Quantitative studies on the positioning of cells

in aggregates

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Acknowledgements

Summary

"Sorting out", that is the grouping of cells according to type and the positioning of cell types relative to the inside and outside in aggregates has been investigated in aggregates formed from mixtures of disaggregated 5 day embryonic chick heart and limb bud cells. The cell types were recognized in aggregates by radioactive labelling. Two quantitative tests for segregation of cells according to type have been used, one attempting to relate to the two cell types used and incorporating corrections for percentage labelling of labelled cell suspensions. In some cases the positioning of cell types within aggregates has been analysed quantitatively.

Data from mixed aggregates of "labelled" and "unlabelled" limb bud cells provided a crucial control. Each experiment has been carried out using reciprocally labelled cell suspensions.

The time course of "sorting out" and the effects of three disaggregation procedures on this process have been examined.

A marked degree of segregation of cell types in aggregates formed after 2 and 4 hours reaggregation in couette viscometers was found. Tentative evidence was obtained that heart cells may be positioned internally in these aggregates formed from EDTA disaggregated cells. When the cells are reaggregated in reciprocating shakers for 42 hours the grouping of cells in resultant aggregates was found to be less segregated than in the aggregates/

aggregates formed after 2 and 4 hours reaggregation. In aggregates formed after 48 hours reaggregation, in gyratory shakers, of cells disaggregated with EDTA, the cells show the same degree of segregation as in aggregates formed from cells similarly disaggregated after 2 hours reaggregation and heart cells were positioned externally.

Cells disaggregated by the three procedures investigated show different degrees of segregation in aggregates formed after 2, 4 and 42 hours reaggregation.

The results have been discussed in relation to theories explaining "sorting out".

INTRODUCTION

"Sorting out" is a term which can be considered to have evolved as the phenomenon it describes has been further investigated. By reference to the highlights in a wealth of literature it is possible to trace historically the discovery of the phenomena which the term "sorting out" defines.

"Sorting out" has been found to occur in an experimental situation; in reaggregates of mixtures of disaggregated cells of different types. Separated frog blastomeres were observed to reaggregate by Roux (1894). Wilson (1907) found that disaggregated sponge cells would reconstitute a sponge when allowed to reaggregate in a glass dish. Wilson realised that there were two hypotheses to explain this reconstitution; that the sponge cells 'redifferentiated' according to their position in the aggregate, or that the sponge cells moved during reaggregation to take up similar positions in the aggregate to those occupied in the intact sponge. Huxley's (1921) finding that only the types of cells allowed to reaggregate were found in the resultant aggregates led to a general acceptance of stability of cell type during reaggregation and the occurence of cell segregation according to cell type.

Townes and Holtfreter (1955) drew attention to the fact that, not only did disaggregated cells segregate into groups of like cells, but that these groups took up a defined position within the aggregate. In a series of experiments using amphibian material, the cell segregation and positioning was documented for various combinations of tissue slices and for the same combinations of disaggregated cells. The behaviour of mixtures of tissue slices and disaggregated cells was shown to follow a definable pattern for/

for the tissue types used.   These studies led to further
investigations of these phenomena, of cell segregation and
positioning of groups of like cells in aggregates, in
sponge, embryonic chick and mouse material.

Thus the occurence of "sorting out": that is, the
segregation of disaggregated cells according to their tissue
(or species) type and the relative positioning of these
segregated groups of cells in aggregates, has been shown in
many combinations of tissues and species. Also Steinberg
(1964) has demonstrated in some combinations of embryonic
chick tissues a 'hierarchy' of "sorting out". This means
that if cells of tissue A segregate internally to cells of
tissue B, and tissue B itself sorts internally to cells of
tissue C, then in mixtures of cells of tissues A and C, cells
of tissue A segregate in an internal position.

Diverse and increasingly sophisticated techniques
have been used to investigate "sorting out". These model
systems of cell interactions might yield information relevant
to normal morphogenesis, during which extensive cell movement,
aggregation and organization of tissues take place, and to
such processes as regeneration and wound healing. However,
the systems used to delimit the behaviour of mixtures of
cells may not be strictly comparable. The evidence for
"sorting out" can only be assessed in relation to the model
systems in which this phenomenon has been reported to occur.

Experimental disaggregation of cells has led to
hypotheses of how cells adhere.   A wide range of treatments
have been found to disaggregate tissues. Mechanical
dispersion alone was used by Wilson (1907) to obtain sponge
cells,/

cells, and usually still is for these animals except for a report by Moscona (1968) of the efficacy of pronase. This technique (mechanical dispersion) has to be used to separate cells from tissues, in which the intercellular adhesions have been weakened by treatment with chemical agents.

Roux (1894) found that in calcium free media, frog blastomeres were more easily separated than in whole salt solution. Ringer (1880) had previously found that calcium was necessary to preserve the normal intercellular contacts in tissue. These findings led to the idea that calcium was involved in the adhesion of cells and the use of calcium chelating agents (such as ethylene-diamine tetra-acetate [EDTA]) in disaggregation of chick and mammalian embryonic tissue. (Zwilling 1954, Anderson 1953). Alkaline pH has also been used to disaggregate tissue (Townes and Holtfreter 1955). Treatments with enzymes such as trypsin, papain etc. were also found to disaggregate tissue (Willmer 1945, Moscona 1951, Easty and Mutolo 1960, Rinaldini 1958). From his work on the enzymic disaggregation of tissues, Moscona (1963 a,b) suggested that cells adhere by means of a trypsin sensitive intercellular binding substance. This substance was presumed to be present in all cell adhesions and not a specialised differentiation product such as a collagen matrix, which is anyway insensitive to pure trypsin. This also led to the semantic and real confusion of the definition of the cell surface. (See Curtis 1967 for discussion).

Many experiments have been performed to discover the basis of cell adhesion. The two views, that of cells sticking together by physico-chemical forces in which calcium may be involved (Pethica 1961, Curtis 1962, Steinberg 1964, Curtis 1967) and that of the presence of intercellular cements (Moscona 1963a), both have adherents at present.

The/

The effects of different disaggregation procedures on cell surfaces and how these may affect cell behaviour have not been investigated systematically. Such changes, as increased "leakiness" of cells (Levine 1960), the inability of amphibian gastrula cells to readhere (Townes 1953), and animalization of sea urchin embryos (Moore 1952) have been reported to occur with trypsin treatments. Alterations in permeability and electrolyte content of cells treated with EDTA or trypsin may have important effects on cell behaviour and morphogenesis (Willmer 1960). However, no definite conclusions can be drawn from these studies as how to compare the behaviour of cell suspensions prepared by different techniques. Part of the aim of the work described here is to provide an answer to this question.

There is some evidence at present suggesting that cell suspensions prepared by different procedures may not be comparable in their behaviour. Curtis (1963) and Curtis and Greaves (1965) found that EDTA disaggregated embryonic chick cells aggregated perfectly well at low temperatures in serum free medium. Moscona and Moscona (1966) using trypsinised embryonic chick cells in serum free medium obtained inhibition of aggregation at low temperatures or with puromycin. Curtis (1967) reported that he had confirmed this inhibition of aggregation of trypsinised cells by low temperature in the absence of serum but this may be due to the use of trypsin. Moscona and Moscona (1967) had countered this idea and suggested that the discrepancy between their results and those of Curtis and Greaves (1965) was due to different criteria for aggregation. Curtis (1970) showed that the adhesiveness of embryonic chick neural retina and liver cells, as measured by the method of Curtis (1969)/

(1969) varied according to whether the cells were disaggregated with EDTA or trypsin.

The possibility that dispersion treatments may differentially affect cell types, either grossly or in the time to 'recover' is interesting in relation to "sorting out". Curtis (1970) has shown that the adhesiveness of trypsin disaggregated embryonic chick liver and neural retinal cells is different and also, varies with time. This clearly could have implications in the time course of "sorting out". (Curtis 1970).

Disaggregation procedures may be selective in the number and types of cells released from a tissue as suggested by Moscona (1965). Whether a selective effect could be produced differentially by different disaggregation techniques has not been investigated.

In much previous work on "sorting out", the presence of small clumps of cells in the initial cell suspensions has not been adequately eliminated. Zwilling (1963) did investigate this problem but only to compare the amount of clumping in the two cell suspensions he later reaggregated together. Any small clumps present in the initial suspensions may have effects on the time course of "sorting out". This problem has been largely solved by the use of cell sieves.

Three main methods of preparing aggregates have been used. Disaggregated cells have been allowed to settle and reaggregate in cavity slides where cell movements and Brownian motion are presumed to form aggregates (Wilson 1907). Conditions at the interface of medium and substrate have/

have been shown to affect cell movement (Rosenberg 1960, Carter 1965) and are difficult to control because of their ill-defined nature. Comparative studies in this system cannot be made. A variation of this method has been to allow cells to reaggregate on the chorio-allantoic membrane of the chick (Weiss and Taylor 1960).

In the other two methods of reaggregation, aggregation of the cells is not dependent on cell locomotion. Disaggregated cells have been pelleted by centrifugation and the pellets cultured on agar (Trinkaus and Lentz 1964). This system is interesting because the cells are already brought together in a potentially quantifiable arrangement. The criticism that the stratification of cell types may occur and thus the initial arrangement of cells is not random (Moscona 1965) need not be restrictive if the arrangement of cell types can be monitored quantitatively at the start and during the course of culture. However, all the cell contacts are not cell to cell and cell to agar attachments may have important effects. (Weston and Abercrombie 1967).

Cells have also been brought together to form aggregates in shaking flasks. This technique was introduced by Gerisch (1960), (he actually used roller tubes), but has subsequently been widely used (e.g. Moscona 1960 onwards, Steinberg 1964). Adhesion to surfaces of the flasks can be minimised by siliconing and cell to cell adhesions alone can be considered. This method overcomes cell type or species type differences in speed or ability to move (Galtsoff 1925) which may be operative in "sorting out" in still systems.

The nature of aggregation in shaker flasks has been reported to be reproducible. Moscona (1962) has used the size and number of aggregates formed in this system under certain standard conditions as a "measure" of the adhesive-ness/

ness of the reaggregating cells. The shearing forces shaking flasks which are a factor controlling aggregate size cannot easily be measured. Curtis (1969) has been able to measure the collision efficiency of cells in couette viscometers where this and other parameters can be calculated accurately.

The need to recognize accurately cells of different origins in mixed aggregates has been realised early on (Wilson 1907) to exclude the possibility of 'redifferent- iation' of cells. Since then, the search for reliable cell markers has continued so that the process of "sorting out" can be more accurately examined. However, the criteria for recognition of cells within aggregates have not always been sufficiently rigorous to exclude the possibility of cell type "redifferentiation" having taken place.

Histological markers such as glycogen in embryonic chick heart cells (Steinberg 1962) or morphological differentiation alone (Moscona 1955 onwards) cannot be assumed to remain stable if the metabolic state of the cell is affected during disaggregation and reaggregation. The efficiency of recognition of single cells of one type in masses of the other type may be rather imprecise on these criteria but could be tested by reference to cells marked by other methods.

Ingenious experiments using cells derived from different species have allowed the use of natural markers, such as the size and staining properties of nuclei (Moscona 1962) to trace the fate of mouse and chick cells in mixed aggregates. However, this technique is of limited application and indentification of individual cells is not always/

always possible due to gradations in nuclear size (Auerbach
and Grobstein 1958).

Burdick and Steinberg (1969) have recently investigated
the "sorting out" of mixtures of embryonic mouse and chick
heart cells using these differences in density of staining
and size of nuclei. Interestingly they checked, in one case,
the impression of the overall distribution of the types of
cells obtained by these morphological criteria by making
autoradiographs of sections of aggregates in which the chick
cells were radioactively labelled. Unfortunately, there
was no precise data correlating the accuracy of cell
identification by the two methods, although the overall
arrangement of cells was apparently the same. Incidentally,
in this work Burdick and Steinberg reported that the chick
and mouse heart cells "sort out"; previously it had been
thought (Moscona 1957, Moscona 1961, Wilde 1959) that
mixtures of mouse and chick cells "sorted out" according to
tissue type rather than species type. Species specific
groupings occur in mixtures of sponges of different species
(Galtsoff 1925, Moscona 1962).

Trinkaus (1963) and Trinkaus and Lentz (1964) have
used the granules of embryonic chick pigmented retinal cells
to recognize this tissue type in mixed aggregates. This is
obviously of limited application. Trinkaus (1963) however
stringently tested the stability and possibility of exchange
of pigment granules and thus produced good evidence that the
patterns he observed in pelleted aggregates were due to
"sorting out". Whittaker (1963) has also shown that pigmented
retina cells do not lose pigmented granules when cultured in
discrete pellets or maintained in organ culture.

The use of artificial markers to follow cell "sorting
out"/

out" has been limited. Okada (1965) used fluorescent antibody labelling techniques to examine "sorting out" in embryonic chick mesonephros and Mintz (1964) took advantage of genetic markers in mice. Trinkaus and Gross (1961) investigated thoroughly the suitability of tritiated thymidine as a cell marker by reference to a natural marker. Although they showed that it was easily detectable, stable, did not apparently affect cell behaviour and exchange could be calculated, this marker has been used by relatively few workers, until recently (Zwilling 1963, 1968, Trinkaus 1961, Weston and Abercrombie 1967, Roth and Weston 1967, Roth 1968, Burdick and Steinberg 1969, Adler 1970) to examine "sorting out" and related phenomena.

Although the techniques in much previous work have been insufficiently exact, the occurence of "sorting out" has been reported in many different combinations of tissue and species cell mixtures. There are a few cases of "non-sorting out" reported (Zwilling 1963, Moscona 1962, Curtis 1962). In the latter case (Curtis 1962), experimental ageing of the cells led to non-sorting, whereas under "normal" conditions, "sorting out" could be obtained (see later). Galtsoff (1925) reported that changes in the alkalinity of the medium in which sponge cells were re-aggregated could affect the patterns of "sorting out". (see later for patterns of "sorting out"). These, as far as I know, are the only reports of grossly different patterns of "sorting out" obtained by the same worker under different experimental conditions.

The question of whether cell type, as well as, or rather than tissue type "sorting out" occurs has not been examined critically by many workers. Okada (1965) showed that epithelial cells of the proximal secretory tubules of/

of chick mesonephros "sorted out" from other cell types
of the mesonephros. Zwilling (1968) discussed experiments
in which cells from embryonic chick limb bud mesoderm and
somites at an early stage of development were reaggregated
together. Although limb bud mesoderm and somites are both
destined to form muscle and cartilage, the cells from these
two sources appeared to segregate at this stage. Fully
differentiated chondrocytes of 8 day chick embryos from
different sources formed chimaeric cartilage when reaggregated
Adler (1970) showed that in aggregates of embryonic chick
neural tube cells, cells which are "differentiating" to form
neuroblasts tend to take up peripheral positions in the
aggregates, compared with neuroepithelial cells. This sort
of approach could help unravel the changing behaviour of
cell populations during morphogenesis.

Leaving aside for the present hypotheses to explain
the mechanism of "sorting out", this process has often
been divided into two phases. (Townes and Holtfreter 1955,
Moscona 1962). At first the cell types adhere in a random
manner to form an aggregate, later "sorting out" occurs,
although sponge cells may not "coalesce" at all with cells
of different species in still culture systems (Galtsoff
1925).

The experimental evidence for the existence of
these two phases will be considered firstly in the re-
aggregation of cells in still culture systems. Early
aggregates of pigmented and colourless amphibian cells have
a speckled appearance. (Townes and Holtfreter 1955). This
speckled appearance is lost as aggregation proceeds. The
first adhesions of amphibian cells have been shown
statistically to be at random (Lucey and Curtis 1959) but
this was an inadequately sized sample (Curtis 1967). In
cultures/

cultures of pelleted cell mixtures "sorting out" from a 'random' arrangement has been reported (Trinkaus and Lentz 1964) although the initial randomness has not been assessed quantitatively.

By extrapolation, it is assumed that aggregates formed in shaker systems at first have a random arrangement of cells. Sheffield and Moscona (1969) have published electron-micrographs of early aggregates of 10 day chick neural retina cells. They recognised from staining properties and morphological features four main 'types' of cells. During the first fifteen minutes, many doublets were formed and may be between like 'types' or unlike 'types'. Unfortunately there is no data on the frequencies of the composition of the doublets. The regions in which the cells adhered could not be related to the polarity of the cells. It should be noted that all the cells used were derived from one tissue which previously has been treated as one 'cell type' in "sorting out" experiments (Steinberg 1962, Stefanelli et al 1961). By 2 hours the aggregates were larger and the orientation of the cells had become ordered. Until more detailed accounts of this work are published with measurements of the composition of small aggregates, this evidence cannot be said to show that cell types adhere at random in early aggregation.

Burdick and Steinberg (1969) looked at 8 hour reaggregates of embryonic chick and mouse heart cells and judged qualitatively the arrangement to be random. They did not check their impression of the overall distribution of cell types using radioactive labelling of one cell type, as they did for $2 - 2\frac{1}{2}$ day aggregates. The existence of a protracted period of indiscriminate adhesion has been challenged by other experiments in shaking systems (Roth and/

and Weston 1967, Roth 1968). Roth (1968) estimated a time period of about four hours during which non-specific adhesion might occur (see later), which suggests that the 8 hour mixed aggregates of Burdick and Steinberg might show some degree of segregation undetectable by their criteria. This emphasises the need for the quantitative treatment of cell segregation.

Adler (1970) is the only worker that I know of, who has attempted to assess an aspect of "sorting out" quantitatively. He has measured the proportions of cells, using radioactive labelling to mark one cell type, in sections through aggregates and has correlated this with the size of the section. This provides an estimate of the positioning of the cell types within aggregates as the smaller sections are assumed to be peripheral parts of the aggregates. He found that the positioning of cells in early aggregates ($1\frac{1}{2}$ hours after the start of reaggregation) was statistically random, whereas in aggregates formed after 1 day there was a difference in the positioning of the two cell types investigated relative to the inside and outside of the aggregates. These measures show therefore that these cells are at first positioned at random, and later take up a definite position relative to the aggregate periphery and centre.

The work I will be describing here examines the time course of cell segregation and positioning of mixtures of two cell types aggregated in shaker systems. The cells are recognised by radioactive labelling and the arrangement of the cells in early and later aggregates has been analysed statistically. In view of the interesting effects of/

of disaggregating agents on the adhesiveness of cells
(Curtis 1970) especially for fairly short periods (up to
about 5 hours), the time course of cell segregation using
different disaggregation procedures to prepare the cell
suspensions has been investigated. These experiments should
solve the majority of the points outlined above.

There are several types of "sorting out" pattern
produced in aggregates of mixed cell types.

One type of tissue (the externally segregating) may
entirely enclose the other type (the internally segregating)
which may form a central single mass. In cases where
several discrete clusters of one tissue type are found in
a mass of the other, the terms discontinuous and continuous
phases are used which may correspond with the internally
and externally segregating tissues. Other patterns, partial
enclosure of one tissue by another or distinct separate
aggregates of each cell type (or species type) have been
recorded. (Review by Curtis 1967 of patterns). The
reproducibility of these patterns for any given mixture of
cell types under defined conditions has been assumed but
partially reported on by Steinberg (1964) alone. Little
work has been repeated under the same conditions, but rather
model systems and techniques have diversified. Little
evidence has been produced to test the assumption that
experiments are repeatable. Variation in positioning has
been reported by Weston and Abercrombie (1967) of two
tissue types under constant conditions, but these were not
aggregates.

Any theory of "sorting out" must account not only
for the preferential grouping of cells of one type, but
also/

also for the positioning of these groupings within the aggregate (Curtis 1962, Curtis 1967). The hierarchy of "sorting out" found by Steinberg (1964) should also be accomodated by the theory.

Chemotaxis has been suggested as a mechanism whereby cells sort out. (Townes and Holtfretor 1955, Stefanelli and Zacchei 1958). Although theoretically attractive in explaining grouping of cells, none of these workers produce any evidence that chemotaxis does occur in their aggregates or that it did not. Observations by Trinkaus and Monahan (1967) on living aggregates, containing pigment cells as one cell type, show that these cells show no directed migration towards cells of like type and in fact often move away. Positive chemotaxis has been shown to occur in the natural aggregation of cellular slime moulds, Dictyosteliaceae (Bonner 1947, Shaffer 1957) but not in their segregation. Although the positioning of segregated cells can be explained on this theory, the existence of a hierarchy of "sorting out" cannot be easily explained. This hypothesis raises the question of how motile cells are within aggregates, which will be discussed later on.

Another early theory suggested that each cell type had a specific mechanism of adhesion which might lead to "sorting out". Considering the specific adhesion theory alone and taken to its logical conclusion separate aggregates of each cell or species type should always result. Steinberg (1958) realising this, qualified his theory of specific adhesion postulating that adhesions between like cell types were strongest. Other workers have reconciled the theory of specific adhesion with experimental findings by combining points/

points of other theories and postulating a temporal lag
of indiscriminate adhesion before the onset of specific
adhesion (Moscona 1962). A discussion of these combined
theories is best left until all the theories have been
outlined, but the evidence for specific adhesion occurring
at all will now be assessed.

Moscona has claimed that the synthesis of an
intercellular cement or the reconstitution of the cell
surface in the broadest sense is necessary for cell re-
aggregation. The extracellular cement demonstrated by
Moscona (1960) was later shown by Steinberg (1963) to be
an artefact of the disaggregation procedure. The evidence
produced by Moscona that cells have to resynthesise a
component of the cell surface before aggregation can take
place, has been shown by Curtis and Greaves (1965) to be
susceptible to other interpretations, though Moscona and
Moscona (1966 and 1967) countered their suggestion. There
is therefore no definite evidence for the existence of these
intercellular binding substances, which Moscona (1962,1963,a)
postulates would be tissue specific and thus lead to "sorting
out".

Humphreys (1963), Moscona (1968) and Lilien (1968)
have isolated supernatants from culture media which are
claimed to increase specifically the adhesiveness of the
sponge species and embryonic chick tissue types with which
the medium has been 'conditioned'. The finding that
'conditioned media' enhance cell aggregation can be
interpreted as being due to the destruction of an aggregation
inhibitor (Curtis and Greaves 1965) and in several other
ways rather than the supplying of an intercellular binding
substance. The specificity of enhancement of aggregation
shown/

shown by Lillen could not be demonstrated by Roth (1968)
in another model system.

Roth and Weston (1967) have introduced a new
model system to study specific adhesion.   Day old
aggregates were prepared of one cell type.   These aggregates
were placed in flasks on gyratory shakers, together with
suspensions of freshly disaggregated (by trypsin) radio-
actively labelled cells. The number of labelled cells
collected by isotypic and heterotypic aggregates was counted
from autoradiographs.   They found that the collection of
cells was markedly isotypic and demonstrated adhesive
selectivity of cell types.   The generality of this phenomenon
found with liver and neural retina cells (Roth and Weston
1967) has been substantiated for some other cell types by
Roth (1968).   Curtis (1970) however, pointed out that only
0.1% of the cells in suspension were collected by isotypic
aggregates.   The implications of these findings (Roth and
Weston 1967, Roth 1968) to theories of "sorting out" will
be discussed later, and at present the possible interpreta-
tions of these experiments will be considered.

Roth and Weston (1967) have assumed that the adhesion
between freshly disaggregated cells and 24 hour aggregates
is essentially similar to that between single cells, or
small groups of cells. Roth (1968) used tissue fragments
rounded up for one day in shaker flasks and tested their
collecting abilities. Selectivity was shown to be the
same for fragments as well as aggregates.   Two points can
be mentioned here.

Firstly, although the fragments show the same
selective properties as aggregates prepared from disaggregated
cells, both "collectors" have been cultured for one day.
The effects this culturing might have on the surface properties
of/

of the external cells are difficult to assess.   The
surfaces of these external cells may well not correspond
with the surfaces of freshly disaggregated cells, which
have been used in "sorting out" experiments.

