combinations of cells from adjacent tissues in the surface tension hierarchy were then produced, either by cell mixing or by aggregate fusions. As shown in Fig. 8, limb bud mesoderm is enveloped by pigmented epithelium which, in turn, is enveloped by heart

### Table 2. Aggregate surface tension values for five embryonic tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of aggregates</th>
<th>Number of compressions</th>
<th>$\sigma$ ± s.e.m. (dyne/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limb bud mesoderm</td>
<td>12</td>
<td>17</td>
<td>20.1±0.5</td>
</tr>
<tr>
<td>Pigmented epithelium</td>
<td>13</td>
<td>21</td>
<td>12.6±0.4</td>
</tr>
<tr>
<td>Heart</td>
<td>12</td>
<td>16</td>
<td>8.5±0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>14</td>
<td>22</td>
<td>4.6±0.1</td>
</tr>
<tr>
<td>Neural retina</td>
<td>11</td>
<td>22</td>
<td>1.6±0.1</td>
</tr>
</tbody>
</table>

![Fig. 7. Aggregate surface tensions are independent of their volume](image-url)
Tissue envelopment and surface tensions

which, in turn, is enveloped by liver which, in turn, is enveloped by neural retina. Thus, just as is the case with ordinary immiscible liquids, in each instance it is the body of lower surface tension (cohesiveness) which spreads over the surface of its partner.

**DISCUSSION**

**Cell aggregate surface tensions and tissue cohesiveness**

The DAH attributes liquid like behavior of cell aggregates to tissue surface and interfacial tensions postulated to arise from the cohesive and adhesive interactions of their component cells. Cell aggregate liquidity arises when an aggregate’s component cells, while cohering, are not bound in fixed positions either to each other or to any rigid matrix but rather are free to slip past each other. In the absence of an externally applied force, such an aggregate, if composed of similar cells with adhesion molecules uniformly distributed around their surfaces, will round up in the manner of a liquid droplet to minimize its surface free energy and therefore its free surface area.

If a sustained external force is applied to such an aggregate, its cells are initially stretched (Phillips et al., 1977). It seems likely that the rapid aggregate relaxation immediately following compression (see Fig. 3) reflects this cell stretching and associated deformations of whatever extracellular matrix might be present. However, the individual cells slowly rearrange and round up toward their original, unflattened shapes even as the aggregate as a whole continues to flatten in response to the applied force (Phillips et al., 1977; Phillips and Steinberg, 1978; Phillips and Davis, 1978). These cell rearrangements are probably reflected in the second, slower phase of aggregate relaxation. The greater the cohesiveness of the cells within such a spherical aggregate, the more energy is required to force them apart to create a unit amount of new aggregate surface area. This is measurable as the aggregate’s surface tension, which is independent of the aggregate’s size. It is measured under an applied force at shape equilibrium, when the interior of the liquid aggregate has relaxed and the applied force tending to flatten the aggregate is precisely balanced by the cohesive forces tending to make it round up. The surface tension is therefore a pure, thermodynamic quantity numerically equal to the aggregate’s specific (per unit area) surface free energy. It is a direct measurement of the intensity of cohesion among the aggregate’s component cells (Foty and Steinberg, 1995; Foty et al., 1996) but it does not distinguish whether this cohesion occurs directly between apposed cell surfaces or is mediated through matrix components.

**Successive compressions to assess aggregate liquidity**

Not all cell aggregates remain liquid indefinitely. In time, their cells can become fixed in position. Such aggregates cannot display cell sorting or tissue spreading behavior nor do they

![Tissue Surface Tension (dyn/cm) Equilibrium Configuration](image)

**Fig. 8.** The five embryonic chick tissues investigated are shown on the left in the order of their decreasing surface tensions. On the right are shown configurations generated, through cell sorting or aggregate fusion, when adjacent tissues in the surface tension hierarchy are combined and allowed to rearrange in vitro. Images are 60 μm optical sections through the resulting, fixed structures. Cells from the two tissue sources were stained with contrasting fluorescent markers (see Materials and Methods), here assigned false colors representing the five tissues. Limb bud mesoderm (green) is enveloped by pigmented retina (red), which in turn is enveloped by heart ventricle (yellow), which in turn is enveloped by liver (blue) which in turn is enveloped by neural retina (orange). In each case, the less cohesive tissue envelops the more cohesive one. The black areas within the aggregates contain cells whose fluorescent signal fell below background in the course of image optimization for contrast, brightness and color saturation.
Table 3. Multiple comparison tests for statistical differences between mean tissue surface tension values