The second point deals with the effect of the size
of the particles in relation to adhesive stability.
Roth and Weston (1967) showed that the size of the collecting
particle affected to the number of single cells collected.
It is interesting to note that in the gyratory shaker the
number of single cells collected varied inversely with the
collecting aggregate size whereas in the reciprocating
shaker the relationship varied directly.   This may have
important effects on the time course of "sorting out"
studied in these two shaking systems.   In gyratory shakers
the size of the collecting aggregate may be limiting the
collection of single cells.   This situation does not seem
comparable with adhesion between single cells.

Roth (1968) has shown that the addition of freshly
disaggregated heterotypic cells reduces the collection of
isotypic disaggregated cells by isotypic aggregates, and vice
versa: disaggregated isotypic cells increasing the collection
of heterotypic cells by isotypic aggregates.   These results
suggest that there is an interaction between the freshly
disaggregated cells of different types which is not specific.
This interaction is abolished if the disaggregated cells are
'aged' in culture for longer than 4 hours before mixing and
collection is tested.

Curtis (1970) has introduced a method of detecting
specific adhesion which does not depend upon the correct
indentification of every cell in aggregates.   He has
measured/

measured the collision efficiencies (related to
adhesiveness, see Curtis 1969) of various proportions
of freshly disaggregated (trypsinised) embryonic chick
neural retina and liver cells. By comparing the plot of
collision efficiencies against the proportions of the two
cell types with a theoretical curve obtained assuming
complete specificity of adhesion he has found that only a
small proportion of these freshly disaggregated cells may
show specific adhesion. Essentially the same result was
obtained using cells which had been 'aged' under experimental
conditions which prevent aggregation and then mixed together.
He could not demonstrate in this way the onset of specific
adhesion of trypsinised cells after a time lag (Roth 1968).

Curtis (1970) has also measured in the same system
the collision efficiencies of suspensions of neural retina
and liver cells when mixed separately with isotypic and
heterotypic aggregates. No evidence for specific adhesion
except in maybe a small proportion of cells was obtained
using EDTA disaggregated cells and trypsin disaggregated
cells.

Curtis (1970) showed that small amounts of trypsin
carried over into the reaggregating system by trypsinised
cell suspensions may affect the adhesiveness of aggregates.
He suggests that this, together with his finding that the
adhesiveness of trypsinised neural retina and liver cell
suspensions change, in fact reverse in strength over a
five hour period, allow a new interpretation of the findings
of Roth and Weston (1967). This work of Curtis (1970)
suggests that temporal changes in adhesiveness of cell
suspensions and aggregates after exposure to trypsin may
affect/

affect cell behaviour, and that cell to aggregate adhesive interactions may not be applicable to the aggregation of cell suspensions.

Discussion of further work on specific adhesive mechanisms will be dealt with after outlining the other hypotheses to account for cell "sorting out". It is worthy of note that a specific adhesive theory alone cannot explain the positioning of cells in aggregates.

Both Steinberg (1962, 1964) and Curtis (1962) have suggested theories to explain "sorting out" which depend on quantitative differences in adhesion between cell types. These theories are attractive because not only can the positioning of cells within aggregates and the existence of a hierarchy of "sorting out" be adequately explained, but also the adhesion of unlike cells at the beginning of aggregation.

Steinberg suggests that "sorting out" takes place so that the system reaches optimal thermodynamic conditions. He considered a system of two cell types, a and b, which are cohesive and motile, if the unlike adhesions are stronger than the average strengths of like adhesions, the cell types will mix.

Mixing $W_{ab} \geqslant \dfrac{W_a + W_b}{2}$ case (1) W = strength of adhesion.

Steinberg stressed that is the only condition when mixing will occur. If unlike adhesions are stronger than the like adhesive strengths of one cell type, segregation will occur.

Concentric masses $W_a \rangle W_{ab} \rangle W_b$ case (2)

If however the average strengths of the like adhesions is much greater than unlike adhesions, the cell types will/

will segregate into separate aggregates.

Separate aggregates $\qquad$ $W_{ab} \leq \dfrac{W_a + W_b}{2}$ $\qquad$ case (3)

Partial enclosure $\qquad$ $W_{ab} < \dfrac{W_a + W_b}{2}$ $\qquad$ case (4)

The patternings postulated on these theoretical considerations have all been experimentally realised. In case (2) the system will be most stable when the free surface area is minimal. Aggregates formed in shakers are usually rounded up after several hours, though ragged aggregates have been reported to occur in some sera (Moskowitz 1963). The free surface should be comprised exclusively of the less adhesive type. Steinberg (1964) showed that in aggregates with 1% of the most adhesive cell type, this cell type was never found at the surface. The boundary areas between "sorted out" regions should have minimal surface area. Clusters of like cells in aggregates have been observed to be compact, or become compacted (Trinkaus and Lentz 1964).

Further support for Steinberg's hypothesis was his elucidation of a hierarchy of positioning. However, Townes and Holtfreter (1955) found in amphibian material that although mesoderm "sorts out" internally in combination with ectoderm or endoderm, in a tertiary tissue mixture mesoderm lies between endoderm and ectoderm.

Steinberg (1964) also showed that fused embryonic chick tissue fragments in hanging drops and in shaker systems would take up the same positions in aggregates as reaggregated cell suspensions. Townes and Holtfreter (1955) also had showed this with amphibian material. Weston and Abercrombie (1967) showed, using more precise marking/

marking techniques, that this enclosure of one tissue by the other occurred when fused embryonic chick fragments were cultured in reciprocating shakers, although some mistakes in positioning did occur. No spreading of one tissue around another occurred when the fused heteronomic fragments were cultured on agar. Bresch (1955) combined fragments of various embryonic chick tissues in organ culture. One tissue tended to spread over the other, but the positioning for any combination of two tissues was variable and was affected by the relative size of the two fragments.

Steinberg's hypothesis depends on cells being freely motile within aggregates. It also suggests that "sorting out" may take place at early stages of aggregation as soon as a choice of adhesions is available. Trinkaus and Lentz (1964) on observations by time lapse cinematography of living pelleted aggregates suggest that segregation in this system might begin after one hour in culture.

As regards motility in aggregates, there is little evidence for its occurence. It is not known whether contact inhibition (Abercrombie and Heaysman 1953, 1954) occurs in three dimensions. Weston and Abercrombie (1967) fused homonomic tissue fragments, the cells of one fragment being labelled, on agar for 24 hours, followed by further culture on agar or in shaking flasks. They showed that in neither case did cells appear to be freely motile, as judged by the absence of labelled cells in the unlabelled part of the fused fragments. Prolonged culturing of heteronomic fused tissue fragments on agar showed that there was little individual cell movement, apart from occasional cells on the surface away from the agar. These experiments provide/

provide evidence that in intact tissue slices there is
little gross movement of cells.

Reaggregating cells may have different motility
capabilities to cells within a tissue. Trinkaus and Lentz
(1964) have studied the potentialities of cell movement
in living aggregates using pigmented cells mixed with
heart cells. Small clusters, indistinguishable from single
cells, were observed to move, but no evidence for movement,
of larger clusters was found. This contrasts with the
finding of De Haan (1964) that clusters of precardiac
cells can migrate in vivo on an endoderm substrate.
Monahan and Trinkaus (1967) in living aggregates, have
not observed movements of more than 30 - 50μ. All these
observations suggest that movement may be very slight in
aggregates and that displacement of cells may occur by
competition of protrusible psuedopodia for the most stable
adhesions. Trinkaus (1966) suggested that this may lead
to incompleteness of "sorting out".

A timing hypothesis in several forms has been
postulated by Curtis (1961, 1962). Essentially, this
suggests that cell adhesiveness can vary differentially
with time according to cell type. He first suggested (Curtis
1961) that the onset of trapping of cells, after dis-
aggregation by contact inhibition could vary between cell
types. There is some evidence that contact inhibition
may occur in aggregates. The outer cell type may become
adhesive before the inner cell type and thus 'herd' the
less adhesive cells, at that time, towards the interior of
the aggregate. This will not necessarily produce a single
internal mass of one type (Curtis 1967) as suggested by
Steinberg (1964). Motility of cells, and the interval
between the onset of trapping of the two cell types may be
limiting./

limiting. On this theory the externally segregating cell type will be most adhesive at first, compared with the less adhesive at any time on the Steinberg hypothesis.

Curtis (1961, 1962) produced evidence of a timing mechanism in "sorting out" by artificially ageing one cell type in culture before addition of the other cell type. In this way he was able to 'confuse' "sorting out" in sponge and amphibian material. His results could be criticized in that the age differences between cells might lead to anomalous results and that his criteria for recognition of cell type may be questionable (Curtis 1967).

The timing hypothesis in a more general form suggests that the adhesiveness of cell types may be differentially affected by the disaggregation procedures or the medium in which reaggregation occurs. That tissue fragments which have not been treated with disaggregating agents also "sort out" (Townes and Holtfreter 1955, Steinberg 1964) argued against the timing hypothesis. Components of the medium, such as the presence of serum, could conceivably alter the adhesiveness of tissues as well as cell suspensions (Curtis 1965).

Trinkaus (1969) commented that there was no evidence that disaggregation procedures differentially affected cell types. Since then, Curtis (1970) has found that the adhesiveness of trypsinised neural retina and liver cells changed in the first five hours after disaggregation and suggested that these temporal changes in adhesiveness might be important in "sorting out".

The timing hypothesis suggests that "sorting out" could be an artefact of the system and does not explain the tendency of mixtures of cells from tissues which have a definite arrangement in organs of intact embryos to mimic this/

this positioning in aggregates. The question of whether induction may take place in such combinations has not been adequately discounted by stringent criteria for cell type recognition. Although Moscona (1962) stressed that in his experiments all tissues used were 'determined' and therefore discounted any inductive effects, the possible effects of disaggregation in relation to stability of cell type have not been investigated. It is well established that cells in tissue culture may change their differentiation properties. (Cahn and Cahn, 1967). Differentiation of cartilage in chick limb buds in vivo has been shown to be dependent on the position of cells within the limb bud. (Searls, 1967).

The timing hypothesis has stimulated many workers to invoke temporal changes in cell behaviour leading to "sorting out". Adherents to the specific adhesion theory have suggested that there is a lag in the onset of this adhesion and Roth (1968) has shown this in one system. This explains the assumption that the first formed adhesions in reaggregation are indiscriminate. Curtis' hypothesis would suggest however that "sorting out" may well occur during reaggregation. This hypothesis that "sorting out" occurs at early stages, would also seem to fit in with Steinberg's theory, although he does not suggest this.

Roth and Weston (1967) have shown in their system isotypic adhesions would be stronger than heterotypic adhesions. On Steinberg's hypothesis this should lead to separate aggregates being formed. It is difficult to reconcile the findings of Roth and Weston with the positioning most commonly reported in aggregates, that of/

of one tissue type surrounding island(s) of another
type. Assumptions of decreased motility of cells with
time may help.

MATERIALS AND METHODS
TISSUE CULTURE

(a) Materials

Hanks's solution

NaCl	8 gm.	)
KCl	0.4 gm.	)
Glucose	1.0 gm.	)
$NaHCO_3$	0.35 gm.	)
KH_2PO_4	0.50 gm.	) in 1000 ml. double
Phenol red	1 ml.	) distilled water.
$CaCl_2$	0.14 gm.	)
$MgSO_4 \cdot 7H_2O$	0.10 gm.	)
$MgCl_2 \cdot 6H_2O$	0.10 gm.	)
Tris	3.0 gm.	)

(hydroxyamino
methylene propane)

pH was adjusted to 7.4 with "Analar" HCl.

CMF Tris buffered saline

NaCl	7.0 gm.	)
KCl	0.37 gm.	)
$Na_2HPO_4 \cdot 12H_2O$	0.30 gm.	) in 1000 ml. of double
KH_2PO_4	0.24 gm.	) distilled water.
D($\div$) Glucose	1.0 gm.	)
Phenol red	2 ml.	)
Tris	3.0 gm.	)

pH was adjusted to 7.8 with "Analar" HCl.

Embryo extract was prepared as described by Paul (1965).

All/

All manipulations in tissue culture methods were carried out under sterile conditions. All solutions were sterilised by passage through Millepore filters (Millepore Corporation, U.S.A.) of pore size 0.22μ, except trypsin solutions which were passed through filters of 0.45μ. All glassware and instruments were sterilised by heating at $160^{o}C$ for two hours. Cell sieves were sterilised by autoclaving at 15 lbs./in^{2} for 15 minutes.

(b) Methods

In each experiment four dozen fertilised hen eggs (Dekalb hens) were incubated at $37^{o}C$. On the third day of incubation, two dozen eggs were windowed by the method of Zwilling (1959). The embryos in the windowed eggs were later "labelled" with tritiated thymidine (see Autoradiography).

On the fifth day of incubation, "labelled" and "unlabelled" embryos were removed aseptically from the eggs and placed separately in Hanks's solution (see previously, Materials). Hearts and limb buds were dissected from the embryos into separate dishes containing Hanks's solution. These four lots of tissues (i.e. "labelled" limb buds, "unlabelled" limb buds, "labelled" hearts, and "unlabelled" hearts) were then disaggregated separately by one of the following techniques.

I   EDTA disaggregation (after Curtis and Greaves 1965).

The tissues were washed three times in calcium and magnesium free saline (CMF, see previously Materials) and then treated with 0.001 M EDTA in CMF (pH 7.8) for ten minutes at $20^{o}C$. After three further washings with CMF,/

CMF, the tissues were mechanically disaggregated in CMF by flushing several times through a fine bore pipette.

II Trypsin, Pancreatin, EDTA disaggregation, (after Steinberg 1963) referred to later as Trypsin and EDTA disaggregation.

The tissues were washed twice in the "disaggregating medium", which was 3% W/V trypsin (Difco 1:250, 1,000 BAEE units of tryptic activity per mg), 1% W/V pancreatin (Sigma) and 0.1% W/V EDTA in CMF, pH 7.6. After a twenty minute incubation at $37^{o}C$ in this medium, the tissues were washed briefly with Hanks's solution containing 50% chick serum (Flow Laboratories) to stop tryptic activity. After a further wash in Hanks's solution, the tissues were mechanically disaggregated (as in procedure I) in Hanks's solution/chick serum (50/50).

III Trypsin disaggregation (after Roth and Weston 1967).

The tissues were washed with Hanks's solution and CMF, prior to a twenty minute incubation with 0.25% trypsin (Difco 1:250, 1,000 BAEE units/mg) solution at $20^{o}C$. The tissues were then rinsed with Hanks's solution and chick serum (50/50) and then with CMF. Disaggregation of tissues was then carried out as in procedure II.

The preparation of cell suspensions was carried out as follows. The cells were centrifuged at 25g for two minutes to sediment any cell clumps. A second "harvest" of cells was sometimes prepared from the pellet obtained in this first centrifugation, by resuspending the pellet in CMF and reflushing through a pipette. The supernatant of the first centrifugation (or the pooled supernatants, when/

when a second harvest was made) was then centrifuged at
300g for ten minutes to pellet the cells. The supernatant
containing cell debris, and maybe a few cells, was dis-
carded. The pellet of cells was then resuspended in a
known volume of "reaggregating" medium or sometimes in
Hanks's solution. The concentration of cells/ml. was
then determined by haemocytometry. The cells were
dispensed, to reaggregate, through cell sieves (nickel
electroformed grid of mesh 22μ, E.M.I. Ltd., Hayes, U.K.),
unless stated to the contrary. This ensured that the
initial reaggregating suspensions contained no clumps
larger than three cells.

The reaggregating medium was Hanks's solution
44.4%, Medium 199 (Glaxo Laboratories) 44.4% and chick
serum 11.2% (i.e. 9 ml of medium was made up with 4ml.
Hanks's, 4 ml. medium 199 and 1 ml. chick serum). This
medium was used for all reaggregations except those in
gyratory shakers, when the medium was Hanks's solution
40%, chick serum 40%, and embryo extract (see previously
Materials) 20% (Steinberg 1963). Reaggregation was
carried out in one of the following ways.

I Flask Shaker Systems

The cells were mixed in desired proportions in
siliconed 10 ml conical flasks. The siliconed surface
to prevent cells sticking to the sides of the flasks was
produced by dipping acid cleaned flasks in 0.1% silicone
fluid MS 1107 (Hopkin and Williams Ltd.) in ethyl acetate
followed by baking for at least half an hour. The flasks,
stopped with silicone bungs, usually contained a minimum
of 1×10^{6} cells/ml. of mixed cells in two to three ml.
of solution; the gas phase was air.

The/

The cells were reaggregated for 2 days or 1 day
in flasks shaken in a reciprocating shaker (Gallenkamp)
at 92 strokes/minute at 37°C. (Curtis and Greaves 1965).
In one experiment the flasks were shaken in a gyratory
shaker (New Brunswick Scientific Co., Inc.) at 80 rpm
for 17 hours, then the rate of gyration was increased to
100 rpm for a further culture period of 31 hours (Steinberg
1962) at 37°C to prevent further fusion of aggregates.

II Couette Viscometers (Curtis 1969)

The cells were mixed in desired proportions in
couette viscometers at a concentration of 1×10^6 cells/ml.
approximately in 15 ml. of medium. The cells were
reaggregated at a shear rate of 8 sec^{-1}. Under these
conditions of low shear fairly large aggregates are
formed in a short time period. Cells were reaggregated
for 2 hours or 4 hours in this way.

HISTOLOGY

Aggregates formed after selected periods of time were transferred into test tubes. Usually all the aggregates from one flask or couette viscometor were placed in one or two test tubes. The aggregates were washed with Hanks's solution prewarmed to 37°C. This washing removes serum and other proteins in the medium, which would precipitate during fixation. The aggregates were then fixed with Bouin's fluid for 10 - 15 minutes. This fixative was chosen because it is compatible with autoradiography provided the picric acid coloration is removed (Rogers 1967). After fixation, the Bouin's fluid was pipetted off the aggregates (see later) and replaced by 70% alcohol, in which the aggregates could be stored prior to subsequent handling.

The handling of small aggregates at first presented a problem. Pipetting of aggregates either between solutions or to embedd, often led to their loss. Aggregates successfully embedded in this way tended to be dispersed throughout the block which made sectioning tedious. It was decided to use a method that allowed easy changes of medium and resulted in a block containing a 'pellet' of aggregates.

The most satisfactory method found was that of Fantin (1964) for protozoa. As already described the aggregates were fixed in test tubes. The aggregates were dehydrated through 90% alcohol and two changes of absolute alcohol in ten minute steps in the tubes. Two ten minute changes of xylene cleared the aggregates. Ten minute changes of 50/50 xylene/paraffin wax (MP 58°C), two changes of pro-filtered/

filtered paraffin wax followed at 60°C in the tubes. In the case of large aggregates, these settled to the bottom of the tube in the ten minute intervals. With small aggregates, which did not settle in this time, gentle centrifugation 'pelleted' the aggregates between each medium change. Centrifugation of aggregates in wax was carried out in jackets of hot water.

For embedding in wax, the aggregates were collected in the bottom of the tubes with gentle centrifugation if necessary. A copper wire handle was placed in the upper part of the wax. The wax was hardened by plunging the tubes into ice. The tube can then be placed in boiling water to melt the wax in contact with the tube and the "block" removed by means of the wire handle.

The rounded block containing the aggregates, usually visible due to slight picric acid coloration, was then trimmed on two sides. 5μ serial sections were then cut on a Jung rotatory microtome (see Counting).

The sections were floated out on "subbed" slides on a warming plate. "Subbed" slides were produced by dipping chromic acid cleaned slides into a filtered 5% W/V gelatin and 0.1% W/V chrome alum solution at room temperature and allowing them to dry in dust free conditions. The "subbed" slides can be produced in bulk and stored at 2°C. The "subbing" of the slides acts as an adhesive for the sections as well as the nuclear emulsion (Rogers 1967). The floating out solution was 50% alcohol. This procedure had two advantages; creased sections were flattened more readily than in water, and the picric acid coloration was removed, a step necessary for subsequent autoradiography (Rogers 1967). The flattened sections were then dried at 37°C overnight.

The/

The sections were then carefully dewaxed in three changes of xylene. It is important to remove all the wax to promote firm cohesion between section and nuclear emulsion (Rogers 1967). The sections were then brought down to distilled water through absolute alcohol (two changes), 70% and 50% alcohol. The sections were then dried at 37°C and ready for application of nuclear emulsion.

AUTORADIOGRAPHY

(a) Dosage and application of isotope.

The dosage of tritiated thymidine needed to "label" chick embryos _in vivo_ depends on the age of the embryo (Weston 1967). Experiments were carried out to discover the optimal dosage of tritiated thymidine (6 - T(n) Thymidine, Radiochemical Centre, Amersham) to "label" five day embryos, using the recommendations of Weston (1967) as a guide to the dosage required. Autoradiographs of 5μ sections of whole "labelled" 5 day embryos, or limb buds or hearts of 5 day embryos were prepared (see later). The trial autoradiographs allowed the effective dosage to be determined as 15μC of tritiated thymidine (specific activity 5.0 curies/mM) per egg, in conjunction with optimal exposure and development time (see later).

Routinely 15μC of tritiated thymidine were pipetted aseptically on to the yolk sac of four day chick embryos in 0.1 ml. of solution made up with Hanks's. After a 24 hour incubation with the labelled thymidine, the tissues were harvested (as earlier).

(b) Application of emulsion to aggregate sections.

All manipulations were carried out in a darkroom with safelight filter "Wratten" series no.1 (Kodak). Ilford nuclear emulsion gel L 4 was used to coat the sections. 20 ml. of emulsion was melted at 40°C and then diluted with 40 ml. of warmed distilled water in measuring cylinders. The diluted emulsion was then poured down the side of a dipping jar, to prevent undue frothing, into the jar. Chromic acid cleaned slides were dipped into the diluted emulsion to clear any bubbles. The dried experimental slides were/

were then dipped into the emulsion, drained, wiped on
the back and placed on a cool tray. A standard rhythm
of dipping was evolved. The slides were then left to dry
for about an hour, after which they were packed into
light tight boxes and sealed. The glassware used in
dipping was cleaned in the manner recommended by Rogers
(1967). The coated sections were then exposed at $2^{\circ}C$.

(c) Development of autoradiographs and staining.

Trial slides of sections of embryos or tissues were
exposed for various lengths of time prior to development.
This, coupled with varying development time, allowed the
optimal times to be determined to give suitable auto-
radiographs for viewing under light field microscopy.
conditions.

Routinely sections were exposed for about 40 days
at $2^{\circ}C$. The slides were developed and fixed under the
darkroom conditions previously mentioned. D19b developer
was made up (Horder 1958) and filtered and stored in
400 ml. aliquots in the dark at $2^{\circ}C$. The slides were
transferred from the boxes to a slide rack. In each rack
an emulsion coated slide which had been exposed to light
was included. The development of this slide provides a
useful check of the state of the developer solution (Rogers
1967). A development time of 7 minutes at $20^{\circ}C$ with no
agitation was found to give optimal visualisation of silver
grains, a large number of grains per "labelled" nucleus
and a low background count under light field illumination.

After development, the slides were passed into a
stop bath of distilled water and then fixed in a filtered
solution of 30% sodium thiosulphate for 10 minutes at $20^{\circ}C$
(Rogers 1967). The slides were then washed for 15 minutes
in/

in slowly running filtered tap water.

The slides were passed through distilled water, 50% and 70% alcohol with a few minutes in each solution. The sections were stained in Gurr's Erhlich's acid haematoxylin (filtered) for 30 minutes - 1 hour. Post-staining with this stain is known not to affect the silver grains (le Blond, Kopriwa and Messier, 1963). Adequate differentiation of the stain (usually a few minutes) was carried out in acid alcohol (1 ml. N HCl in 100 ml. 70% alcohol). "Blueing" was accomplished in Scott's Tap Water Substitute (0.7% W/V sodium bicarbonate, 4.0% W/V magnesium sulphate (crystalline) in distilled water). The sections were dehydrated by passage through 90% alcohol (2 minutes) and two passages of 2 minutes in absolute alcohol. The slides were cleared to remove any air locks, in cedar oil/absolute alcohol (50/50) for one hour followed by xylene/Canada Balsam (50/50) for another hour (le Blond, Kopriwa and Messier, 1963). The slides were mounted with Chance No.1. coverslips with neutral thin Canada Balsam. The slides were allowed to dry and then cleaned with xylene.