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Fisher PLSD</th>
<th>Scheffé F-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural retina vs. liver</td>
<td>1.009</td>
<td>14.776</td>
</tr>
<tr>
<td>Neural retina vs. heart</td>
<td>1.100</td>
<td>68.018</td>
</tr>
<tr>
<td>Neural retina vs. pigmented epithelium</td>
<td>1.021</td>
<td>200.859</td>
</tr>
<tr>
<td>Neural retina vs. limb bud</td>
<td>1.081</td>
<td>506.110</td>
</tr>
<tr>
<td>Liver vs. heart</td>
<td>1.100</td>
<td>22.277</td>
</tr>
<tr>
<td>Liver vs. pigmented epithelium</td>
<td>1.021</td>
<td>107.609</td>
</tr>
<tr>
<td>Liver vs. limb bud</td>
<td>1.081</td>
<td>357.505</td>
</tr>
<tr>
<td>Heart vs. pigmented epithelium</td>
<td>1.111</td>
<td>23.655</td>
</tr>
<tr>
<td>Heart vs. limb bud</td>
<td>1.166</td>
<td>171.011</td>
</tr>
<tr>
<td>Pigmented epithelium vs. limb bud</td>
<td>1.092</td>
<td>81.259</td>
</tr>
</tbody>
</table>

According to both of the statistical tests employed, the differences in mean surface tension values measured for all of the above tissue pairs are significant at the 99% level.

Effect of culture duration on apparent aggregate surface tension

Some time ago we observed that heart and liver reaggregates cultured for only half a day envelop both older reaggregates and undissociated fragments of the same tissue. This was interpreted to mean that the cohesiveness of recently formed cell aggregates (1) is lower than that of the undissociated tissues from which they were derived and (2) can increase for a time in culture (Wiseman et al., 1972). Our finding that, in several of the aggregate types investigated here, surface tension increased for a day or two in culture before reaching a plateau confirms that interpretation. We believe that while some of this increase may be due to a slow restoration of the full complement of cell surface and/or extracellular adhesion molecules, some of it may also be due to a progressive increase in the area over which cells within the aggregate cohere, as intercellular spaces are eliminated.

The ‘second increase’ in apparent surface tension of certain aggregates cultured for a longer time period, particularly pronounced here in heart aggregates (Fig. 6), presumably has a different explanation. In every case, the force required to flatten these more differentiated aggregates by a unit amount has increased at the same time that the aggregates have lost their liquidity, a second, greater compression yielding a higher value for the apparent surface tension. These elevated values not being area-independent, they are not true surface tensions. This means that flattening such aggregates requires work other than that involved in expanding the surface area reversibly through cell rearrangements. Most likely, this energy is expended in stretching newly deposited extracellular matrix components and/or cells which have become immobilized as they have undergone differentiation.

Tissue surface and interfacial tensions as determinants of multicellular organization

The present experiments constitute a direct, physical test of the DAH on the basis of its requirement that one cell population which spreads over another must have the lower surface tension of the two. This relationship holds true regardless of the intensity of adhesion between the two populations, providing this is not so great as to cause them to intermix and form a single mixed tissue phase (Steinberg, 1963a, 1964, 1978). It has the corollary that tissue spreading relationships must be transitive. The tissues used here to test this relationship were chosen because their mutual spreading relationships had previously been investigated (Collins, 1966; Steinberg, 1970) and a hierarchy in their mutual spreading preferences demonstrated. The same tissue spreading preferences were observed in the present experiments although the culture medium differed from the ones employed previously.

We earlier established that the mutual spreading preferences of chick embryonic limb bud mesoderm, heart ventricle and liver correspond with these three tissues’ relative tissue surface tension values (Phillips and Steinberg, 1969). However, numerical surface tension values were not obtained in those experiments. Moreover, for a series of only three tissues, there is a significant probability that the observed correspondence between relative surface tension and mutual spreading tendency was due to chance alone. This can be calculated as follows. If these two properties were unrelated, the probability that the tissue which segregates internally to both of the others also possesses the highest surface tension (of three tissues) is 1/3. For the remaining two tissues, the probability that the one which segregates internally to both of the others also possesses the highest surface tension is 1/2. Thus, the probability that the envelopment hierarchy and the sequence of surface tension values (Phillips and Steinberg, 1969) of these five chick embryonic tissues and compared their sequence with these three tissues’ mutual spreading tendencies. A perfect correspondence between these two properties has here been demonstrated, the tissue of lower surface tension always tending to envelop the tissue of higher surface tension. The probability that this correspondence is due to chance alone is, for a series of five tissues, 1/5 × 1/4 × 1/3 × 1/2 = 1/120. Equal to 0.008, this is well below the 0.05 level required to establish the statistical significance of this correlation.

It is important to understand the succession of causal relationships that produce the rearrangements displayed by two (or more) mobile cell populations brought into contact. The immediate determinants of tissue immiscibility and spreading behaviors are forces, acting between mobile cells, which generate in the combined cell populations measurable surface and interfacial free energies. These forces arise from the interactions of cell adhesion molecules on and between cell surfaces. Equivalent forces, had they arisen through the cells’ possession of some other set of adhesion molecules, would have produced the same results. Thus direct morphogenetic causality here arises from the physics of the situation. Many reports have documented the association of particular adhesion molecules with particular morphogenetic events during embryogenesis and
elsewhere. However, a rigorous understanding of the roles of these adhesion molecules in these events will require an equally rigorous quantitative appraisal of the intensities of binding between and among all of the molecules involved, which summate to generate the morphogenetic forces. We have begun such an appraisal (Foty et al., 1996).