COUNTING

A. The use of tritiated thymidine as a cell marker.

(1) Criterion for recognition of a "labelled" cell.

A cell was considered to be labelled if there were five or more grains over the nucleus, as the labelling intensity was high and background low. Baserga and Malamud (1969) suggest an arbitary lower limit of four to five grains per cell to distinguish "labelled" from "unlabelled" cells under these conditions.

Although I did not know of this paper at the time and have not used his method, Stillström (1963) has suggested a less arbitary and therefore preferable way of estimating the proportion of "labelled" cells in a population. By reference to control autoradiographs of unlabelled tissues the proportion of cells which have a higher number of grains than background can be calculated. This method, however, assumes a uniform background count which is practically rarely achieved.

(2) Percentage labelling of the "labelled" cell suspensions.

Identification of cell type by radioactive labelling can only be certain if all the cells of the cell type which is labelled have taken up the label. It is important for recognition of cell type to determine the percentage of cells which are labelled in the "labelled" cell suspension, i.e. the percentage labelling.

It was decided to determine the percentage labelling of "labelled" cell suspensions in a manner that could directly be related to aggregates of mixed cell types. An estimate of the percentage labelling, if determined from/

from cell smears or short term coverslip cultures of
"labelled" cell suspensions, cannot be extrapolated to
aggregate sections without considerations of section
thickness and penetrance of β emissions.

(a) Digression.

With β emitters, such as tritium, the distance
between the source and emulsion is critical because the
pathlength is short.   Any inert layer between the source
and emulsion will effectively screen the percentage of
source emitters registering in the emulsion. With short
pathlength β emissions (tritium) the thickness of the
source can affect the efficiency. Above certain thicknesses
of source, approximately equal to the pathlength, self
absorption occurs. In other words, the emissions are
"quenched" by the source before reaching the emulsion.
The degree of "quenching" of β emissions will depend on
the source thickness. Smears of cells and coverslip
cultures are therefore not comparable in autoradiographic
efficiency with 5μ sections.   The autoradiographic
efficiency is "the number of grains produced in the nuclear
emulsion per radioactive disintegration in the source"
(Rogers 1967).

In a section of 5μ thickness, the self-absorption
of $H^3\beta$ particles is higher than in thinner sections (see
Rogers (1967) for a table of autoradiographic efficiency
as a function of section thickness with a source uniformly
labelled with tritium). However, the flattening of wax
sections becomes more critical, the thinner the section.
Steedman (1960) quotes the compression after flattening
as 28% with 3μ and 19% with 6μ wax sections. Estimates
of the maximum pathlength of β particles from tritium in
media of higher density than air vary. Rogers (1967)
gives/

gives 3μ and Lathja and Oliver 8μ as the maximum path-length.

If the section thickness is greater than the limit of penetrance of β emissions from tritium only a proportion of the labelled nuclei and fragments will produce autoradiographic images. Simnett (1968) derives a correction factor which can be applied, if all other parameters are determined, to give the total number of labelled nuclei per section.

The number of unlabelled nuclei in a section can be calculated from the fragments of nuclei observed, provided the relationship between nuclear diameter and section thickness is considered (Abercrombie 1946, Marrable 1962). It is therefore possible to express all counts of fragments of unlabelled nuclei observed and of autoradiographic images observed in terms of the total number of labelled and unlabelled nuclei per section, i.e. in the same terms. The advantage of correcting counts of unlabelled and labelled nuclei in this way is that any differences in the "efficiency" of observing unlabelled and labelled nuclei in the section can be eliminated (see later). However, in segregation tests the position of one cell type relative to another in the plane of section observed are scored. This means that observational counts must be used. It was therefore decided not to convert any counts of the number of labelled and unlabelled nuclei observed into the total number of nuclei per section.

(b) Method of determining percentage labelling.

Aggregates were prepared solely of "labelled" cell suspensions in the routine manner. Aggregates of "labelled" limb/

limb bud cells and of "labelled" heart cells were sectioned at 5μ and autoradiographs prepared. Roth and Weston (1967) used this method to determine percentage labelling.

Aggregates formed after 42 hours reaggregation were prepared from "labelled" heart cells disaggregated with EDTA. Similarly aggregates of "labelled" limb bud cells were produced from cells disaggregated by all three techniques. This provides a check that each disaggregation procedure releases a similar proportion of "labelled" cells from limb buds. Ideally the proportion of "labelled" cells released from hearts should also be checked with each disaggregation technique although this was not done.

Cells were reaggregated for 42 hours, so counts of these aggregates would yield a percentage labelling relevant to aggregates formed from two cell types in 42 hours. It is possible that some dilution of label due to cell division during two days in culture might take place. This may cause over-correction when the estimate of percentage labelling from aggregates formed after 42 hours is applied to aggregates formed from two cell types in shorter time periods. Whether this is the case could be checked by aggregating "labelled" heart or limb bud cell suspensions for equivalent time periods, although this was not done.

Random fields of every third section of randomly chosen aggregates (see later) was examined at x900 (oil immersion) magnification. Cells, in randomly chosen squares (by means of a numbered square grid eye-piece and tables of random numbers) were scored as labelled or un-labelled. Baserga and Malamud (1969) suggest the counting of the number of labelled cells in a total of 1,000-2,000 cells is sufficient for a crude estimate of the percentage of labelled cells.

(c) Results.

Labelled limb bud aggregates:

	% labelled	total number of cells counted
(a) EDTA disaggregation	93.2	884
(b) EDTA + TRYPSIN dis-aggregation	94.3	820
(c) TRYPSIN disaggregation	92.3	908
average =	93.27	2,612

proportion of unlabelled cells in "labelled" limb bud aggregates   =   0.0673

Labelled heart aggregates:

	% labelled	total number of cells counted
EDTA disaggregation	91.08	897

proportion of unlabelled cells in "labelled" heart aggregates   =   0.0892

(3) The problem of exchange of label between cells.

Trinkaus and Gross (1961) estimated exchange of label from labelled cells to unlabelled cells, by reference to a second natural marker, pigment granules in pigmented retinal cells.   A reliable independent marker for heart or limb buds could not be found.   Steinberg (1962) recognised heart cells in aggregates by staining with Bulmer's periodic acid - dimedone-Schiff procedure (Bulmer 1959).   This stain was found to be compatible with autoradiography if staining was carried out before application of nuclear emulsion.   Labelled 5 day limb bud sections showed no staining.   The uptake of stain by heart cells in sections of 5 day labelled heart was found to be patchy and not reliable in my hands.

Duplicate/

Duplicate experiments were therefore performed.
In one case "labelled" heart cells were mixed with
"unlabelled" limb bud cells; in the other "labelled"
limb bud cells were mixed with "unlabelled" heart cells,
i.e. reciprocally labelled experiments (referred to
hereafter as reciprocal experiments). If no appreciable
exchange of label takes place the arrangement of cells
in reciprocal experiments will be the same irrespective
of which cell type is labelled. A difference in
arrangement of cells in reciprocal experiments can
therefore indicate that exchange of label is taking place
or that the "labelling" of the cell suspension affects
the behaviour of the cells.

(4) The effect of labelling on cell behaviour.

The effect of labelling on cell behaviour was
investigated by Trinkaus and Gross (1961). They detected
qualitatively no difference in the behaviour of labelled
cells in regard to migration, "cell affinities" and
morphogenesis compared to that of unlabelled cells of the
same type. It is desirable to have a direct test of
the effect of labelling in a "sorting out" system. This
test can be readily performed by mixing together "labelled"
and "unlabelled" cells of the same cell type. Aggregates
of "labelled" and "unlabelled" limb bud cells were pre-
pared and counted in the routine manner (see later).
Ideally similar aggregates of heart cells should be pre-
pared but this was not done.

Assuming that labelling does not affect cell
behaviour, it would be expected that the "labelled" and
"unlabelled" cells in these aggregates would be randomly
arranged if, also, there is no gross exchange of label
between cells. A small divergence from a random pattern
of/

of cells in these aggregates was found and the implica-
tions of this will be discussed (see control results).
The analysis of these aggregates (referred to as control
aggregates) form a crucial control for studying the
arrangement of cells in aggregates of two cell types.

B. Measures of the arrangement of cells in aggregates.

The arrangement of cells in aggregates has been studied quantitatively with regard to two features; the degree of segregation, i.e. the grouping of cells according to type, and the positioning of cells relative to the inside and outside of the aggregate.

Introductory Digression -

Aggregates are three dimensional entities. Serial sections represent the aggregates in two dimensional slices taken at random, since the aggregates are orientated and cut at random. Measures of positioning and segregation are carried out therefore in two dimensions. The relation of these measurements to the three dimensional aspect of aggregates is discussed later.

The positioning of cells relative to the inside and outside of aggregates has only been examined briefly. The degree of segregation of cells according to type has been investigated more thoroughly and these methods and results will be presented first.

(a) TESTS FOR SEGREGATION OF CELLS ACCORDING TO TYPE.

TEST I.   Elton's Method (Elton to be published).

Consider an aggregate of two cell types A and B, target cells are selected at random. The nearest surrounding cells are scored according to type. Two points can be mentioned here for clarity.

Firstly, the scores of cells surrounding target cells of type A and B are recorded separately. Two sets of data are thus obtained so that there is no "averaging out" effect. If one cell type does occur sparsely in masses of the other cell type, the chance of picking one as a target cell is small; but, if, occasionally, such a cell is randomly chosen as a target, the score of the surrounding cells can give an indication of "misplacings" of cells.

Secondly, the number of surrounding cells scored for each target needs some discussion. The surrounding cells could be considered to be "nearest neighbours" to the target cell.   This terminology has been used by plant ecologists (Pielon 1961, Clark and Evans 1955) where the distances between plants can be measured and the nearest neighbour ascertained. "Nearest neighbour" measures of segregation cannot be applied to two dimensional sections as the distance between cells in a packed array cannot easily be measured.

The simplest approach is to assume that the cells form a close packed structure, such as that described by Blumenson (1967) for the packing of spherical beads in a column.   This type of packing contains the minimum amount of void space.   If, assuming for simplicity, that the centres of the cells all lie on the same plane, each cell is/

is surrounded by six neighbouring cells.

A further assumption for this model is that the cells of each type are of the same size. Unlabelled heart and limb bud cells were measured with an eye piece scale (previously calibrated) in sections of aggregates formed after 2 hours, with x900 (oil immersion) magnification. The mean diameter of cells as seen in section surface was 6.60μ (s = 1.23μ) for heart cells and 6.61μ (s = 1.27μ) for limb bud cells.

Therefore the six nearest cells to a target cell were scored according to whether they were "labelled" or "unlabelled".

The general theory will now be presented and a discussion of corrections incorporated into the calculations will follow.

If a two dimensional array of _equal_ proportions of cells of type A and type B is considered, it would be expected that, if the cells are randomly arranged, the proportion of cells of type B in the six surrounding cells of targets A would be 0.5; and likewise, the proportion of cells of type A in the six surrounding cells of targets B, would be 0.5. Put in general terms, the proportion of cells of type A surrounding target cells, B, will equal the proportion of A cells in the mixture, for a range of mixture proportions, _if_ the cells are arranged randomly. Similarly, the proportion of B cells surrounding A targets will equal the proportion of B cells in the mixture _if_ the cells are randomly arranged.

We can also consider the proportion of A cells surrounding targets, A, and the proportion of B cells surrounding targets, B. Again, these proportions will equal the proportions of A and B cells respectively in the/

the mixture when the cells are randomly arranged. We can
consider in a random array of cells that the type of
target cell does not "determine" the types of cells
surrounding it.

When the cells are segregated, the proportion of
cell type A around targets of type B will be lower in value
compared with that expected in a random array, i.e. the
proportion of A in the mixture; likewise the proportion of
cell type B around targets A will be similarly lower in
value. These decreases in the proportions of surrounding
cells compared with those expected in a random array, can
give a measure of segregation.

Let the "unlabelled" cell suspension be of type A
" " "labelled" " " " " " B
Considering the theoretical situation of aggregates of
type A and type B cells,

let the proportion of A cells $= \theta$

then " " " B " $= (1 - \theta)$

If the cells are randomly arranged

the proportion of A cells around B targets $= \theta$

and " " " B " " A " $= (1 - \theta)$

If the cells are segregated, the proportion of A cells
around targets B will be lowered and the proportion of B
cells around targets A will be similarly lowered. We
can designate this lowering as α, which will equal 1,
when the cells are randomly arranged.

We can therefore write, introducing α,

the proportion of A cells around B targets $= \alpha\theta$

and " " " B " " A " $= \alpha(1 - \theta)$

Since the proportions of the two cell types
around a <u>given</u> type of target cell must summate to give 1,
we/

we can also write

the proportion of A cells around A targets $= 1 - \left[\alpha(1 - \theta)\right]$

and " " " B cells " B " $= 1 - \alpha\theta$

From aggregates we have the following observations (see later)

x = proportion of unlabelled targets

y = " " " cells round labelled targets

z = " " labelled " " unlabelled "

We cannot use the values of x and y directly to calculate α, since unlabelled and labelled cells are not in fact simply of type A and type B cells respectively. This is because a proportion of B cells are not in fact labelled (see percentage labelling).

The proportion of B cells, which are not labelled was determined as

p = 0.0673 when B = limb bud cells

p = 0.0892 " " = heart cells

and p is assumed to be a constant.

Elton has expressed the observational estimates of x, y and z in terms of θ and p to obtain a value of which refers to cell types A and B.

Let us consider labelled targets; we know that these are all of type B.

We have y = proportion of unlabelled cells surrounding labelled targets.

We are therefore observing here the proportion of A cells around B targets $(\alpha\theta)$ plus the proportion of unlabelled cells of type B (p), of the proportion of B cells surrounding B targets $(1 - \alpha\theta)$

expected value of y, $\eta = \alpha\theta + p(1 - \alpha\theta)$ (1)

Let us now consider the more complicated situation of observed proportion of labelled cells surrounding unlabelled targets (z).

The/

The unlabelled target cells consist of the proportion of target cells of type A and the proportion of target cells, which are in fact B, but unlabelled. So the observed proportion of labelled cells surrounding unlabelled targets (z) represents the contribution of the proportion of labelled cells around unlabelled targets of type A, and the contribution of the proportion of labelled cells around unlabelled targets, which are in fact type B.

These contributions from observations of surrounding cells of unlabelled targets A and of surrounding cells of unlabelled targets, which are in fact B, depend on the proportion of unlabelled targets that are A and the proportion of unlabelled targets that are B.

We can express the proportion of unlabelled targets of type A, and the proportion of unlabelled targets of type B in terms of p and θ .

Proportion of A targets $= \theta$

Proportion of B targets $= p(1-\theta)$

which are unlabelled

Total proportion of unlabelled targets $= \theta + p(1-\theta)$

The proportion of target cells of type A observed among the total of unlabelled targets

$$= \frac{\theta}{\theta + p(1-\theta)}$$

The proportion of target cells of type B observed among the total of unlabelled targets

$$= \frac{p(1-\theta)}{\theta + p(1-\theta)}$$

Now we can consider the proportion of labelled cells around unlabelled targets, which are contributed to z from these proportions of unlabelled target cells which are of type A and B.

A proportion of z comes from the observations of surrounding/

surrounding cells of a proportion of unlabelled targets which are of type A i.e.

$$\frac{\theta}{\theta + p(1 - \theta)}$$

The proportion of labelled cells round targets A

$$= (1 - p) \alpha (1 - \theta)$$

is the proportion of B cells that are labelled $(1 - p)$ of the proportion of B cells round A targets $(\alpha(1 - \theta))$.

So the contribution to z of observations of labelled cells round A targets

$$= \frac{\theta(1 - p)\alpha(1 - \theta)}{\theta + p(1 - \theta)}$$

A proportion of z comes from the observations of surrounding cells of a proportion of unlabelled target cells which are of type B i.e.

$$\frac{p(1 - \theta)}{\theta + p(1 - \theta)}$$

The proportion of labelled cells around target cells, B which are actually unlabelled

$$= (1 - p)(1 - \alpha\theta)$$

is the proportion of B cells that are labelled $(1 - p)$ of the proportion of B cells around B targets $(1 - \alpha\theta)$.

So the contribution to z of observations of labelled cells around B targets which are unlabelled

$$= \frac{p(1 - \theta)(1 - p)(1 - \alpha\theta)}{\theta + p(1 - \theta)}$$

So the expression for the expected value of z, ζ, in terms of α, θ and p is

$$\zeta = \frac{\theta(1 - p)\alpha(1 - \theta)}{\theta + p(1 - \theta)} + \frac{(1 - p)(1 - \alpha\theta)p(1 - \theta)}{\theta + p(1 - \theta)} \qquad (2)$$

The/

The solution of equations (1) and (2) for α and θ is

$$\theta = \frac{(1 - p)\eta - p\varsigma}{(\eta + \varsigma)(1 - p)} \qquad (3)$$

and

$$\alpha = \frac{\eta - p}{\theta(1 - p)} \qquad (4)$$

The observed values of y and z can be substituted for η and ς respectively in equations (3) and (4) and estimates of α and θ can be calculated (see later).

Elton has considered the expected value, ξ, of x (the proportion of unlabelled cells) from target cell counts.

$$\xi = \theta + p(1 - \theta) \qquad (5)$$

The proportion of unlabelled cells will be the proportion of A cells (θ) plus the proportion (p) which are unlabelled of the proportion of B cells ($1 - \theta$).

When the estimate of θ from the data is substituted into (5) the value of ξ was in the majority of cases higher than the observed value of x (see later for the distribution of surrounding cells). The most probable explanation of this discrepancy was that the unlabelled cells were being underestimated in target cell counts. It is possible to explain this underestimation on considerations of relative "counting efficiency" of labelled and unlabelled cells. An unlabelled cell is only recognizable if part of the nucleus is at the surface of the section. A labelled nucleus, on the other hand, may produce an autoradiographic image even if it is not on the surface of the section (see previously: discussion of β particle penetrance).

A third unknown parameter was introduced, let q be the proportion of unlabelled target cells observed.

From/

From observations x = proportion of unlabelled targets

$$\xi = \frac{\text{proportion of unlabelled targets observed}}{\text{total proportion of targets observed}}$$

The proportion of unlabelled targets observed

$$= q(\theta + p(1 - \theta))$$

is the proportion observed (q) of the proportion of unlabelled targets ($\theta + p(1 - \theta)$).

The total proportion of targets observed

$$= q(\theta + p(1 - \theta)) + (1 - p)(1 - \theta)$$

is the proportion of unlabelled targets observed plus the proportion of labelled targets observed.

$$\therefore \xi = \frac{q(\theta + p(1 - \theta))}{q(\theta + p(1 - \theta)) + (1 - p)(1 - \theta)} \tag{6}$$

which reduces using equation (3) to

$$= 1/(1 + \zeta/\eta)$$

or

$$q = \frac{\xi \zeta}{\eta(1 - \xi)} \tag{7}$$

replacing ξ, ζ and η by x, z and y respectively, we have

$$q = \frac{x z}{y(1 - x)}$$

Elton has investigated the possible effect of a similar underestimation of unlabelled cells in counts of surrounding cells. Since the target cells and surrounding cells are counted by different methods (see later) the underestimation of unlabelled cells in surrounding cell counts was not considered to be the same parameter as q. A fourth parameter was introduced, let r be the proportion of unlabelled surrounding cells observed. This parameter can be assumed to be the same for both labelled and unlabelled target cells.

We/

We can calculate from x, y, z and p using equations (3), (4) and (8) values of α, θ and q assuming r = 1. How do values of r different from 1 affect α, θ and q?

If $r \neq 1$, the observed values of y and z differ from the true values y^1 and z^1 of the proportion of unlabelled cells round labelled targets, and the proportion of labelled cells round unlabelled targets respectively.

$$y = \frac{ry^1}{ry^1 + 1 - y} \qquad \text{if } r = 1 \quad y = y^1$$

$$z = \frac{z^1}{z^1 + r(1 - z^1)} \qquad \text{if } r = 1 \quad z = z^1$$

To summarize, Elton expresses α and θ as functions of y, z, p and r; and q as a function of r. When he plotted these functions for real data he found that the value of α is not affected very much by small changes in r, in the region r = 1. In practice, he concluded that α may be decreased slightly by this effect if the true value of $r < 1$, but this is not probably important.

If we use the observed values x, y and z instead of ξ, η and ς we can estimate q, θ and α.

$$q = \frac{x \, z}{y(1 - x)}$$

$$\theta = \frac{(1 - p)y - pz}{(1 - p)(y + z)}$$

$$\alpha = \frac{y - p}{(1 - p)}$$

A number of samples provide replicate estimates of each parameter under given conditions, which are used in significance tests.

Mechanics/

Mechanics of Counting

(1) Sampling

5/μ serial sections of embedded aggregates were available for counting. The procedure of Roth and Weston (1967) was adopted. They examined every third section of the ribbon to avoid counting the same labelled cell twice.

If we consider a "sorted out" aggregate, it can be seen that the proportion of cell types will vary along the sections.
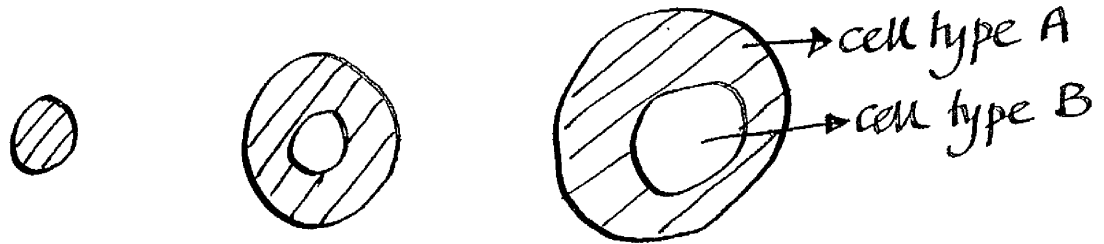
In fact Adler (1970) has used this variation of proportion of cell types with aggregate size as a measure of "sorting out" (see also discussion on positioning). The degree of segregation will also vary along the sections.

We could consider the segregation of cell types in arbitrarily chosen regions of the aggregate either by selecting to count certain sections or areas of aggregates within sections. This method was not used because such a choice of regions would be rather subjective. The other alternative, which was the method used, is to examine the whole aggregate in serial sections and to obtain a value of segregation in these two dimensional sections at "all levels" within the aggregate. With this method, a measure of the proportions of cells in the random areas of the aggregate analysed for segregation (see later) is obtained/

obtained since this measure is calculated from target cell counts. In this approach, although the whole aggregate is sampled in two dimensional slices, a measure of segregation of "the whole" can be obtained.

This method is only applicable to aggregates formed after 42 hours' reaggregation, when embedded sparsely because the aggregates formed after shorter time periods are too small. A single aggregate can then be recognised in each third section throughout its "length". Counts can then be made at all levels of sectioning. The material analysed in this way is referred to as Aggregate, where a whole aggregate has been examined.

The counting of whole aggregates would not yield sufficient data if small early aggregates were examined in the same way. In sections of these aggregates every third section can be distinguished on the slide, although individual aggregates cannot easily be traced. In these cases, the material can still be analysed for the degree of segregation in terms of "whole" aggregates. A random sample of a number of aggregates in every third section is taken. This data is referred to as Sample. In this way, all "levels" of sections are considered together and thus the degree of segregation refers to that of total aggregate population rather than certain areas of aggregates. Some aggregates formed after two days were also counted in samples. The aggregate method of collecting data provides useful information on the homogeneity of α between aggregates formed in the same experiment (see later). Homogeneity would add justification to the use of the sample method.