Adhesive interactions are only one class of morphogenetic determinants. The spreading movements with which we have here been concerned require cell mobility – an ability to change cellular partners in response to local forces. Cell populations in this regime can flow and behave in many respects as liquids. However, mobile cells may also change position in response to external attractive or repulsive chemotactic signals (Armstrong, 1985; Kennedy and Tessier-Lavigne, 1995). Adhesion molecules themselves can act as receptors in signal transduction pathways and, when activated by a ligand, alter the states and the behaviors of the cells which bear them (Rosales et al., 1995). Cell populations that are fixed in their mutual attachments cannot undergo liquidlike rearrangements but changes in the shapes of their component cells, brought about by cytoskeletal restructuring, may result in corresponding changes of an epithelial rudiment – thickening and narrowing, thinning and widening or bending and folding (Phillips et al., 1977; Ettensohn, 1985; Leptin, 1994; Schoenwolf, 1994; von Kalm et al., 1995). Both cartilage and bone are solidified by embedment in a solid extracellular matrix. We have here documented the passage of several kinds of cell aggregates from a ‘liquid’ to a solidified state in the course of time. Cell proliferation and apoptosis can also contribute to morphogenesis (Saunders and Fallon, 1967; Rosales et al., 1995). Given the existence of these many avenues for affecting cell behavior, it may seem surprising that the relative values of a single physical parameter – tissue surface tension – should alone be capable of explaining all of the cases of tissue spreading that have been investigated. However, it should be realized that no matter what other properties cells may have or how these properties may change due to cell interactions, if the cells remain mobile their cohesive and adhesive interactions will constantly act as a set of unavoidable physical determinants impelling them to shift positions at every opportunity to increase their binding intensities. Through repetition of this process, the cell population approaches a configuration in which its interfacial free energy represents at least a local minimum.

**Adhesion-guided assembly as a normal morphogenetic mechanism**

The rules governing the spreading behavior of apposed tissues could only be deduced by methodical perturbations of tissues’ contact relationships. These studies included the systematic combination of embryonic tissues that never normally encounter each other and the intermixing, in different proportions, of their dissociated cells. Most recently, we have combined cells genetically engineered to express predetermined adhesion molecules in differing amounts (Steinberg and Takeichi, 1994). These studies revealed that tissue spreading is only one manifestation of a wider syndrome of cell population behaviors. This syndrome includes not only (1) the spreading of one tissue mass over the surface of another but also (2) the rounding-up of irregularly shaped tissue fragments toward a spherical shape; (3) the sorting-out of heterotypic cell mixtures to approach a particular anatomical configuration by the coalescence of smaller islands to form larger ones, demonstrable at appropriate cell ratios; (4) the approach to the same final anatomical configuration by alternative pathways, e.g. cell sorting and tissue spreading; (5) the hierarchical ranking of tissues’ tendencies to envelop one another; and (6) the perfect correspondence between the sequence of these spreading potentials and that of the tissues’ measured surface tension values.

Disclosure of these linked behaviors (Steinberg and Poole, 1982) focused attention upon the only system known to display them: liquids, whose rearrangements were known to arise through the global maximization of inter-subunit binding energies. Because this principle operates to guide the system as a whole toward some most-stable arrangement, its operation leads to the self-assembly of a given structure from any number of different, initial configurations. Acting in any assemblage of mobile, cohesive subunits, the principal of system-wide maximization of binding between subunits explains the ability of embryonic cell populations to self-organize into semblances of the ‘proper’ structure through pathways not followed in the course of normal development. The demonstration that disassociated, intermixed and reggregated cells of amphibian gastrula and neurula tissues can reassemble into quite normal structures (Townes and Holtfreter, 1955; Phillips and Davis, 1978) leaves little doubt that those structures represent a most-stable arrangement. Direct, physical measurements on rounded-up fragments of these germ layer tissues have established that this arrangement, like those studied in the present work, conforms with the relative values of the tissues’ surface tensions (Davis, 1984; Davis et al., unpublished data).

It should be recognized that if a tissue complex can self-assemble, this implies that the properties underlying this behavior specify not a particular pathway of rearrangement but rather a process of rearrangement, leading toward the achievement of a particular, most-stable anatomical structure. The pathway followed in this process, similar though it may be from individual to individual, will nevertheless be an opportunistic one, leading ‘downhill’ to the favored structure following a route determined not by genetics but by the local tissue geometry. Investigations of the ability of various embryonic structures to self-assemble via novel pathways such as sorting-out can provide important insights into the mechanisms governing their normal morphogenesis.

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