(2)/

(2) Scoring

To obtain the counts used in the equations to
obtain α the following procedure was adopted. Aggregates
were selected for counting by either of the two methods,
outlined above, i.e. as <u>aggregates</u> or <u>samples</u> at x100
magnification.

Then the magnification was changed to x900 (oil
immersion) and the field obtained was used for counting.
This field was assumed to be a random selection of part of
an aggregate or group of aggregates. Target cells were
selected by means of a Chalkley grid eye-piece with 25
dots randomly arranged (Curtis 1960). The type of each
target was noted and the six nearest cells were scored
according to whether they were labelled or unlabelled.
When the labelled cells were the smaller proportion (this
was the case in most experiments) the counting of
surrounding cells was particularly easy. In these cases,
the number of labelled cells surrounding the target were
counted and the remainder of the surrounding cells were
assumed to be unlabelled.

Counts of surrounding cells were therefore obtained
in two columns, one referring to labelled target cells and
the other to unlabelled target cells. The percentage of
the labelled and unlabelled cells in aggregates or samples
of aggregates can be calculated from target cell counts,
and is included in the results tables, L being the pro-
portion of labelled targets and U being the proportion of
unlabelled targets (x in equations). The frequency of
the numbers of surrounding cells of the same type as the
target cells can be drawn up. The distribution of the
numbers/

numbers of cells surrounding a target of given type will be briefly examined later.

The proportion of unlabelled cells round labelled targets and the proportion of labelled cells round unlabelled targets can be calculated. Equations (3),(4) and (8) can be used to calculate q, θ, and α using observed values of y and z.

$$q = \frac{x \, z}{y(1 - x)}$$

p = proportion of unlabelled cells in "labelled" cell population.

$$\theta = \frac{(1 - p)y - pz}{(1 - p)(y + z)}$$

q = proportion of unlabelled targets observed.

$$\alpha = \frac{y - p}{\theta(1 - p)}$$

θ = proportion of unlabelled cells of the unlabelled cell type.

α = degree of segregation.

SEGREGATION TESTS

TEST I: Results

Controls LB*LB   ("labelled" limb bud cells mixed with
"unlabelled" limb bud cells)

LB*LB   (1), disaggregated with TRYPSIN, cells mixed
together without passage through cell
sieves, aggregated for 42 hours.

Aggregate number	total	TARGET CELLS							
		L	U	y	z	q	θ	α	1.
I	205	0.30	0.70	0.57	0.30	1.21	0.63	0.85	
II	338	0.34	0.66	0.67	0.23	0.67	0.73	0.89	
III	311	0.31	0.69	0.68	0.23	0.76	0.73	0.91	
IV	306	0.32	0.68	0.69	0.20	0.91	0.76	0.89	

mean α = 0.88

s^2 =   0.0006.

1. Note: for clarity figures are rounded off to 2 decimal
places, although four decimal places were used
for calculations.
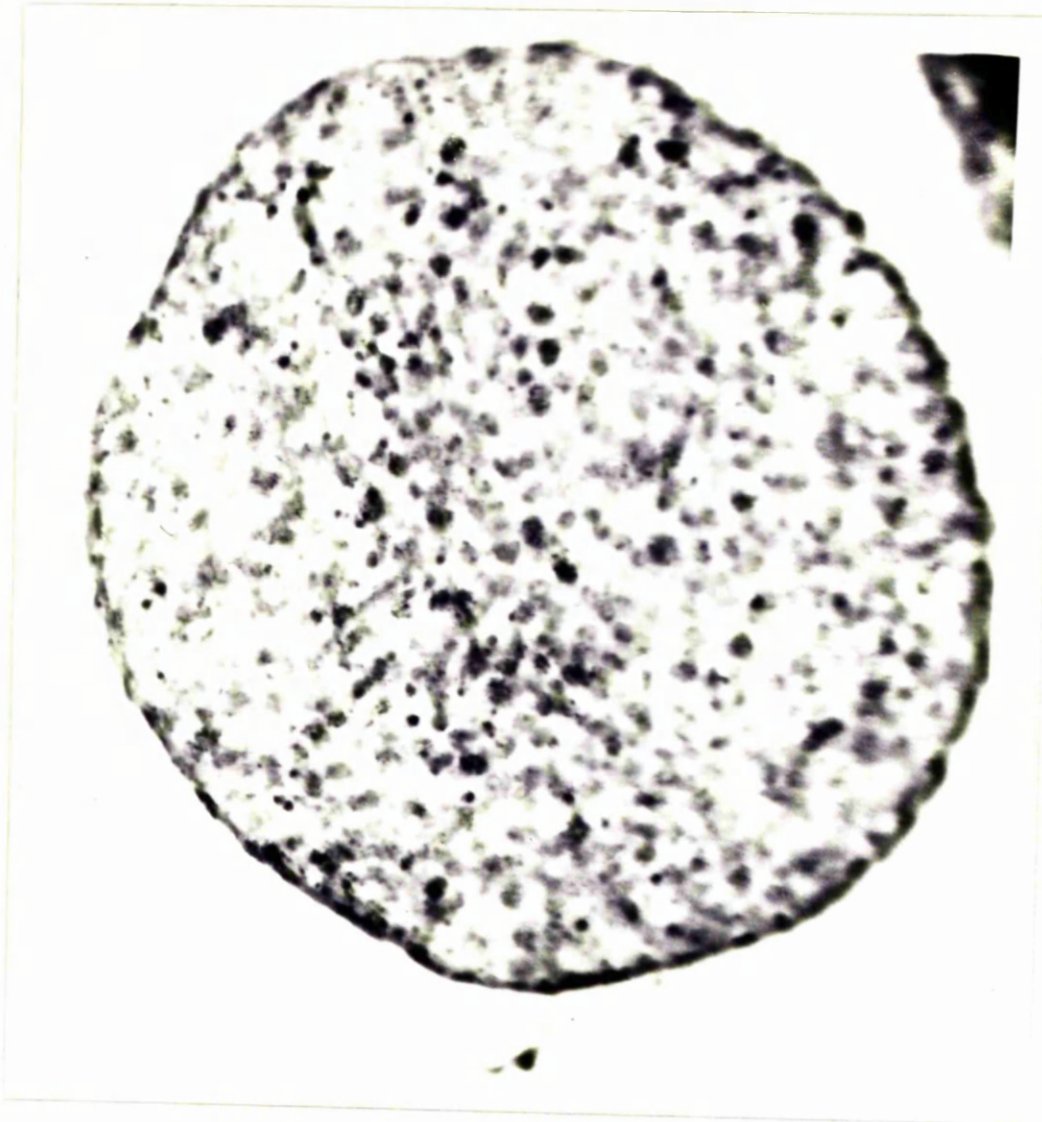
FIGURE Ia

CONTROL:LB*LB,disaggregated with TRYPSIN,cells mixed together
without passage through cell sieves,aggregated for 42 hours.

The arrangement of labelled and unlabelled cells approaches
randomness.

Light field illumination.
Magnification:X440.
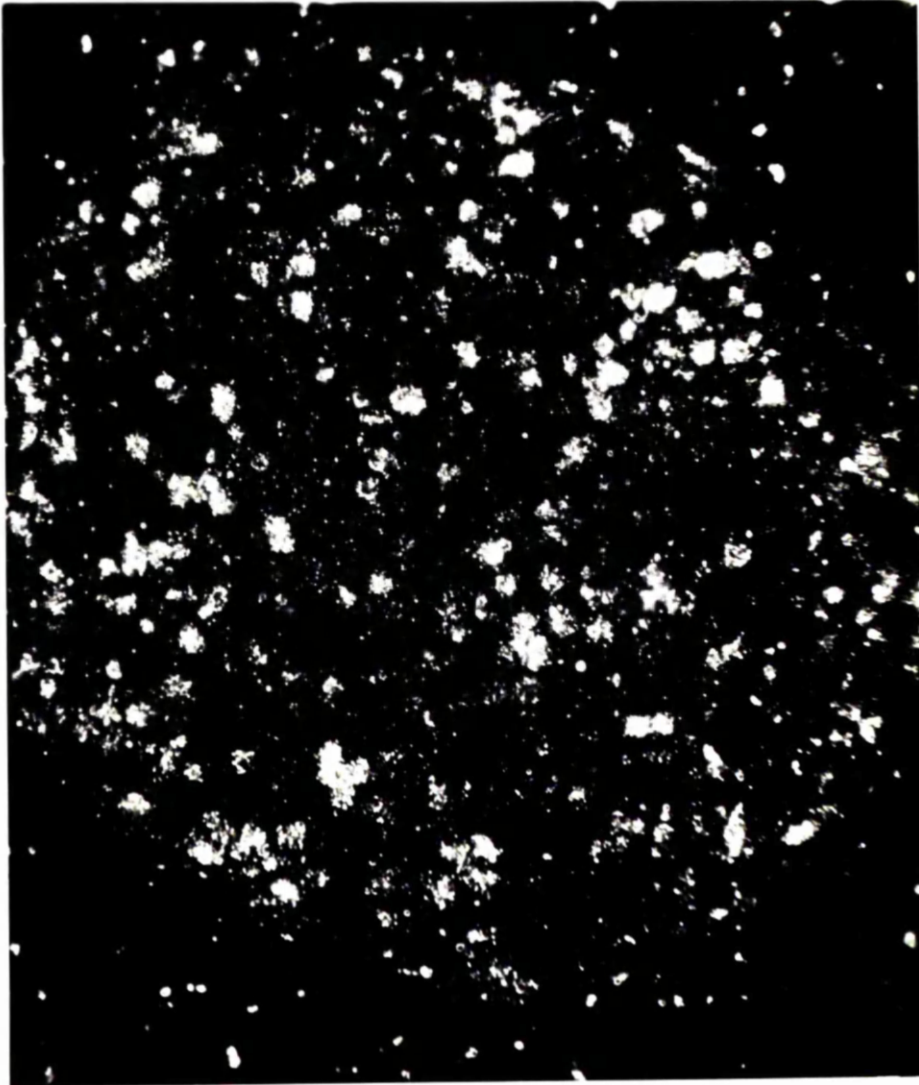
FIGURE Ib

CONTROL:LB* LB,disaggregated with TRYPSIN,cells mixed together without passage through cell sieves,aggregated for 42 hours.

Similar aggregate to that in figure Ia.

Dark field illumination.
Magnification:X440.

LB*LB (2), disaggregated with EDTA, cells "sieved",
aggregated for 24 hours.

Sample number	total	TARGET CELLS $\underline{L}$	$\underline{U}$	$\underline{Y}$	$\underline{z}$	$\underline{q}$	$\underline{\theta}$	$\underline{\alpha}$
I	101	0.62	0.38	0.41	0.48	0.70	0.43	0.87
II	162	0.57	0.43	0.45	0.54	0.92	0.41	0.99
III	222	0.54	0.46	0.44	0.49	0.97	0.44	0.92
IV	236	0.58	0.42	0.44	0.52	0.86	0.42	0.95

$$\text{mean } \alpha = 0.93$$
$$s^2 = 0.0025$$

LB*LB (3), disaggregated with EDTA, cells sieved and aggregated for 42 hours.

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	437	0.31	0.69	0.67	0.22	0.73	0.73	0.88
II	226	0.28	0.72	0.72	0.21	0.77	0.76	0.92
III	181	0.24	0.76	0.73	0.23	1.01	0.74	0.95
IV	366	0.30	0.70	0.70	0.24	0.81	0.73	0.93

$$\text{mean } \alpha = 0.92$$
$$s^2 = 0.0009$$

In a random mixture of cells $\alpha = 1$

LB*LB compared with $\alpha = 1$ (One tailed 't' test)

LB*LB (1) t = -9.618   df 3   p: $0.0025 > p > 0.001$ positive segregation at 1% level

LB*LB (2) t = -2.678   df 3   p: $0.05 > p > 0.025$ positive segregation at 5% level

LB*LB (3) t = -4.505   df 3   p: $0.025 > p > 0.01$ positive segregation at 5% level.

Comparison of controls with each other.

(i)   We could consider "time course"

Aggregates (EDTA, with cell sieves)

LB*LB (2) (reaggregated for 24 hour) compared with
LB*LB (3) (reaggregated for 42 hour)

$$\delta = 0.041 \quad t = 0.3686 \quad df\ 6 \quad p: 0.8 > p > 0.5$$

(ii) The effects of cell "sieving"

LB*LB (1) (trypsin, without cell sieves, reaggregated for
42 hour)

      (a) compared with LB*LB (3) (EDTA, "cell sieved",

                            reaggregated for 42 hour)

$$\delta = 0.027 \quad t = -2.003 \quad df\ 6 \quad p: 0.1 > p > 0.05$$

      (b) compared with LB*LB (2) (EDTA, "cell sieved",

                            reaggregated for 24 hour)

$$\delta = 0.0389 \quad t = -1.779 \quad df\ 6 \quad p: 0.2 > p > 0.1$$

Comments on Controls

The degree of segregation of labelled and unlabelled cells in aggregates LB*LB (1), in which the initial cell suspensions were not sieved is significantly greater than that expected if there were a random arrangement (N.B. the greater the degree of segregation the lower the α value). The numerical value of α for LB*LB (1) is 0.8842, which therefore shows that the cells in these aggregates are segregated significantly but to a small extent according to whether they are "labelled" or "unlabelled". This could be explained by the presence of cell clumps in the original cell suspension.

The finding that this "clumping" effect persists into aggregates formed after 42 hours can be predicted on the hypothesis of Steinberg (1964) that "sorting out" occurs by exchange of weak for stronger adhesions. In these aggregates all the cells are of the same "tissue type" and differ only in some being "labelled". If "labelling" has no effect on cell behaviour one would expect "labelled" cell adhesions to be the same strength as "unlabelled" cell adhesions of the same "tissue type", and thus no exchanges of adhesions would be predicted. Incidentally it might be predicted that small clumps of like cells in initial cell suspensions for mixed reaggregations of two cell types would enlarge or disperse during two days' culture in reaggregates. So in long term aggregates (of a few days) of different cell types the presence of cell clumps in the initial cell suspensions should not affect the final arrangement of cells. I have assumed in this argument that the degree of segregation in LB*LB (1) is due only to clumps in the unsieved initial.suspensions. That/

That this may only be partially true is suggested by the fact that the degrees of segregation of cells in LB*LB (2) and (3) aggregates are not statistically significantly different from that in LB*LB (1), even though these aggregates were prepared from "sieved" cell suspensions.

In control aggregates, prepared with cell sieved suspensions, the cells show a small but probably significantly different arrangement from random. This segregation could be due to cell divisions occurring during the culture period, leading to groups of like cells being produced; or to the "labelling" in some way affecting the behaviour of the cells. Mitotic figures have been observed in aggregates formed after 42 hours reaggregation of mixed heart and limb bud cells, so it seems likely that cell division could adequately account for this small departure from a random arrangement of cells in LB*LB (2) and LB*LB (3). It would be expected, if this were the case, that this segregation effect would be more marked in aggregates formed after 42 hours in which more cell divisions have taken place than in aggregates reaggregated for 24 hours. The degree of segregation, although numerically higher (and therefore nearer random) in aggregates formed after 24 hours is not significantly different to that in aggregates formed after 42 hours. Even so, it can reasonably be assumed that "labelling" of a cell suspension does not alter the behaviour of cells in aggregates more than slightly if at all.

This small degree of segregation, probably due to cell division, will also occur in mixed aggregates of different cell types, in addition to any segregation of cells according to type. It is the latter segregation we are/

are interested in. When testing whether the mixed aggregates show any significant segregation according to cell type, the α values of the control LB*LB (3) aggregates are used as the expected values for a random arrangement of cells, thus including the correction for any psuedo-segregation due to cell division. In this way, we can test whether there is any significant segregation due only to the mixing of two different cell types.

Ideally we should have controls like LB*LB (3) for aggregates formed in shorter times. It had been hoped that LB*LB (2) aggregates (formed after 24 hours) would show that the degree of segregation obtained in control aggregates was due to cell division. Testing α values from mixed aggregates formed after 2 hours and 4 hours against the α values of LB*LB (3) is probably over-estimating in these cases the degree of segregation due to cell division.

EDTA DISAGGREGATION

(a) Aggregates formed after 2 hours reaggregation.

RECIPROCAL EXPERIMENTS

(1) LB*H ("labelled" limb bud cells mixed with "unlabelled" heart cells)

Sample Number	TARGET CELLS total	L	U	y	z	q	θ	α
I	392	0.70	0.29	0.26	0.51	0.85	0.29	0.71
II	506	0.65	0.35	0.28	0.40	0.80	0.37	0.62
III	446	0.69	0.31	0.26	0.41	0.70	0.34	0.61
IV	370	0.67	0.33	0.25	0.43	0.86	0.32	0.60
Total sample[1]	1,714	0.68	0.33	0.26	0.43	0.80	0.33	0.63

mean α = 0.64

s^2 = 0.0027

[1] "Total Sample" means that all the data was treated as one sample as a check, but is not included in mean α.
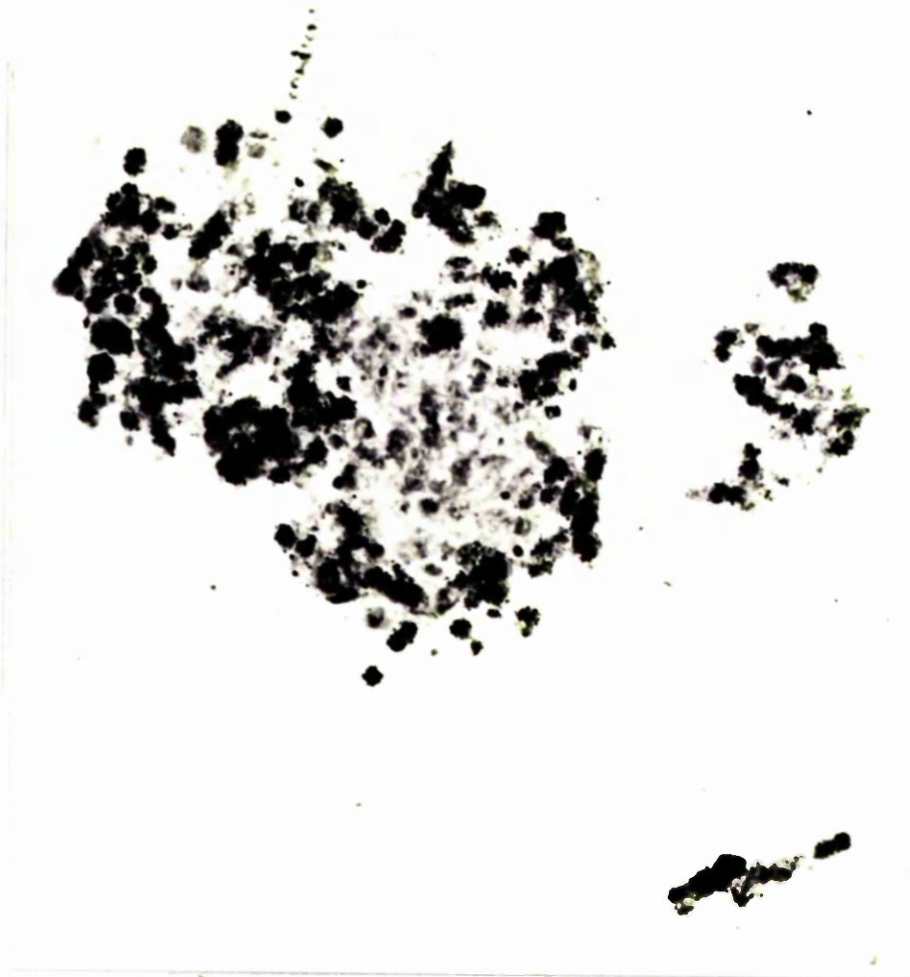
FIGURE IIa

LB H:cells disaggregated with EDTA,reaggregated for 2 hours.

This shows segregation of cell types.
Note also labelled cells,limb bud,appear to be positioned externally.

Light field illumination.
Magnification:X440.

FIGURE IIb

LB$^+$H:cells disaggregated with EDTA,reaggregated for 2 hours.

This is the same aggregate as in figure IIa under dark field illumination to provide a reference for dark field photographs.

Magnification:X440.

(EDTA disaggregation continued)

(a) Aggregates formed after 2 hours reaggregation.

    (ii) H*LB ("labelled" heart cells + "unlabelled" limb bud cells)

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	215	0.35	0.65	0.47	0.14	0.57	0.74	0.56
II	233	0.22	0.78	0.54	0.14	0.92	0.78	0.64
Total sample	448	0.28	0.72	0.50	0.14	0.72	0.76	0.59

$$\text{mean } \alpha = 0.60$$
$$s^2 = 0.0033$$

LB*H compared with H*LB

$$\sigma = 0.053 \qquad t = 0.741 \qquad df \quad 4 \qquad p \doteq 0.5$$

Note: df = number of degrees of freedom and is the total number of samples in both groups - 2.
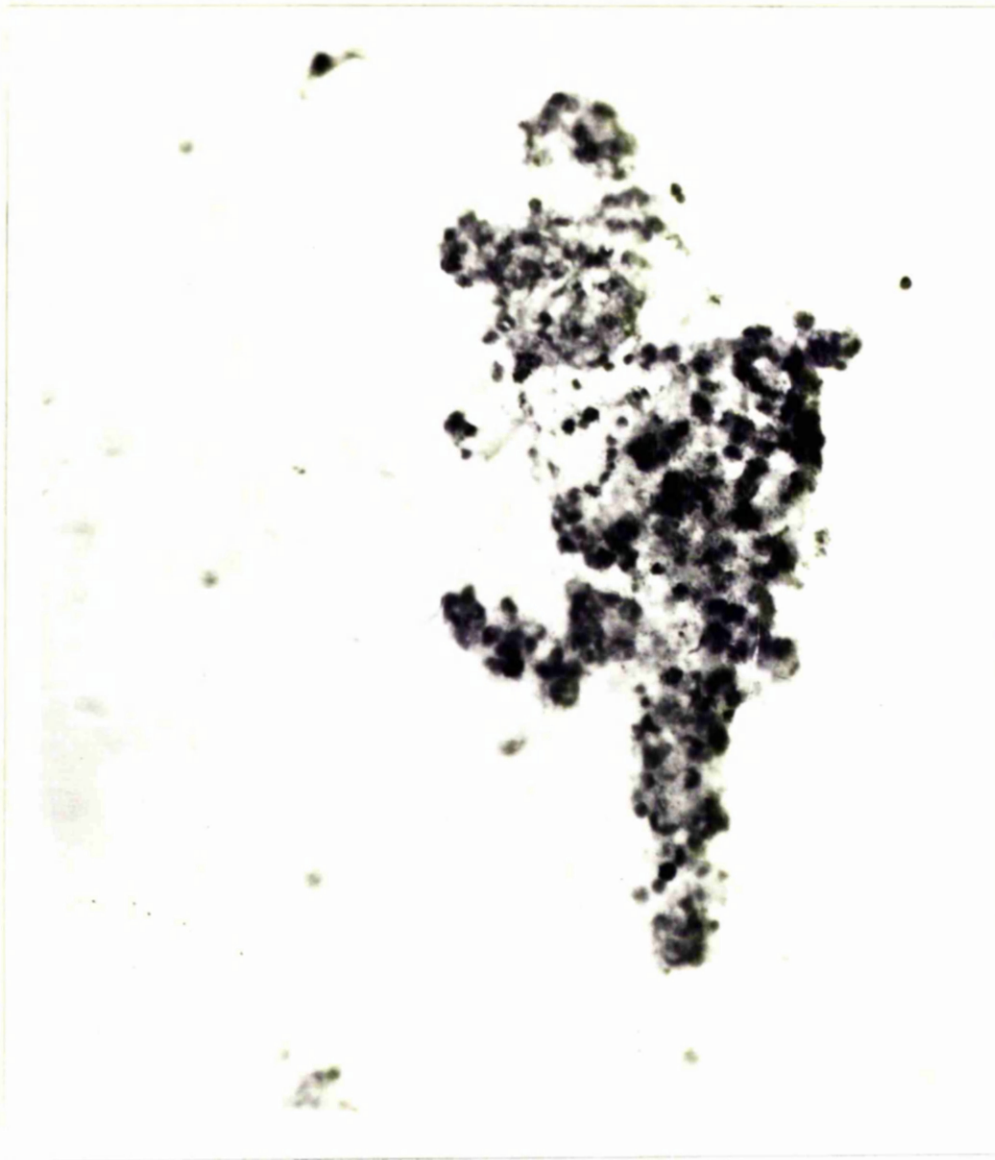
FIGURE III

H[*]LB:cells disaggregated with EDTA,reaggregated for 2 hours.
A central mass of labelled cells,heart,can be seen with tapering
aggregations of limb bud cells. Aggregates of this type show a marked
degree of segregation. The autoradiograph is of rather poor quality.

Light field illumination.
Magnification:X480

(EDTA disaggregation continued)

(b) Aggregates formed after 4 hours reaggregation.

RECIPROCAL EXPERIMENTS

(i) LB*H

Sample Number	TARGET CELLS total	L	U	y	z	q	θ	α
I	380	0.74	0.26	0.25	0.47	0.66	0.30	0.64
II	312	0.69	0.31	0.32	0.43	0.61	0.38	0.70
III	458	0.79	0.21	0.23	0.47	0.53	0.28	0.63
IV	414	0.78	0.22	0.21	0.47	0.62	0.26	0.59
Total sample	1,564	0.76	0.24	0.26	0.46	0.57	0.32	0.66

mean α = 0.64

s^2 = 0.0021

(ii) H*LB

Sample Number	TARGET CELLS total	L	U	y	z	q	θ	α
I	651	0.07	0.93	0.65	0.02	0.35	0.97	0.63

LB*H compared with H*LB

$t = 0.5465$ df 3 p: $0.8 > p > 0.5$ (2 tailed)

(EDTA disaggregation continued)

(c)   Aggregates formed after 42 hours reaggregation in
       reciprocating shakers.

RECIPROCAL EXPERIMENTS

(i) LB*H

Aggregate number	TARGET CELLS total	L	U	y	z	q	θ	α
I	196	0.67	0.33	0.40	0.54	0.67	0.39	0.93
II	392	0.70	0.30	0.35	0.53	0.66	0.35	0.84
III	267	0.69	0.33	0.40	0.55	0.66	0.38	0.94
IV	226	0.49	0.51	0.56	0.36	0.66	0.58	0.91

$$\text{mean } \alpha = 0.91$$
$$s^2 = 0.002$$

(ii) H*LB

Aggregate number	TARGET CELLS total	L	U	y	z	q	θ	α
I	214	0.11	0.89	0.85	0.03	0.26	0.97	0.86
II	404	0.13	0.87	0.78	0.06	0.47	0.93	0.82
III	360	0.13	0.87	0.78	0.08	0.67	0.90	0.85

$$\text{mean } \alpha = 0.84$$
$$s^2 = 0.0006$$

LB*H compared with H*LB

$$\delta = 0.038 \quad t = 2.280 \quad df\ 5 \quad p: \ 0.1 > p > 0.05$$

Note in (c) LB*H aggregate IV has different proportions
       of cells to aggregates I, II and III but has a
       similar   .
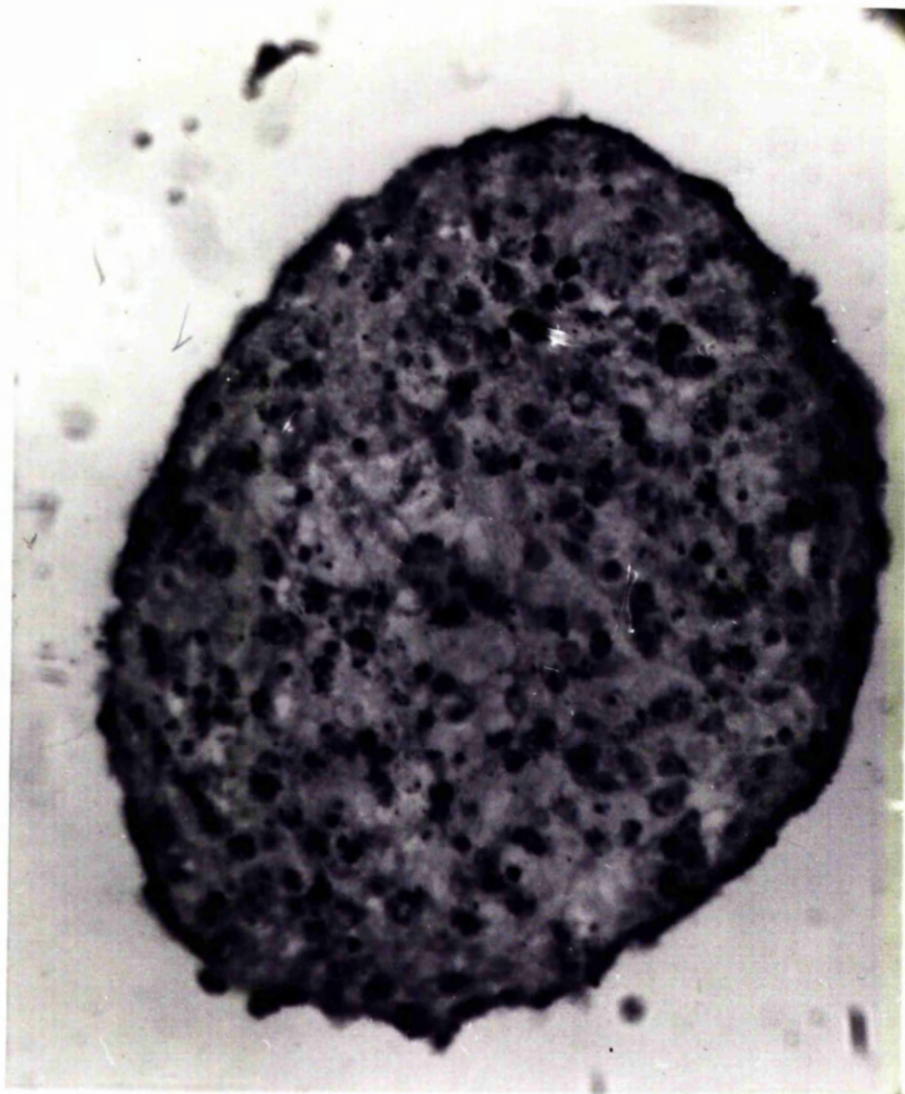
FIGURE IV

LB*H:cells disaggregated with EDTA, reaggregated for 42 hours in reciprocating shakers.

Note this depicts an almost random arrangement of labelled and unlabelled cell types at this proportion of 3:1, L:U.

Light field illumination.
Magnification:X440.

(EDTA disaggregation continued)

(d) Aggregates formed after 48 hours reaggregation in gyratory shaker.

RECIPROCAL EXPERIMENTS

(i) LB*H

Aggregate number	TARGET CELLS total	L	U	y	z	q	θ	α
I	150	0.85	0.15	0.17	0.53	0.55	0.18	0.58
II	254	0.70	0.30	0.21	0.53	1.05	0.23	0.66
III	248	0.77	0.23	0.20	0.57	0.82	0.21	0.70
Sample IV	452	0.73	0.27	0.19	0.50	0.97	0.22	0.59

mean α = 0.63

s^2 = 0.0035

(ii) H*LB

Sample Number	TARGET CELLS total	L	U	y	z	q	θ	α
I	583	0.16	0.84	0.67	0.10	0.82	0.86	0.74
II	293	0.18	0.82	0.58	0.11	0.91	0.82	0.66

mean α = 0.70

s^2 = 0.0032

LB*H compared with H*LB

$$\sigma = 0.059 \qquad t = 1.345, \qquad df \ 4 \qquad p: \ 0.5 > p > 0.2.$$
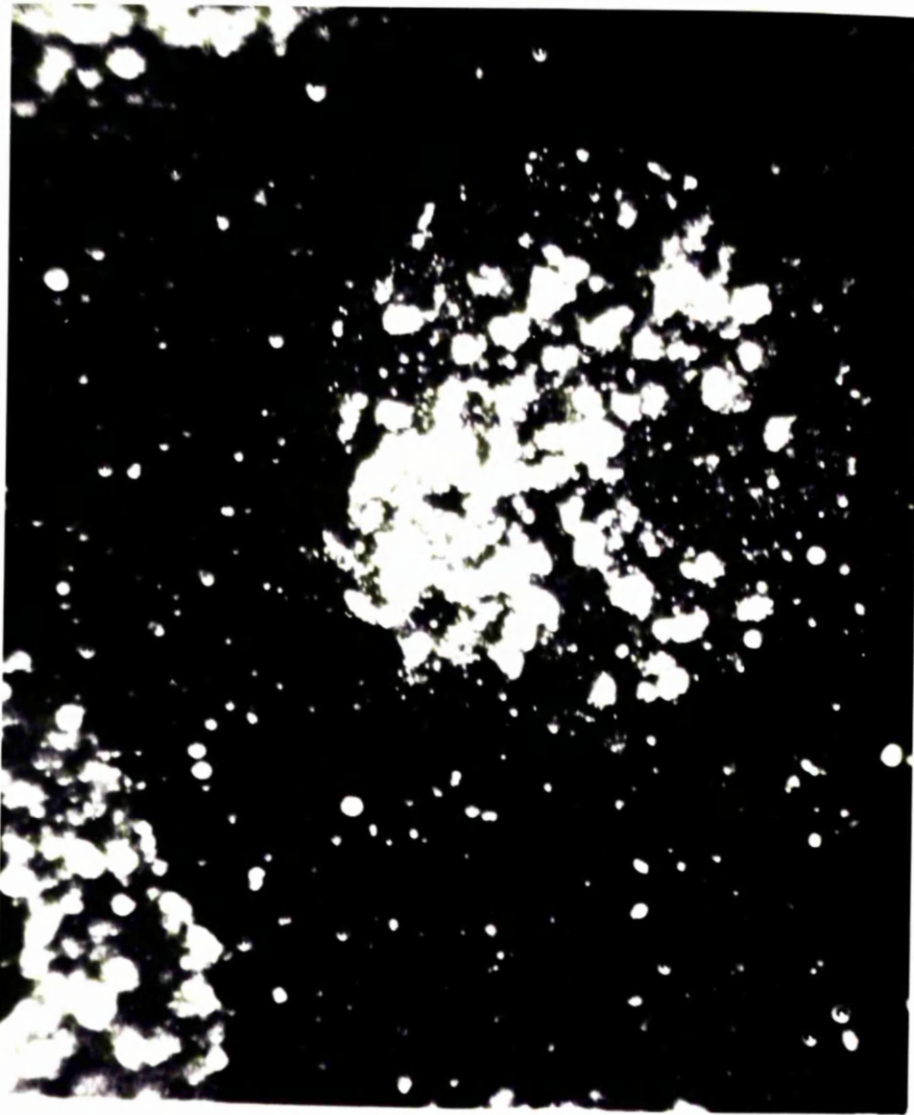
FIGURE Va

LB H:cells disaggregated with EDTA,reaggregated for 48 hours in gyratory shaker.

The cell types are markedly segregated,and the labelled cell type limb bud,is positioned internally;note therre are some limb bud cells at the edge of the aggregate. This section probably represents an "end"of an aggregate.

There is a heavy background.

Dark field illumination.

Magnification:X480.
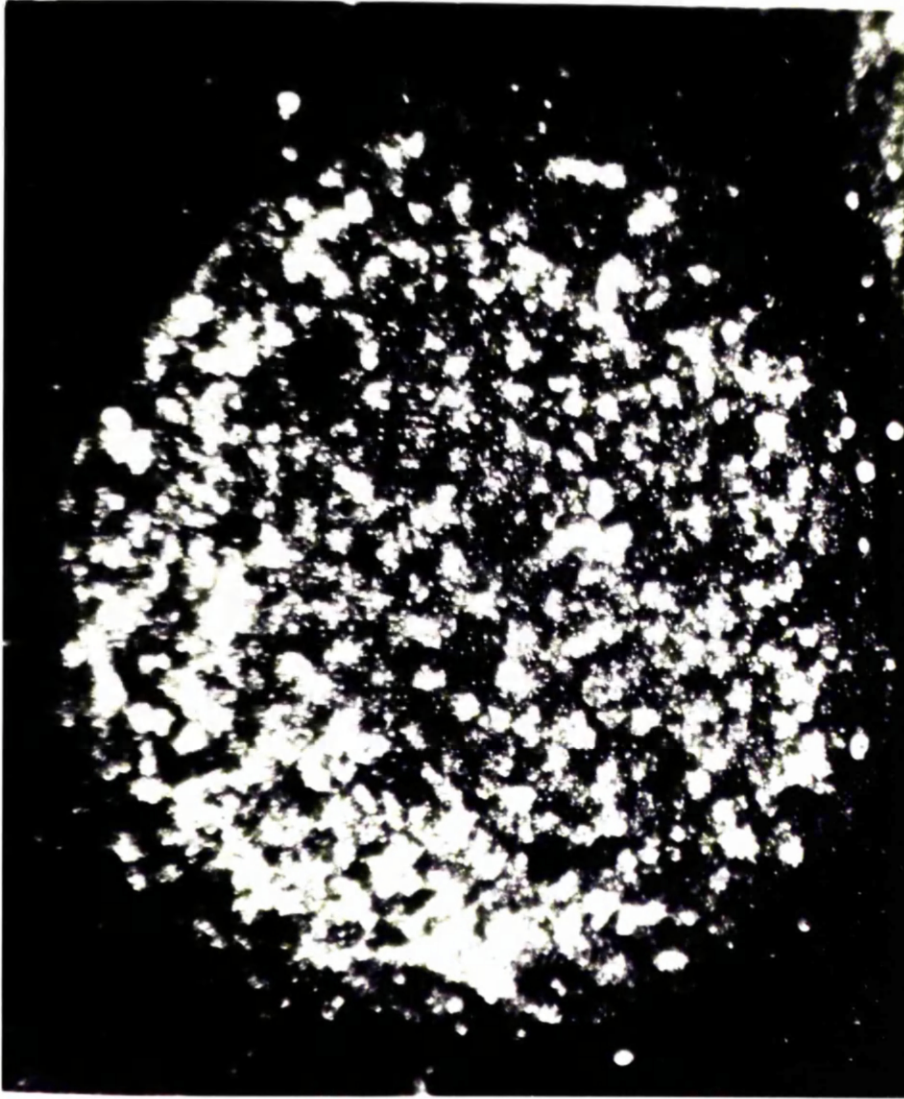
FIGURE Vb

LB H:cells disaggregated with EDTA,reaggregated for 48 hours in gyratory shaker.

More median section of an aggregate formed in the same experiment as aggregate shown in figure Va. Note segregation of cell types and thin external layer of the unlabelled cell type,heart.

Dark field illumination.

Magnification:X480.
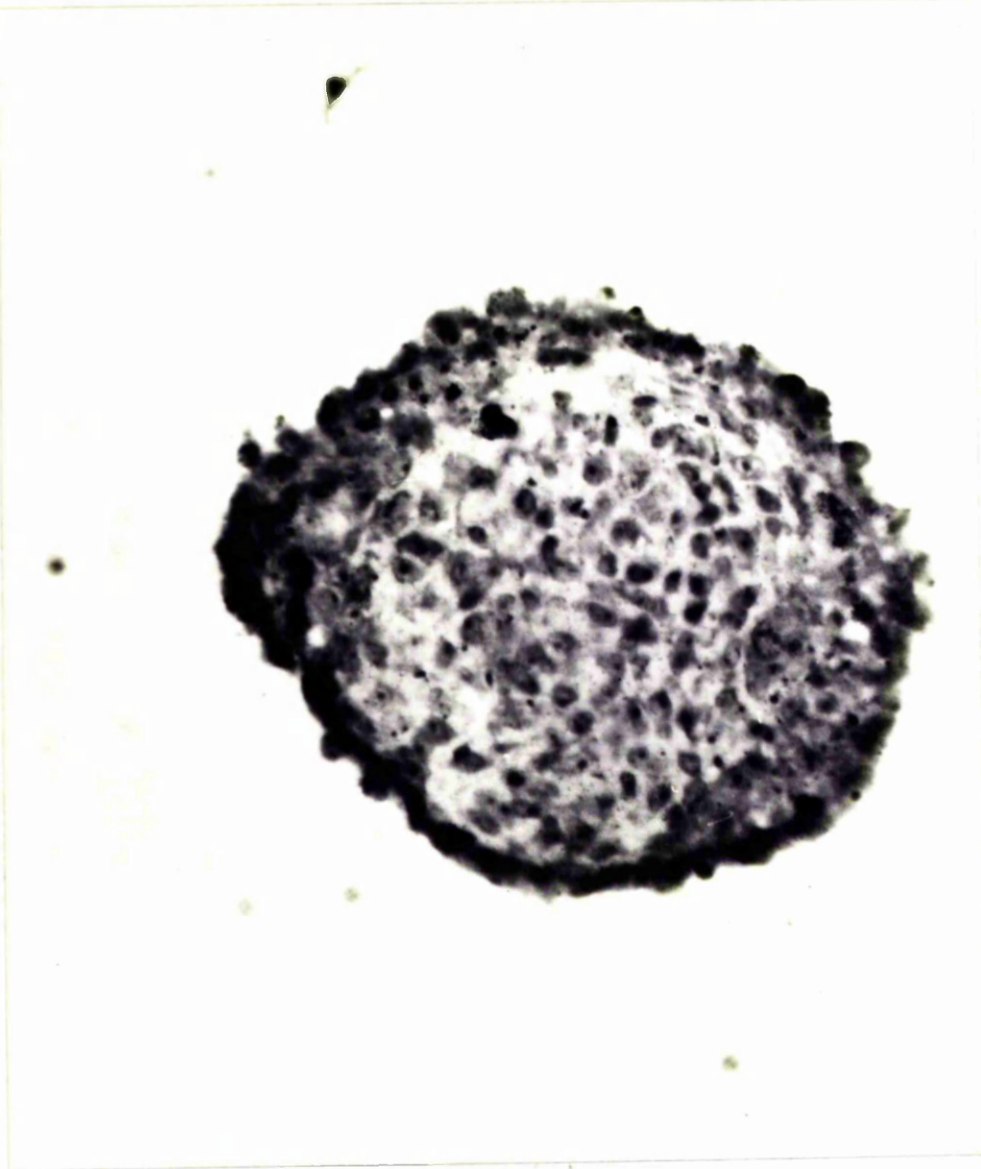
FIGURE VIa

H LB:cells disaggregated with EDTA,reaggregated for 48 hours in gyratory shaker.

A few cells of the labelled cell type,heart,can be seen at the edge of the aggregate. Internally lies a mass of the unlabelled cell type,limb bud.

Light field illumination.

Magnification:X480.
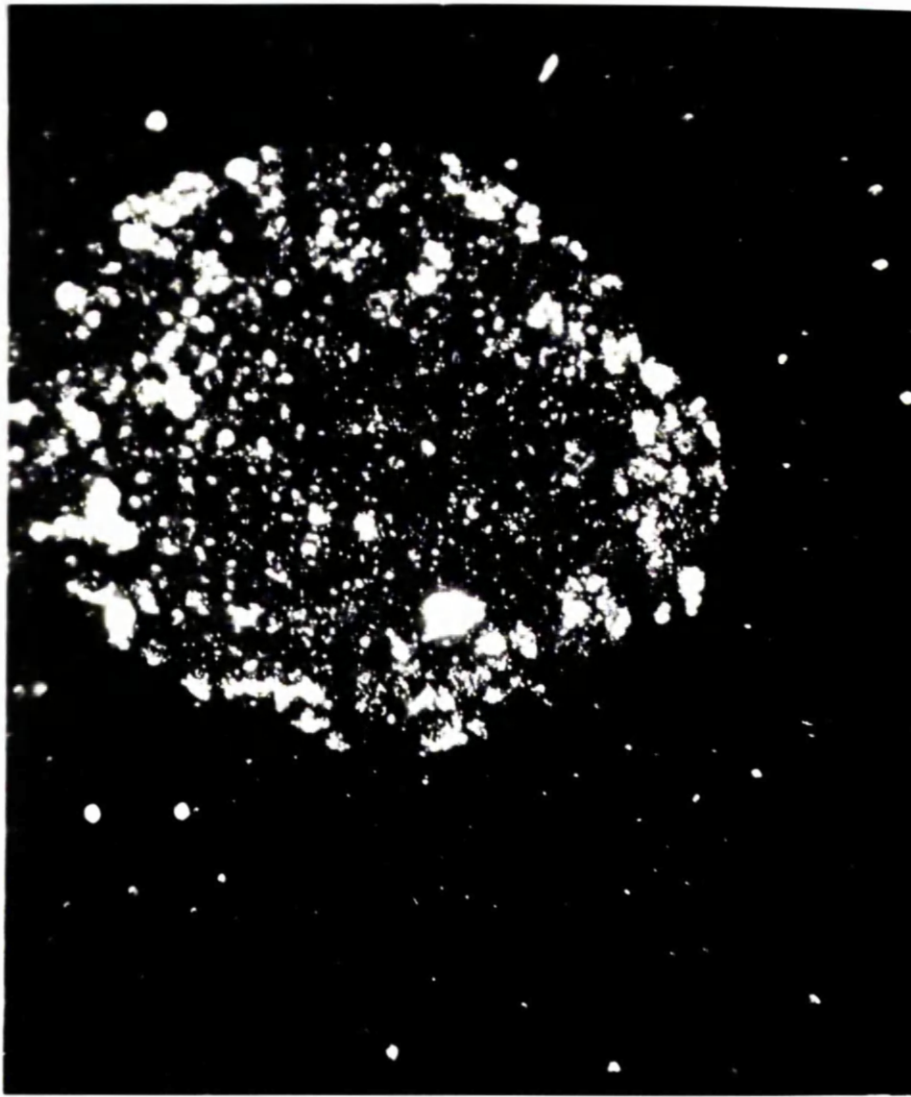
FIGURE VIb

H[*]LB:cells disaggregated with EDTA,reaggregated for 48hhours
in gyratory shaker.

Same aggregate section as in figure VIa. Note external position-
ing of heart cells.

Dark field illumination.
Magnification:X480.

EDTA and TRYPSIN DISAGGREGATION

(a) Aggregates formed after 2 hours reaggregation.

RECIPROCAL EXPERIMENTS

(i) LB*H

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	335	0.67	0.33	0.28	0.38	0.66	0.38	0.61
II	348	0.66	0.34	0.28	0.42	0.76	0.36	0.64
III	330	0.68	0.32	0.29	0.37	0.59	0.40	0.60
IV	323	0.70	0.30	0.24	0.44	0.80	0.31	0.61.
total sample	1,331	0.68	0.32	0.28	0.40	0.70	0.36	0.61

mean α = 0.61

s^2 = 0.0004

(ii) H*LB

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	378	0.14	0.86	0.65	0.08	0.79	0.87	0.71
II	353	0.15	0.85	0.69	0.05	0.40	0.93	0.71
III	290	0.21	0.79	0.59	0.08	0.51	0.87	0.64
IV	297	0.16	0.84	0.72	0.07	0.55	0.90	0.77
V	323	0.15	0.85	0.61	0.06	0.60	0.90	0.64
VI	376	0.16	0.84	0.59	0.05	0.46	0.91	0.60
Total sample	2,017	0.16	0.84	0.64	0.07	0.55	0.90	0.68

mean α = 0.68

s^2 = 0.0039

LB*H compared with H*LB

σ = 0.051   t = 1.958   df 8   p: $0.1 > p > 0.05$.

(EDTA and TRYPSIN continued)

(b) Aggregates formed after 4 hours reaggregation.

RECIPROCAL EXPERIMENTS

(i) LB*H

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	280	0.81	0.19	0.19	0.55	0.67	0.21	0.65
II	403	0.76	0.24	0.19	0.60	1.02	0.19	0.71
III	529	0.79	0.21	0.19	0.55	0.77	0.21	0.65
IV	553	0.77	0.23	0.19	0.54	0.85	0.21	0.65
total sample	1,765	0.79	0.21	0.19	0.56	0.78	0.20	0.66

mean α = 0.66

s^2 = 0.0009

(ii) H*LB

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	320	0.16	0.84	0.73	0.07	0.52	0.90	0.78
II	316	0.19	0.81	0.71	0.11	0.68	0.85	0.80
III	360	0.17	0.83	0.62	0.08	0.67	0.87	0.67
IV	676	0.19	0.81	0.65	0.07	0.49	0.89	0.69
total sample	1,672	0.18	0.82	0.67	0.08	0.57	0.88	0.73

mean α = 0.74

s^2 = 0.0040

LB*H compared with H*LB

σ = 0.050   t = -2.074   df 6   p: $0.1 > p > 0.05$.

(EDTA and TRYPSIN continued).

(c) Aggregates formed after 42 hours reaggregation.
RECIPROCAL EXPERIMENTS

(i) LB*H

Aggregate number	TARGET CELLS total	L	U	y	z	q	θ	α
I	170	0.65	0.35	0.29	0.50	0.96	0.32	0.74
II	267	0.63	0.37	0.39	0.50	0.76	0.40	0.86
III	71	0.59	0.41	0.36	0.43	0.82	0.42	0.76
IV	101	0.66	0.34	0.34	0.47	0.70	0.38	0.76

mean α = 0.78

s^2 = 0.0031

Duplicate experiment

I	517	0.89	0.11	0.11	0.75	0.90	0.06	0.68
II	564	0.88	0.12	0.12	0.70	0.78	0.09	0.68

mean α = 0.68

s^2 = 0.000

Comparison of experiment with duplicate

σ = 0.048   t = 2.399   df 4   p: $0.1 \rangle p \rangle 0.05$.

LB*H Two experiments

Total mean α = 0.75

s^2 = 0.0046.

(ii) H*LB

Aggregate number	TARGET CELLS total	L	U	y	z	q	θ	α
I	490	0.12	0.88	0.67	0.08	0.91	0.88	0.73
II	329	0.09	0.91	0.80	0.06	0.76	0.92	0.84

mean α = 0.79

s^2 = 0.007

LB*H compared with H*LB

σ = 0.070   t = 0.705   df 6   p: $0.8 \rangle p \rangle 0.5$.
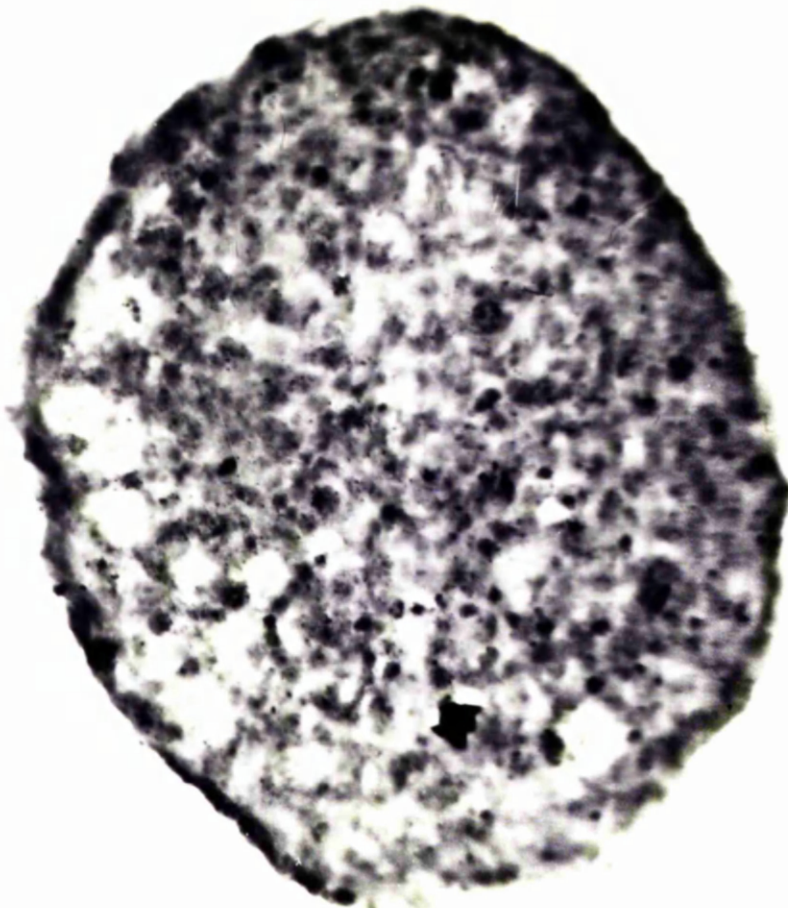
FIGURE VII

LB H:cellsdisaggregated with EDTA and TRYPSIN,reaggregated for
42 hours in reciprocating shaker.

The cell types are not markedly segregated.

Light field illumination.
Magnification:X480.

TRYPSIN DISAGGREGATION

(a) Aggregates formed after 2 hours reaggregation.

RECIPROCAL EXPERIMENTS

(i) LB*H

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	632	0.83	0.17	0.13	0.70	1.11	0.10	0.70
II	563	0.85	0.15	0.13	0.74	1.02	0.09	0.75
Total sample	1,195	0.84	0.16	0.13	0.72	1.07	0.09	0.72

mean α = 0.72

s^2 = 0.0014

(ii) H*LB

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	331	0.22	0.78	0.70	0.09	0.44	0.88	0.77
II	345	0.20	0.80	0.67	0.09	0.54	0.87	0.73
Total sample	676	0.21	0.79	0.69	0.09	0.49	0.88	0.75

mean α = 0.75

s^2 = 0.0006

LB*H compared with H*LB

δ = 0.0311   t = -0.8109   df 2   p $\simeq$ 0.5.

FIGURE VIII

LB*H:cells disaggregated with TRYPSIN,reaggregated for 2 hours.

A small clump of the unlabelled cell type,heart,can be seen even though this cell type is in the smaller proportion.

Light field illumination.
Magnification:X480.
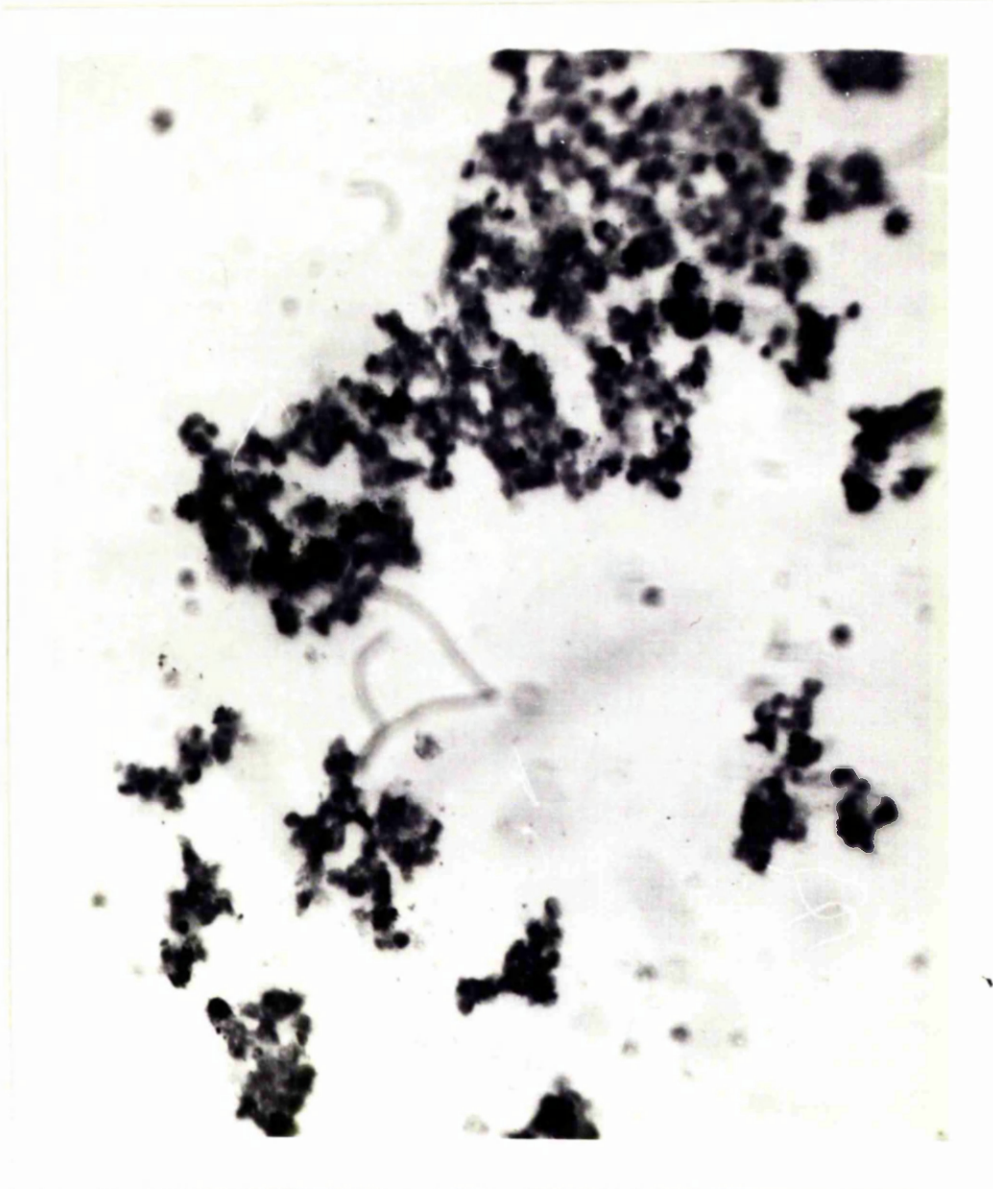
FIGURE IX

H* LB:cells disaggregated with TRYPSIN,reaggregated for 2 hours.

Note groups of labelled cell type,heart.

Light field illumination.

Magnification:X480.

TRYPSIN DISAGGREGATION (continued)

(b) Aggregates formed after 4 hours reaggregation.

RECIPROCAL EXPERIMENTS

(i) LB*H

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	190	0.82	0.18	0.13	0.70	1·17	0.10	0.71
II	273	0.82	0.18	0.13	0.67	1·18	0.10	0.65
III	212	0.83	0.17	0.13	0.74	1·20	0.09	0.77
IV	220	0.82	0.18	0.14	0.69	1·10	0.10	0.71
Total sample	895	0.82	0.18	0.13	0.70	1·16	0.10	0.71

mean α = 0.71

s^2 = 0.0025

(ii) H*LB

Sample number	TARGET CELLS total	L	U	y	z	q	θ	α
I	378	0.14	0.86	0.70	0.08	0·66	0.89	0.75
II	389	0.16	0.84	0.77	0.07	0·50	0.91	0.82
Total sample	767	0.15	0.85	0.73	0.07	0·56	0.90	0.79

mean α = 0.78

s^2 = 0.0029

LB*H compared with H*LB

$\acute{0}$ = 0.051   t = -1.637   df 4   p: 0.2 > p > 0.5.

TRYPSIN DISAGGREGATION (continued)

(c)   Aggregates formed after 42 hours reaggregation
     in reciprocating shaker.

RECIPROCAL EXPERIMENTS

   (i)   LB*H

Aggregate number	TARGET CELLS total	L	U	y	z	q	θ	α
I	161	0.60	0.40	0.35	0.49	0.94	0.38	0.80
II	456	0.67	0.33	0.32	0.54	0.84	0.32	0.82
III	383	0.72	0.28	0.34	0.52	0.61	0.35	0.83
IV	359	0.69	0.31	0.33	0.55	0.75	0.33	0.85

mean α = 0.83
s^2 = 0.0005

Duplicate experiment.

I	586	0.53	0.47	0.47	0.37	0.70	0.52	0.82
II	606	0.50	0.50	0.48	0.36	0.75	0.54	0.83

mean α = 0.82
s^2 = 0.000

Comparison of experiment and duplicate:

δ = 0.018   t = 0.069   df 4   p $>$ 0.8.

Two experiments total mean = 0.8
s^2 = 0.0003

Note that there are different proportions of labelled and
unlabelled cells in the two experiments giving similar
α's.
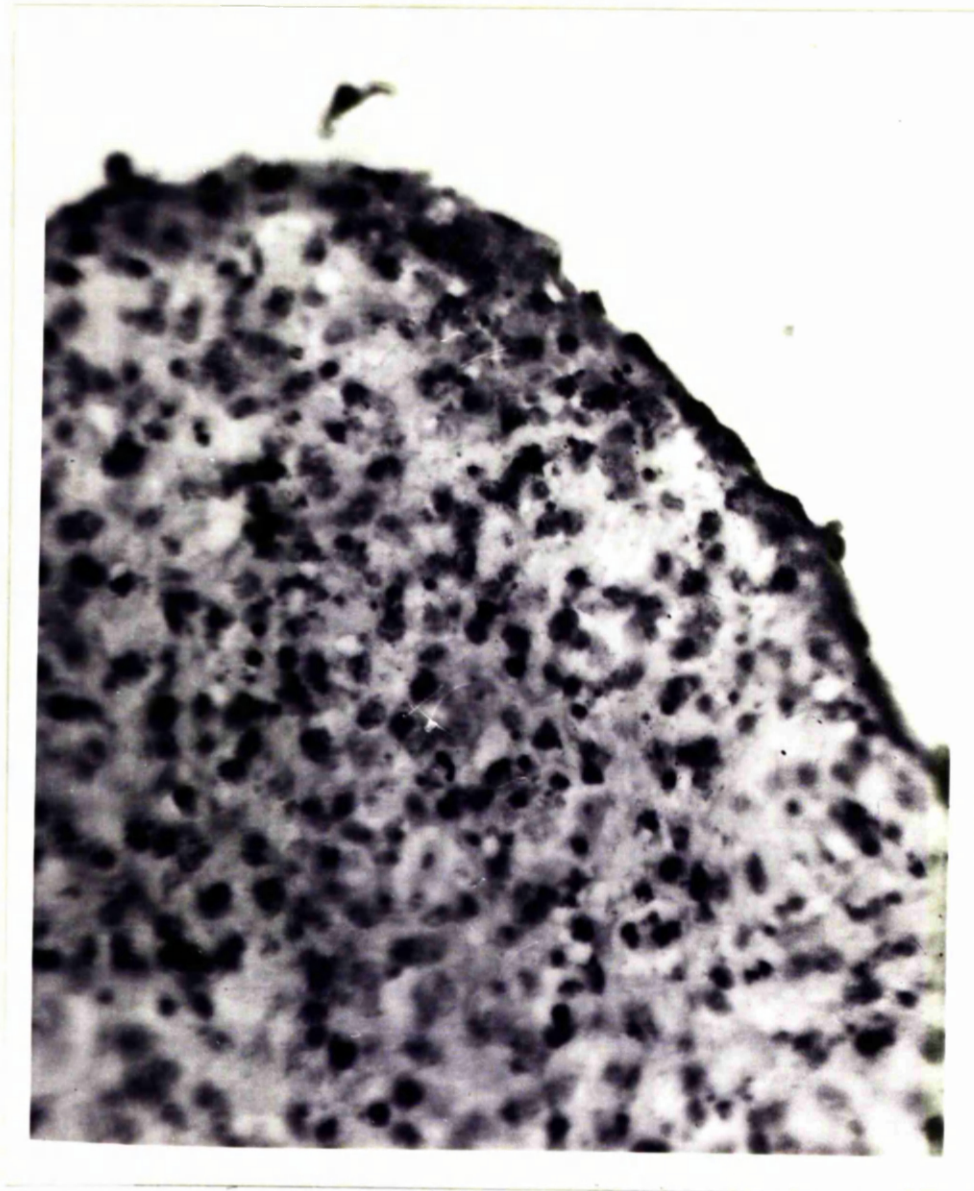
FIGURE X

LB H:cells disaggregated with TRYPSIN, reaggregated for 42 hours
in reciprocating shaker.

Note small degree of segregation.

Light field illumination.
Magnification:X440.

TRYPSIN DISAGGREGATION ((c) continued)

(ii) H*LB

Aggregate number	TARGET CELLS total	L	U	y	z	q	θ	α
I	506	0.12	0.88	0.70	0.08	0.78	0.89	0.75
Sample II	417	0.16	0.84	0.72	0.17	1.25	0.79	0.88

mean α = 0.82

s^2 = 0.0087

Duplicate experiment.

Sample I	487	0.08	0.92	0.85	0.04	0.54	0.95	0.87

Comparison of experiment and duplicate:

$t = 0.967$ df 1   p: $0.8 > p > 0.5$ (2 tailed).

Two experiments: total mean $\alpha = 0.84$

$s^2 = 0.005$

Comparison of total data of LB*H with total data of H*LB

$\sigma = 0.041$ $t = -0.394$ df 7   p: $0.8 > p > 0.5$.

To test whether the aggregates show significant segregation of cell types.

The degree of segregation (α) of aggregates prepared by each procedure at each time interval was compared with the α value of the control LB*LB (3)(aggregated for 42 hours and "cell sieved"). In each case, all the values obtained for α in reciprocal and duplicate experiments (when performed) were used.

Comparison with LB*LB (3), mean $\alpha = 0.92$, $s^2 = 0.0009$.

Type of aggregates

Dis-aggregated with	Re-aggregated for	total mean α	total s^2	σ	t	df	p
EDTA	2 hour	0.62	0.0026	0.044	10.51	8	p<0.001[+]
EDTA	4 hour[1]	0.64	0.0016	0.036	11.80	7	p<0.001[+]
EDTA(recip)[1]	42 hour	0.88	0.0024	0.044	1.59	9	p=0.2-0.1[-]
EDTA(gyrat)[2]	48 hour	0.65	0.0040	0.053	7.86	8	p<0.001[+]
EDTA + TRYPSIN	2 hour	0.65	0.0034	0.052	8.76	12	p<0.001[+]
EDTA + TRYPSIN	4 hour	0.70	0.0036	0.053	6.91	10	p<0.001[+]
EDTA + TRYPSIN	42 hour	0.76	0.0045	0.059	4.65	10	p<0.001[+]
TRYPSIN	2 hour	0.74	0.0009	0.030	8.93	6	p<0.001[+]
TRYPSIN	4 hour	0.73	0.0034	0.050	5.84	8	p<0.001[+]
TRYPSIN	42 hour	0.83	0.0015	0.037	4.24	11	p=0.002-0.001[+]

1 reciprocating shaker used for reaggregation.

2 gyratory " " " "

+ significant segregation.

- not significant segregation.

All groups of aggregates except those prepared by EDTA, aggregated for 42 hours in a reciprocating shaker, have α values which are significantly lower than the α values of the control LB*LB (3) aggregates, and therefore the cells are segregated according to type.

Time Course of "segregation"

(1) EDTA disaggregation

Comparison of

(a) Reaggregated for	mean α	(b) Reaggregated for	Mean α	σ	t	df	p	Significantly different +/−
2 hour	0.62	4 hour	0.64	0.046	−0.52	9	p:0.8≫0.5	−
2 hour	0.62	42 hour (recip)[1]	0.88	0.050	9.24	11	p<0.001	+
2 hour	0.62	48 hour (gyrat)[2]	0.65	0.057	0.86	10	p:0.5≫0.2	−
4 hour	0.64	42 hour (recip)	0.88	0.046	9.02	10	p<0.001	+
4 hour	0.64	48 hour (gyrat)	0.65	0.054	0.43	9	p:0.8≫0.5	−
42 hour (recip)	0.88	48 hour (gyrat)	0.65	0.056	−7.30	11	p<0.001	+

Time Course of "segregation"(continued)

In aggregates formed after 2 hours and 4 hours reaggregation the arrangement of cells is the same, and in both cases more segregated than the arrangement of cells in aggregates formed after 42 hour reaggregation in a reciprocating shaker,

[1   aggregated in reciprocating shaker   *see previous table*]
[2   "   " gyratory   "   ]

which in fact shows no segregation.   The arrangement of cells in aggregates formed after 48 hours in the gyratory shaker is not significantly different from that in aggregates formed after 2 hours and 4 hours reaggregation.

Time Course of "segregation" (continued)

(2) TRYPSIN and EDTA disaggregation

Comparison of

(a) Reaggregated for	mean α	(b) Reaggregated for	mean α	δ	t	df	p	Significant difference +
2 hour	0.65	4 hour	0.70	0.057	-1.77	16	p: $0.1 > P > 0.05$	− at 5% level
2 hour	0.65	42 hour	0.76	0.062	3.54	16	p: $0.005 > P > P 0.002$	+
4 hour	0.70	42 hour	0.76	0.064	1.77	14	$p \simeq 0.1$	−

Time Course of "segregation"(continued)

The arrangement of cells in aggregates formed after 2 hours and 4 hours reaggregation is not significantly different. The degree of segregation of cells in aggregates formed after 2 hours reaggregation is significantly greater than that in aggregates formed after 42 hours reaggregation, whereas that of aggregates formed after 4 hours reaggregation is not significantly lower than that of aggregates formed after 42 hours.re-aggregation. This suggests that the degree of segregation of aggregates formed in 4 hours is intermediate between that of aggregates formed in 2 hours and in 42 hours.

Time Course of "segregation"(continued).

(3) TRYPSIN disaggregation

Comparison of

(a) Reaggregated for	mean α	(b) Reaggregated for	mean α	σ	t	df	p	Significant difference
2 hour	0.74	4 hour	0.73	0.050	0.04	8	p$>$0.8	-
2 hour	0.74	42 hour	0.83	0.037	-4.19	11	p:0.002$>$p$>$0.001	+
4 hour	0.73	42 hour	0.83	0.048	-3.74	13	p:0.005$>$p$>$0.002	+

Time Course of "segregation" (continued)

The cells in aggregates formed after 2 hours and
4 hours reaggregation show the same degree of segregation.
Testing the degree of segregation of aggregates formed in
2 hours and 4 hours with that of aggregates formed in 42
hours shows a significant difference.

Time Course irrespective of disaggregation procedure.

Conclusions:

(1)   The cells are highly segregated in aggregates produced
      after a few hours.

(2)   The cells show a progressively more random arrangement
      with time in culture, when aggregates are formed under
      certain conditions in reciprocating shakers.

(3)   A segregation of EDTA disaggregated cells, similar to
      that in early aggregates is shown when aggregates are
      produced in gyratory shakers in 48 hours.

Comparison of disaggregation techniques.

(1) Aggregates formed after 2 hours reaggregation.

Comparisons of

(a) Disaggregated with	mean α	(b) Disaggregated with	mean α	σ	t	df	p	Significant difference +
EDTA	0.62	TRYPSIN	0.74	0.044	-3.96	8	p:0.005>p>0.002	+
TRYPSIN	0.74	TRYPSIN + EDTA	0.65	0.051	2.82	12	p:0.02>p>0.01	+ at 5% level
EDTA	0.62	TRYPSIN + EDTA	0.65	0.055	-0.96	14	p:0.5>p>0.2	-

The segregation of cells in aggregates formed in 2 hours compared to the disaggregation procedure can be written thus:

EDTA $\rangle$ TRYPSIN + EDTA $\rangle$ TRYPSIN.

Comparisons of disaggregation techniques (continued)

(2) Aggregates formed after 4 hours reaggregation.

Comparisons of

Disaggregated with (a)	mean α	Disaggregated with (b)	mean α	σ	t	df	p	Significant difference + at 5% level
EDTA	0.64	TRYPSIN	0.73	0.051	-3.12	9	p:0.02〉p〉0.01	+
TRYPSIN + EDTA	0.70	EDTA	0.64	0.052	2.07	11	p:0.1〉p〉0.05	-
TRYPSIN + EDTA	0.70	TRYPSIN	0.73	0.058	-1.12	12	p:0.5〉p〉0.2	-

In aggregates formed in 4 hours, cells disaggregated with EDTA and TRYPSIN again seem to show an intermediate degree of segregation between cells treated with EDTA and cells disaggregated with TRYPSIN

EDTA 〉 EDTA + TRYPSIN 〉 TRYPSIN.

(3) Aggregates formed after 42 hours in a reciprocating shaker.

Comparisons of

	(a)		(b)						Signi-ficant difference +
Disaggregated with	mean α		Disaggregated with	mean α	σ	t	df	2	
EDTA	0.88		EDTA + TRYPSIN	0.76	0.060	4.01	13	p:0.002>0.001	+
EDTA	0.88		TRYPSIN	0.83	0.044	2.29	14	p:0.05>0.02	+ at 5% level
TRYPSIN	0.83		EDTA + TRYPSIN	0.76	0.054	2.78	15	p:0.02>0.01	+ at 5% level

Cells in aggregates, previously disaggregated with EDTA show no significant segregation at all. Cells in aggregates, previously disaggregated with EDTA and TRYPSIN show the highest degree of segregation in this group.

EDTA and TRYPSIN $\rangle$ TRYPSIN $\rangle$ EDTA (no segregation).
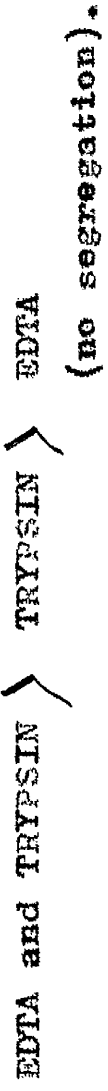
Comparison of disaggregation techniques (continued)

(4) Comparison of aggregates reaggregated for 48 hours
    in a gyratory shaker (mean α = 0.65) with aggregates
    reaggregated for 42 hours in a reciprocating shaker.
Compared with

disaggregated with	mean α	δ	t	df	p	Significantly different +
EDTA (recip)[1]	0.88	0.056	7.30	11	<0.001	+
EDTA + TRYPSIN	0.76	0.066	-2.92	12	p:0.02>0.01	+ at 5% level
TRYPSIN	0.83	0.050	-6.71	13	p<0.001	+

Aggregates prepared in gyratory shakers show a
significantly higher degree of segregation than any aggreg-
ates reaggregated for a comparable length of time in
reciprocating shakers.   It should be remembered that the
medium in which reaggregation was carried out was different
in gyratory shakers.

1 reaggregated in reciprocating
   shaker.

Comparison of aggregates produced from cell suspensions initially disaggregated by various techniques.

Conclusions

Conclusions early aggregates (formed after 2 hours and 4 hours) the degree of segregation can be expressed as:

$$EDTA > TRYPSIN + EDTA > TRYPSIN.$$

(2) In the aggregates formed after 42 hours reaggregation in reciprocating shakers, the degree of segregation can be expressed as:

$$TRYPSIN + EDTA > TRYPSIN > EDTA$$

(3) In gyratory shaker produced aggregates (cells disaggregated with EDTA) the degree of segregation is significantly higher than in aggregates reaggregated in reciprocating shaker for comparable time periods.

Distribution of cells from observations in segregation test I.

The frequency distribution of the number of cells of one type around a target cell of a given type can be examined where the cells are recognized by being "labelled" or "unlabelled".

If the cells are randomly arranged the frequency distribution of cells of one type surrounding targets of a given type would be expected to be binomial. The binomial distribution is obtained by expansion of the term $(p + q)^n$

Where n is the maximum possible number of surrounding cells, i.e. 6

p is the probability that the cells are of one type i.e. the proportion of that cell type in the aggregate.

q is the probability that the cells are of the other type i.e. the proportion of this cell type in the aggregate and $q = (1 - p)$.

E.g. When considering "labelled" cells round "labelled" targets, p is the proportion of "labelled" cells in the aggregate and q is the proportion of "unlabelled" cells in the aggregate.

$(p + q)^6$ expanded thus

$$q^6 + 6pq^5 + 15p^2q^4 + 20p^3q^3 + 15p^4q^2 + 6p^5q + p^6$$

We can calculate the binomial distribution of the expected frequencies of 0, 1, 2, 3, 4, 5, 6 cells of one type surrounding a target cell of given type when the cells are randomly arranged as p and q are given by the proportion of/

of target cells of each type. These parameters can be
readily calculated from the counts of "unlabelled" and
"labelled" target cells by simple proportion using the
correction for percentage labelling. We can then test
the observed frequency of 0, 1, 2, etc. "labelled" cells
surrounding "labelled" targets and of "unlabelled" cells
surrounding "unlabelled" targets against the calculated
binomials by means of a χ^2 test. If the cells are
randomly arranged, "labelled" and "unlabelled" cells should
be distributed not significantly differently from this
binomial distribution regardless of percentage labelling
(see later).

> LB*LB (3) (disaggregated with EDTA, cell "sieved"
>   and reaggregated for 42 hours.

Sample III $\alpha = 0.95$

Corrected for percentage labelling: proportion of targets
                                       of labelled cell
                                       type $= 0.26$

                                       proportion of targets
                                       of unlabelled cell
                                       type $= 0.74$

(i) "labelled" cells surrounding "labelled" targets.

   $p = 0.26$

   $q = 0.74$

Number of surrounding cells	Observed frequency	Expected frequency	
0	4	7.03	
1	17	14.48	
2	14	12.42	
3	7	5.68	
4	1	1.47	⎫
5	0	0.20	⎬ 1.68[1]
6	0	0.01	⎭

$\chi^2 = 2.25$ df^2 4   p: $0.7 > p > 0.5$.

[1] classes summated to give expectation of not less than
     1 (Cochran 1954)

df^2 the number of degrees of freedom is the number of
     classes − 1.

LB*LB (3)   Sample III (continued)

(ii) "unlabelled" cells surrounding "unlabelled"
targets.

$$p = 0.74$$
$$q = 0.26$$

Number of "unlabelled" surrounding cells	Observed frequency	Expected frequency	
0	0	0.05)	
1	0	0.85)	5.61
2	4	4.71)	
3	15	18.24	
4	40	39.87	
5	48	46.47	
6	31	22.56	

$$\chi^2 = 4.24 \quad df\ 4 \quad p: 0.5 > p > 0.3.$$

In LB*LB (3)   Sample III the frequency distribution
of the number of "labelled" cells surrounding "labelled"
targets and that of the number of "unlabelled" cells
surrounding "unlabelled" targets fits a binomial calculated
from the proportion of target cells of each type in the
aggregate.   This provides additional evidence that labelled
and unlabelled cells are distributed at random (see later)
in this sample of aggregates.

From the data of the mean number of surrounding
"labelled" or "unlabelled" cells of "labelled" or "un-
labelled" targets we can calculate a binomial distribution
$(p + q)^n$

where n = 6   (as before)
      p = proportion of one type cell around a
          given target, as recognized by being
          labelled or unlabelled.
      q = (1 - p).

We/

We have from segregation measures, the proportion (y)
of unlabelled cells round labelled targets and the pro-
portion (z) of labelled cells round unlabelled targets.

Therefore the proportion of labelled cells = $(1 - y)$
    round labelled targets

      and the proportion of unlabelled   = $(1 - z)$
      cells round unlabelled targets

In LB*LB (3) Sample III the labelled and unlabelled
cells are randomly arranged. The proportion of the
labelled target cells in the aggregate should approximately
equal $(1 - y)$ and the proportion of unlabelled target cells
approximately equal $(1 - z)$

Corrected proportion   =   0.26 $(1 - y) = 0.27$
  of labelled targets

Corrected proportion   =   0.74 $(1 - z) = 0.77$
  of unlabelled targets

Binomial distributions calculated from the data
of the mean number of surrounding cells can be fitted to
the observed distribution of cells.

      LB*LB (3) (disaggregated with EDTA, cell "sieved"
          and reaggregated for 42 hours)

    SAMPLE IV $\alpha = 0.99$

(1)   Binomial distribution calculated from the corrected
proportions of cells in aggregates to test whether the
cells are arranged randomly.

    (i) "Labelled" cells surrounding "labelled" targets
        p = 0.32 (corrected proportion of "labelled"
                              targets)

        q = 0.68 ("   "   " "unlabelled"
                                   targets)

LB*LB (3) Sample IV (continued)

Number of surrounding cells	Observed frequency	Expected frequency
0	11	10.77
1	35	30.05
2	35	34.92
3	20	21.64
4	6	7.55
5	1	1.40 ⎫ 1.51
6	0	0.11 ⎭

$$\chi^2 = 1.44 \qquad df\ 5 \qquad p: 0.95 \rangle p \rangle 0.9.$$

(ii) "Unlabelled" cells surrounding "unlabelled"
targets
p = 0.68
q = 0.32

Number of surrounding cells	Observed frequency	Expected frequency
0	0	0.26 ⎫ 3.60
1	2	3.35 ⎭
2	6	18.03
3	26	51.70
4	74	83.41
5	107	71.78
6	43	25.72

$$\chi^2 = 51.48 \qquad df\ 5 \qquad p \lll 0.001.$$

The/

LB*LB (3) Sample IV (continued)

The distribution of the number of "labelled" cells surrounding "labelled" targets is not significantly different from the binomial expected if the cells are randomly arranged. The distribution of "unlabelled" cells round "unlabelled" targets is however significantly different from the binomial expected if the cells are randomly arranged. The frequencies of high numbers of "unlabelled" cells surrounding "unlabelled" targets are greater than expected. The frequencies of low numbers of "labelled" cells surrounding "labelled" targets are greater than expected. These "skews" in the observed distributions could be partially explained by the fact that a proportion of the "labelled" cell type does not take up the label, although the real situation is more complicated.

(2)   Binomial distribution calculated from an estimate
       of p from the data on surrounding cells.
       LB*LB (3) Sample IV
       (1) "Labelled" cells surrounding "labelled" targets
       p = 0.30
       q = 0.70 (y)

Number of surrounding cells	Observed frequency	Expected frequency
0	11	12.9
1	35	32.7
2	35	34.9
3	20	19.7
4	6	6.2
5	1	1.04 } 1.1
6	0	0.06

$$\chi^2 = 0.44 \quad df^{[1]} \quad 4 \quad p: 0.98 > p > 0.95.$$

[1]   degrees in freedom in this case where p and q are
      calculated from data is the number of classes − 2.

LB*LB (3)   Sample IV (continued)

(ii) "Unlabelled" cells surrounding "unlabelled" targets.

p = 0.76

q = 0.24   (z)

Number of surrounding cells	Observed frequency	Expected frequency
0	0	0.26) 1.03
1	2	0.77)
2	6	6.97
3	26	30.44
4	74	73.53
5	107	94.69
6	43	50.83

$$\chi^2 = 4.50 \quad \text{df } 4 \quad p: 0.5 > p > 0.3.$$

Although the distribution of "unlabelled" cells round "unlabelled" targets is significantly different from that expected if the cells were randomly arranged, the frequency of the number of surrounding cells is distributed binomially.

A binomial distribution of "labelled" cells around "labelled" targets was calculated from the data for LB*H Sample IV of aggregates formed from cells disaggregated with EDTA in 2 hours and compared with the observed distribution.

LB*H Sample IV $\alpha = 0.67$

p = 0.75

q = 0.25 (y)

LB*LB (3)   Sample IV (continued)

Number of surrounding cells	Observed frequency	Expected frequency
0	1	0.03 ⎫
1	1	0.89 ⎬ 8.66
2	10	7.74 ⎭
3	34	31.98
4	67	73.32
5	81	87.58
6	52	44.03.

$$\chi^2 = 2.67 \qquad df \ 3 \qquad p: \ 0.5 > p > 0.3.$$

Even when the cell types are segregated the number
of "labelled" cells round "labelled" targets is distributed
binomially.   This was found to be the general case.   In
other words, the experimental data on proportions of cells
surrounding targets show no more variation than expected
in a binomial distribution.

Further points on Segregation test I

We can test how homogeneous the observed proporti
y and z from aggregates prepared under the same conditio
As an illustration aggregates LB*LB (1) (disaggregated
with trypsin, not cell "sieved" reaggregated for 42 hours)
have been examined briefly.

If the proportions of "labelled" and "unlabelled"
target cells in the four aggregates are not statistically
different the frequency distribution of the number of
"labelled" cells round "labelled" targets should also not
be statistically different.

(a) The proportions of "labelled" and "unlabelled"
    targets.

2 x 4 Contingency table

	"labelled" targets	"unlabelled" targets
Aggregate I	62	143
" II	114	224
" III	97	214
" IV	97	209

$\chi^2 = 0.86$ df 3   p: $0.9 > p > 0.8$ not significantly different.

(b) "Labelled" cells surrounding "labelled"
    targets.

	number of surrounding cells						
	0	1	2	3	4	5	6
Aggregate I	4	14	8	19	12	5	0
" II	18	25	31	25	12	2	1
" III	11	24	34	21	6	1	0
" IV	13	26	34	15	7	1	1

$\chi^2 = 30.32$

treated as one class
so expected frequency
not less than 1.

df ^{1} = 15

p: $0.05 > p > 0.01$

not significantly different at 1% level

df ^{1} df = (rows - 1) (columns - 1)

The surrounding cell frequencies may show significant variation over and above random. This may not be important and data has been treated as homogeneous. It has been pointed out in the results, where applicable, that α values do not seem to depend on the proportion of the two cell types in aggregates, in the range of proportions obtained.

TESTS FOR SEGREGATION OF CELLS ACCORDING TO TYPE.

TEST II   Pielou's (1962) Line transect method.

The general theory (after Pielou 1962).

We can consider two cell types A and B mixed together in an aggregate.   If we take line transects across the aggregate recording the type of each cell in succession we can obtain a distribution of run lengths for each cell type.

Following Pielou (1962) the theoretical situation is:-

Let the probability of encountering individuals of A be a.

    "   "   "   "   "   "   " B be b.

The probability of obtaining an uninterrupted sequence of r individuals of A is $a^{r-1}b$.

The probability of obtaining an uninterrupted sequence of s individuals of B is $b^{s-1}a$.

So for both A and B the distribution of run lengths is geometric.

Considering the runs of A's,

    let mean run length $=$ $\bar{m}_A$

Considering the runs of B's,

    let mean run length $=$ $\bar{m}_B$

the estimator of b, $\hat{b}$ $= \dfrac{1}{\bar{m}_A}$

the estimator of a, $\hat{a}$ $= \dfrac{1}{\bar{m}_B}$

Let θ be the proportion of A cells, then $(1 - \theta)$ is the proportion of B cells.

    If/

If the cells are randomly arranged

$$\frac{1}{\bar{m}_A} + \frac{1}{\bar{m}_B} = 1$$

and $\hat{b} = \dfrac{1}{\bar{m}_A}$ = proportion of B cells

and $\hat{a} = \dfrac{1}{\bar{m}_B}$ = proportion of A cells,

so we can write

$$\frac{1}{\bar{m}_A} = (1 - \theta)$$

and $\dfrac{1}{\bar{m}_B} = \theta$

therefore in a random array we can check

$$\frac{1}{\bar{m}_A} + \frac{1}{\bar{m}_B} = (1 - \theta) + \theta = 1.$$

If the cells are segregated, the mean run lengths of A and B will be increased. $\dfrac{1}{\bar{m}_A}$ and $\dfrac{1}{\bar{m}_B}$ will be lowered from that expected from the proportions of B and A in the mixture.

We can introduce a term β, to describe this lowering. $\beta = 1$, when the cells are randomly arranged because in this case $\dfrac{1}{\bar{m}_A} = (1 - \theta)$ and $\dfrac{1}{\bar{m}_B} = \theta$.

Introducing/

Introducing β

$$\frac{1}{\bar{m}_A} = \beta(1 - \theta) \qquad \frac{1}{\bar{m}_B} = \beta\theta$$

so the sum of $\frac{1}{\bar{m}_A}$ and $\frac{1}{\bar{m}_B}$ expressed in terms of β is

$$\frac{1}{\bar{m}_A} + \frac{1}{\bar{m}_B} = \beta \quad (\beta = 1, \text{ when cells randomly arranged})$$

Pielou (1962) is interested in the value of

$$1 - \left(\frac{1}{\bar{m}_A} + \frac{1}{\bar{m}_B}\right)$$

to detect segregation. If the cells are randomly arranged $\frac{1}{\bar{m}_A} + \frac{1}{\bar{m}_B} = 1$, and the difference is 0. When the cells are segregated $\alpha \to 0$ and the difference $\to 1$.

If the number of observations of runs of A (n_A) is sufficiently high $\hat{b}$ will have an approximately normal distribution.

The variance of this distribution can be given as

$$S_b^2 = \frac{1}{n_A} \cdot \frac{\bar{m}_A - 1}{\bar{m}_A{}^3}$$

likewise

$$S_a^2 = \frac{1}{n_B} \cdot \frac{\bar{m}_B - 1}{\bar{m}_B{}^3}$$

If A and B are unsegregated it follows that with a 95% probability

$$\frac{1}{\bar{m}_A} + \frac{1}{\bar{m}_B} = \hat{b} + \hat{a} = 1 \pm 1.96 \sqrt{S_a^2 + S_b^2}$$

since the run lengths are independent of each other.

We/

We can test whether $\dfrac{1}{\bar{m}_A} + \dfrac{1}{\bar{m}_B}$ is significantly less than

unity and, thus, if the cells are segregated.

We have the following observations

$\bar{m}_L$ = mean run length of labelled cells.

$\bar{m}_U$ = " " " " unlabelled cells.

From these observations β has been calculated and compared with 1 ± 2 standard deviations, ignoring corrections for percentage labelling. The problem of assessing the degree of segregation in terms of the cell types, rather than "labelled" and "unlabelled" cells is rather complicated as "unlabelled" runs will be heterogeneous in respect to cell type.

Counting methods

Fields were selected for counting, as in the previous method, of samples of aggregates. An eye piece square graticule was used to obtain a line along which cells were scored according to whether they were labelled or unlabelled at x900 magnification. In early aggregates the line could be fitted across aggregate sections in one field. With larger aggregates the field was moved so that the end cell was brought to the beginning of the line for the next field. This was repeated until the aggregate had been traversed. In Pielou's (1962) transects of forest trees the first and last runs in a transect sample were not used as these could be parts of longer runs. In aggregates with a definite edge all runs can be used.

SEGREGATION TESTS

TEST II: Results

(1) Control LB*LB (3), disaggregated with EDTA, cells "sieved", reaggregated for 42 hours

$$\hat{l} \qquad \hat{u} \qquad \beta \qquad 1-\beta \qquad \overset{\hat{l}+\hat{u}}{\sqrt{s_l^2 + s_u^2}} \qquad 1 \overset{\pm}{} 1.96 \sqrt{s_l^2 + s_u^2}$$

$$0.28 \quad 0.69 \quad 0.97 \quad 0.03 \qquad 0.003 \qquad 1 \overset{\pm}{} 0.114.$$

The cells are randomly arranged in these control aggregates. In this case $\hat{l} \doteq$ the proportion of labelled cells and $\hat{u} \doteq$ the proportion of unlabelled cells in aggregates LB*LB (3). This can be checked by reference to target cell proportions obtained in TEST I segregation measures, L being the proportion of "labelled" targets and U being the proportion of "unlabelled" targets.

	$\hat{l} = 0.28$	$\hat{u} = 0.69$
Sample I	L = 0.31	U = 0.69
Sample II	L = 0.28	U = 0.72
Sample III	L = 0.24	U = 0.76
Sample IV	L = 0.30	U = 0.70

(2) Aggregates formed after 2 hours, disaggregated with EDTA.

Aggregates	$\hat{l}$	$\hat{u}$	β	$1-\beta$	$\overset{\hat{l}+\hat{u}}{\sqrt{s_l^2 + s_u^2}}$	$1 \overset{\pm}{} 1.96\sqrt{s_l^2 + s_u^2}$
LB*H	0.40	0.23	0.63	0.37	0.08	1± 0.156
H*LB	0.30	0.37	0.67	0.33	0.05	1± 0.105

In these aggregates the cells show positive segregation.

We/

We can compare the value of β, in effect the degree of segregation of "labelled" and "unlabelled" cells in aggregates with a similar value from TEST I of segregation. We cannot compare β directly with α, as α is corrected for percentage labelling and refers directly to the two cell types.

We have in TEST I

y = proportion of unlabelled cells round labelled targets.

z =   "   "   labelled   "   "   unlabelled   "

We could write

$y = \alpha' U$ where U = proportion of "unlabelled" cells

$z = \alpha'(1 - U)$ α' = degree of segregation of "unlabelled" and "labelled" cells.

therefore $y + z = \alpha'$

We can therefore compare values of β and α' in significance tests, calculating α' as above.

(1) Control LB*LB (3)

    $\beta = 0.97$ mean $\alpha' = 0.93$

                                  $s^2 = 0.0007$

    $t = -3.03$ df 3 $p: 0.1 > p > 0.05$.

                          no significant difference at 5% level.

(2) Aggregates reaggregated for 2 hours, disaggregated with EDTA

    (i) LB*H

      $\beta = 0.63$ mean $\alpha' = 0.70$

                                $s^2 = 0.001$

      $t = 3.25$ df 3 $p: 0.05 > p > 0.02$.

                          no significant difference at 1% level.

    (ii)/

(ii) H*LB

$$\beta = 0.67 \qquad \alpha' = 0.65$$

$$s^2 = 0.0024$$

$$t = -0.73 \qquad df \ 1 \qquad p: \ 0.8 > p > 0.5$$

no significant difference.

The degree of segregation of "unlabelled" and "labelled" cells of two cell types is probably not statistically different as calculated by TEST I and TEST II.

If the value α' is examined it is found that approaches the real value α when the cells are randomly arranged, this is because regardless of percentage labelling, the labelled and unlabelled cells will be distributed just as 'randomly' as the two cell types.

The distribution of run lengths.

From Pielou (1962) the nature of the distribution
of run lengths can be tested on various models. "The
runs of cells may fit a geometric distribution even
when segregated, although the expected sum of the parameters
of the two series will not equal one." Pielou assumes
"that we are dealing with a Markov chain with fixed
transition probabilities."

The expected proportion of runs of length x of a
geometric distribution is in each case $p^{x-1}q$ $(p + q = 1)$
and the estimator of q, $\hat{q} = \dfrac{1}{\bar{m}}$ where $\bar{m}$ is mean run
length.

The expected distribution of run lengths can then
be compared with observed distribution by means of a χ^2
test.

Control LB*LB (3) (disaggregated with EDTA, cells "sieved", reaggregated for 42 hours).

UNLABELLED CELLS			LABELLED CELLS		
Run length	Observed frequency	Expected frequency	Run length	Observed frequency	Expected frequency
1	15	17.47	1	39	39.14
2	11	12.55	2	13	12.26
3	8	9.01	3	3	3.84
4	9	6.47	4	1)$_2$	) 1.58
5	8	4.65	5	1)^2	)
6	3	3.34			
7	3	2.40			
8	3	1.72			
9	1)				
10	0)2^1	2.12			
11	1)				

$\hat{q} = 0.28$ $\hat{q} = 0.69$

$\hat{p} = 0.72$ $\hat{p} = 0.31$

$\chi^2 = 5.2$ $\chi^2 = 0.34$

$df^2 = 7$ $df = 2$

$p: 0.7 > p > 0.5$ $p: 0.9 > p > 0.8$

[1] classes pooled to give an expected value not less than 1 (Cochran 1954)

df^2 number of degrees of freedom is the number of classes – 2.

LB*H (Aggregates formed after 2 hours, disaggregated
with EDTA)

UNLABELLED CELLS			LABELLED CELLS		
Run length	Observed frequency	Expected frequency	Run length	Observed frequency	Expected frequency
1	20	17.60	1	9	14.72
2	10	10.56	2	14	11.33
3	6	6.34	3	10	8.72
4	4	3.80	4	10	6.71
5	0	2.81	5	6	5.17
6	0	1.37	6	3	3.98
7	1		7	5	3.07
8	1		8	2	2.36
9	1 } 4	1.52	9	0	1.82
10	0		10	1	1.40
11	0		11	2	1.08
12	1		12	0	3.63
			13	0 } 2	
			14	1	
			15	1	

$\hat{q} = 0.4$

$\hat{p} = 0.6$

$\chi^2 = 8.59$

df 5

p: $0.2 > p > 0.1$

$\hat{q} = 0.23$

$\hat{p} = 0.77$

$\chi^2 = 9.74$

df = 10   p: $0.5 > p > 0.3$

H*LB   (Aggregates formed after 2 hours, disaggregated
with EDTA)

UNLABELLED CELLS			LABELLED CELLS		
Run length	Observed frequency	Expected frequency	Run length	Observed frequency	Expected frequency
1	13	12.21	1	10	10.87
2	9	7.63	2	9	7.59
3	5	4.85	3	4	5.30
4	1	3.05	4	3	3.70
5	1	1.92	5	3	2.58
6	0	1.21	6	4	1.80
7	0)		7	1	1.26
8	2)		8	0)	
9	2)4	2.13	9	0)2	2.91
10	0)		10	1)	
11	0)		11	1)	

$\hat{q} = 0.37$ $\qquad\qquad\qquad$ $\hat{q} = 0.30$

$\hat{p} = 0.63$ $\qquad\qquad\qquad$ $\hat{p} = 0.70$

$\chi^2 = 4.98$ $\qquad\qquad\qquad$ $\chi^2 = 4.22$

df $= 5$ $\qquad\qquad\qquad$ df $= 6$

p: $0.5 > p > 0.3$ $\qquad\qquad$ p: $0.7 > p > 0.5$

In all three kinds of aggregates examined, the
distribution of run lengths fits a geometric distribution
even where the cells are segregated. Therefore the occur-
ences of "labelled" and "unlabelled" cells along a line
form a Markov chain. The type of cell in a given position
is influenced by the nature of the adjoining cell. However
we should expect that cells which do not fall on the transect
might influence the type of cell in a given position and also
the position of the cell relative to the inside and outside
of the aggregate (see Positioning).

C. MEASURES OF POSITIONING.

Segregation of cells according to type may lead
to positioning. "Positioning" has been defined relative
to the inside and outside of an aggregate and has been
considered to be characteristic for a given combination of
tissue types, e.g. limb bud precartilage segregates
internally when mixed with heart (Steinberg 1963a).

Aggregates formed from cells disaggregated with
EDTA after 2 hours and 48 hours (in gyratory shakers) have
similar degrees of segregation. From individual aggregates
(see figures II & III) it appears that in the aggregates formed
after 2 hours, heart segregates internally, whereas in
aggregates formed after 48 hours, it is limb bud cells
which are apparently internal (see figures V & VI). To test
the generality of these impressions and present data from
a large number of aggregates it was decided to apply some
quasi-quantitative measures of "positioning" and record
these in a pictorial fashion.

Consider a typically "sorted out" aggregate (Steinberg
case 2).
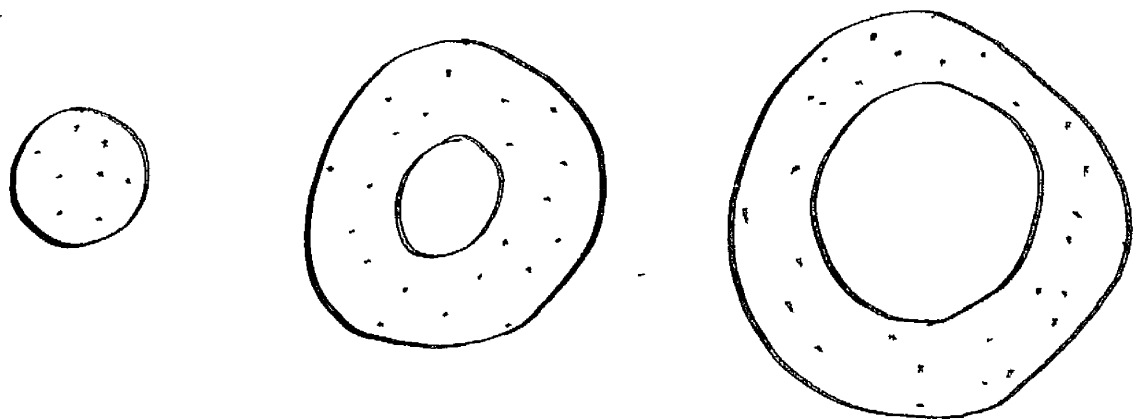
Adler/

Adler (1970) has compared the relative proportions of the two cell types with the area of the section. In my material this method would be difficult to apply for two reasons. It is not always possible to trace a single aggregate in this way. With small aggregates (formed after 2 hours) of 33μ - 110μ in diameter cut into 5 sections and counting every third section the amount of data is limited.

I. Aggregates formed after 2 days reaggregation.
Methods

An eye-piece graticule grid of squares, of side 26.7μ at x400 magnification, was fitted to the widest part of the aggregate which would give a complete number of squares. Under dark field illumination the number of "labelled" cells per square was counted across the aggregate. The data was kept separate for various "square widths" of aggregate. The small "widths" will represent either small aggregates or the "ends" of "sorted out" aggregates. Since, in the gyratory shaker, the aggregates produced are of relatively uniform size, it is most likely that the "small widths" are in fact "ends" of aggregates. In this case, it would be expected that the smaller aggregate widths show a more uniform distribution of cells than larger aggregate widths.

Histograms of the mean number of labelled cells per square have been drawn for aggregates reaggregated for 2 days in gyratory shakers of "labelled" limb bud and "unlabelled" heart cells, and the reciprocally labelled aggregates. The histograms show a reciprocal distribution of "labelled" cells (see Histograms 1 and 2) and that heart/

LB R-cells disaggregated with EDTA, reaggregated for 2 days in gyratory shaker.

Mean number of labelled cells/square across aggregate sections of various diameters (5-11 samples/aggregate size class)
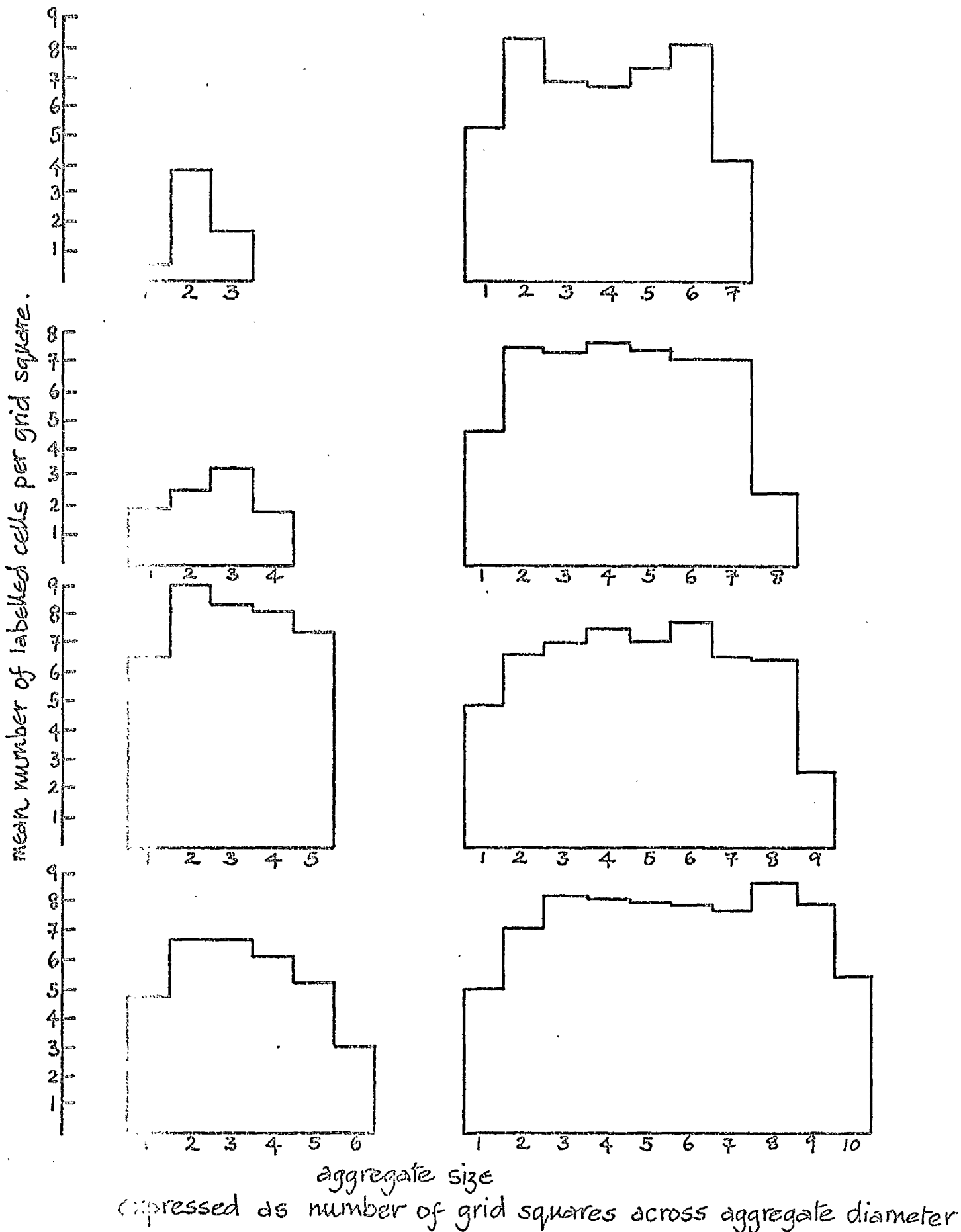
mean number of labelled cells per grid square.

aggregate size

expressed as number of grid squares across aggregate diameter

H*LB:cells disaggregated with EDTA,reaggregated for 2 days in gyratory shaker.

Mean number of labelled cells/square across aggregate sections of various diameters(3-19 samples/aggregate size class)

aggregate size
expressed as number of grid squares across aggregate diameter
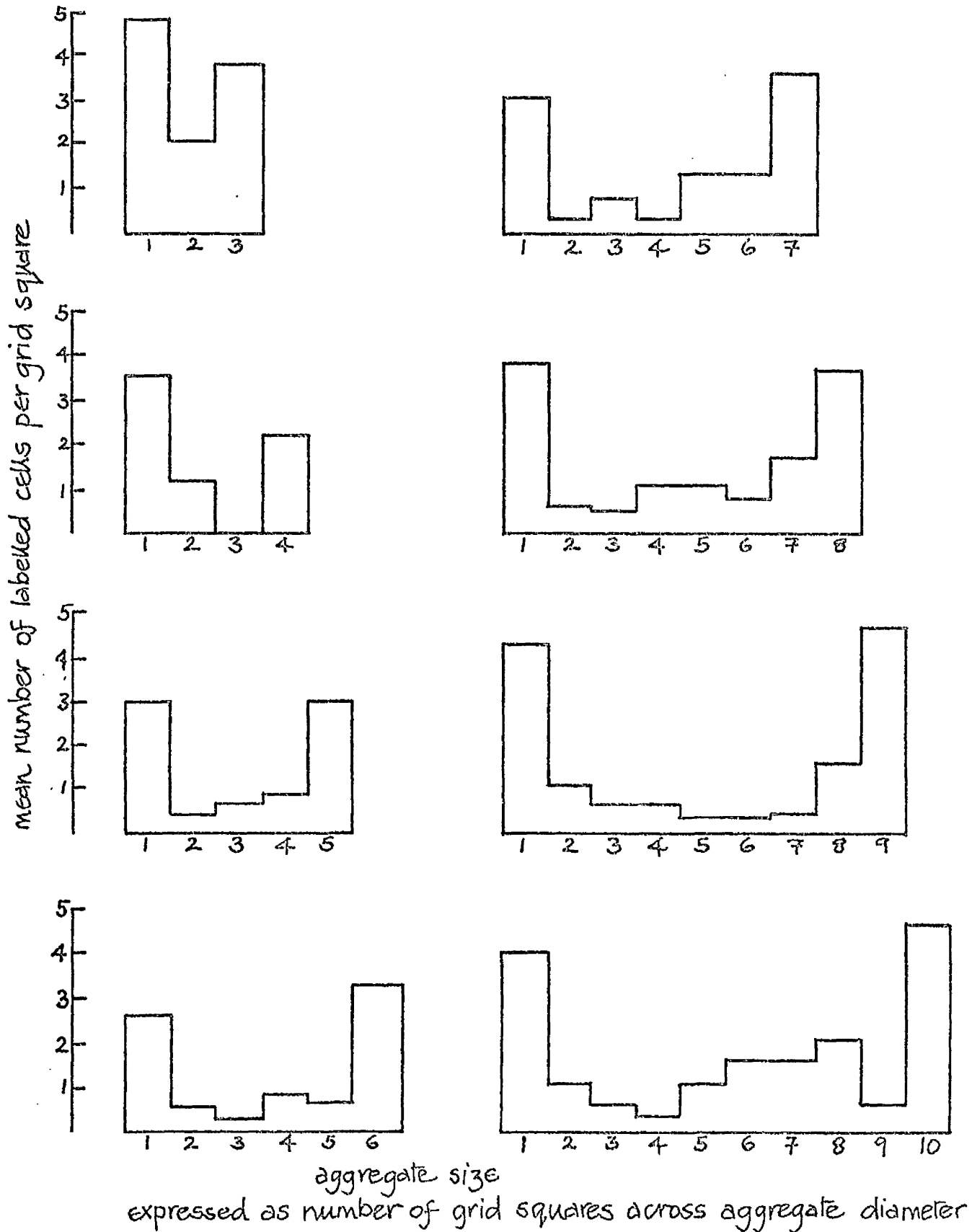
heart is positioned externally. No corrections were
made for percentage labelling, as this should not affect
the results grossly.

As a control, histograms of the mean number of
labelled cells per square across Control aggregates,
LB*LB (3) (of "labelled" and "unlabelled" limb bud cells)
have been made (see Histogram 3).

Histograms of the total number of cells per square
were then analysed by means of a χ^2 test to show whether
the distribution of "labelled" cells across aggregates formed
in 2 days, was statistically significant. The total
number of "labelled" cells from all squares was divided
by the number of squares to give the average number of
cells per square. If the cells are distributed randomly
across the aggregate, the average number of cells per
square will not be significantly different from the actual
scores for each square. The total number of "labelled"
cells per each square were therefore tested against the
expected number per square (i.e. the average number per
square) on the hypothesis that the "labelled" cells are
distributed randomly.

Results

Control LB*LB (3) (EDTA disaggregated, "cell sieved",
reaggregated for 2 days)

Aggregate size (in square widths)	χ^2	df^1	p	significant difference +
4	1.38	3	p: 0.8$\rangle p \rangle$0.7	—
5	1.12	4	p: 0.9$\rangle p \rangle$0.8	—
6	8.88	5	p: 0.2$\rangle p \rangle$0.1	—
7	4.36	6	p: 0.7$\rangle p \rangle$0.5	—
8	4.20	7	p: 0.8$\rangle p \rangle$0.7	—
9	4.61	8	p: 0.8$\rangle p \rangle$0.7	—
10	10.60	9	p: 0.5$\rangle p \rangle$0.3	—
11	17.90	10	p: 0.1$\rangle p \rangle$0.05	— at 5% level
12	3.60	11	p: 0.99$\rangle p \rangle$0.98	—

df^1, the number of degrees of freedom = number of squares − 1

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HISTOGRAM 3

CONTROL LB*LB:disaggregated with EDTa, cells sieved and reaggregated for 42 hours in reciprocating shaker.

Mean number of labelled cells/square across aggregate sections of various diameters (3-15 samples/aggregate size class)

mean number of labelled cells per grid square
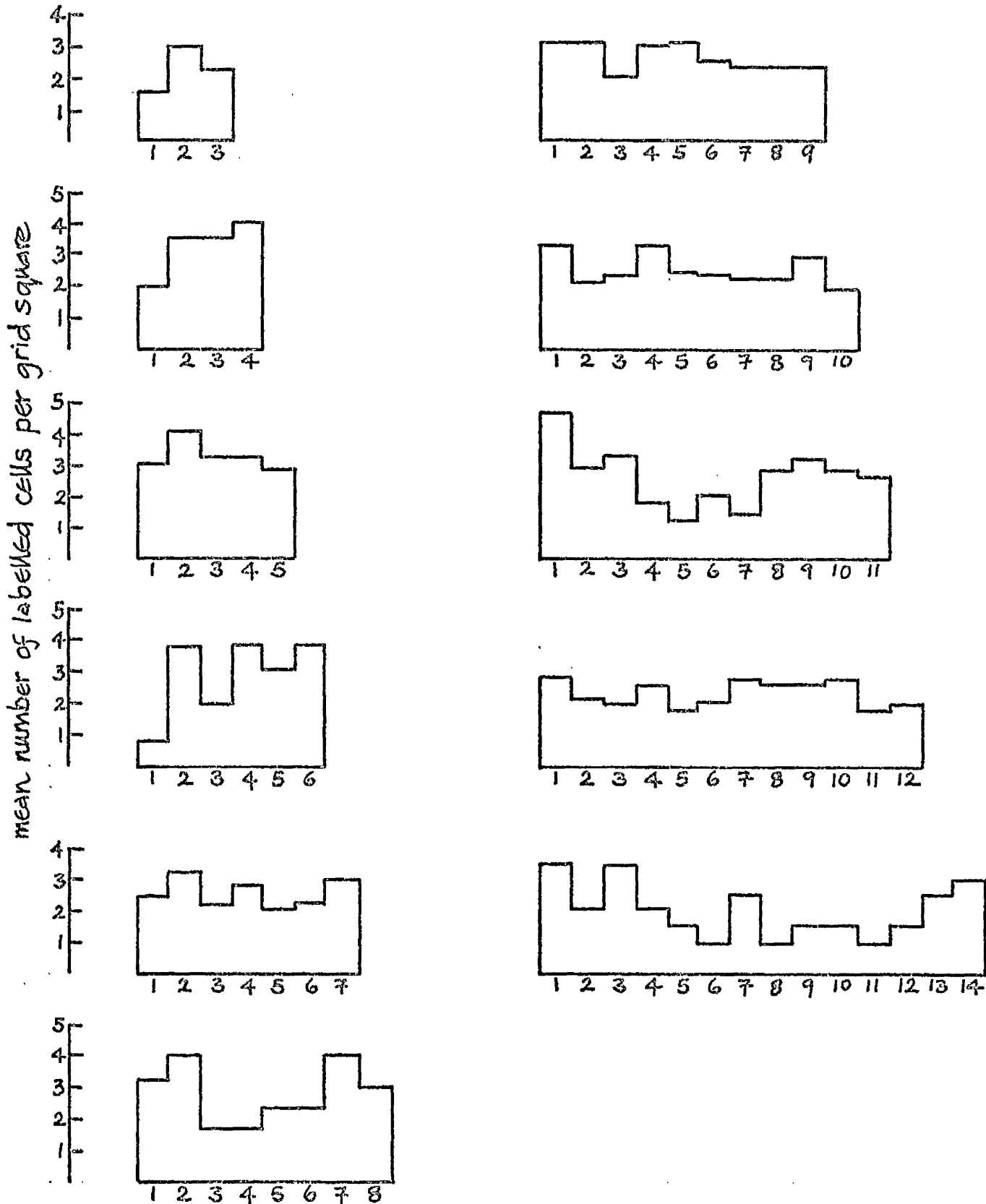
aggregate size
expressed as number of grid squares across aggregate diameter

In all aggregate sizes the distribution of "labelled" cells across the aggregates was not significantly different from that expected from a random arrangement across aggregates.

LB*H (EDTA disaggregated, reaggregated for 2 days in gyratory shaker).

Aggregate size (in square widths)	X^2	df	p	Significant difference +
3	13.40	2	$p < 0.001$	+
4	2.44	3	$p: 0.5 > p > 0.3$	−
5	8.83	4	$p: 0.1 > p > 0.05$	− at 5% level
6	15.32	5	$p: 0.01 > p > 0.001$	+
7	12.55	6	$p: 0.1 > p > 0.05$	− at 5% level
8	38.79	7	$p \ll 0.001$	+
9	31.02	8	$p \ll 0.001$	+
10	19.82	9	$p: 0.02 > p > 0.01$	+ at 5% level.

The distribution of "labelled" cells in the larger aggregate section (6 - 10 squares wide) is probably significantly different from a random distribution. In smaller aggregates (except 3 squares wide) the "labelled" cells are distributed more randomly. The mean number of "labelled" cells per square is lower in small aggregate sections than in large sections (see Histogram (1)).

H*LB (EDTA disaggregated, reaggregated for 2 days
in gyratory shakers)

Aggregate size (in square widths)	X^2	df	p	Significant difference ±
3	3.16	2	p: 0.3≫0.2	-
5	87.3	4	p≪0.001	+
6	104.8	5	p≪0.001	+
7	38.8	6	p≪0.001	+
8	90.5	7	p≪0.001	+
9	130.9	8	p≪0.001	+
10	32.4	9	p<0.001	+

In all sizes of aggregate sections (except the
smallest size) the "labelled" cells are distributed
across the aggregate in a manner significantly different
from random.

From these results, it is clear that in those
aggregates reaggregated for 2 days in gyratory shakers,
heart cells are positioned externally.

This method for determining the positioning of cell
types in aggregates formed after 2 days could be commented
on further. The number of "labelled" cells only, per
square has been counted. The aggregates were examined
at x400 magnification under dark field illumination so
that "labelled" cells only could be scored. It is
therefore assumed that the total number of cells per square
is fairly constant at x400 magnification. This assumption
was tested by measuring the total number of "labelled"
cells per square in aggregates of "labelled" limb bud
cells/

cells only, ignoring the percentage labelling effect.
The mean number of "labelled" limb bud cells per square
was found to be 10.01 (s^2 = 3.67). The percentage of
"labelled" cells in LB*LB (3) aggregates had been
determined by counts of target cells in segregation test I
as approximately 30%. The mean number of cells per
square in LB*LB (3) aggregates is 2.91. This suggests
that the distribution of "labelled" cells across aggregates
can give a fairly true indication of the positioning of
cell types. This suggestion is also borne out by the
reciprocal relation of the histograms of LB*H and H*LB.

II Aggregates formed after 2 hours reaggregation.

These aggregates are too small to be examined at
x400 magnification in the same way as aggregates formed
after 2 days. The number of complete squares fitting
across the widest part of the aggregates is small. The
distribution of cells across aggregates formed after 2 hours
was measured at x900 (oil immersion) magnification; the
side of a square being 11.4μ. The mean number of cells
possible per square was 5.78 (s^2 = 2.33) counting both
"labelled" and "unlabelled" cells under light field
illumination.

As the possible number of cells per square is
small and cells were counted under light field at this
magnification, the total number of cells per square was
scored according to the number "labelled" and "unlabelled".
The cell types were recognized by being "labelled" or
"unlabelled" with no corrections for percentage labelling.

Histograms/

Histograms were constructed of the percentage of "labelled" cells per square in widths across reciprocally "labelled" aggregates formed from cells disaggregated with EDTA and reaggregated for 2 hours, (see Histograms 4 and 5). The distribution of cell types across these aggregates was analysed by a χ^2 test. Contingency tables were drawn up for the total number of "labelled" and "unlabelled" cells per each square, thus giving tables with two rows of data. The variation of the proportion of "labelled" and "unlabelled" in squares of a transect can be tested against a null hypothesis that all squares have equal proportions of "labelled" and "unlabelled" cells, i.e. that the cells of both types are distributed randomly across the aggregate. The formula of Brandt and Snedecor was used (Bailey 1964). That no expected number was less than 1, was checked where necessary (Cochran 1954).

Results

LB*H

Aggregate size in squares x900	χ^2	df	p	Significant difference +
10	32.92	9	$p < 0.001$	+
8	37.99	7	$p < 0.001$	+
6	14.78	5	$p: 0.02 > 0.01$	+ at 5% level.
5	13.60	4	$p: 0.01 > 0.001$	+

The distribution of cell types across these aggregates is significantly different from random in probably all the aggregate sizes examined.

H*LB/

LB*H:cells disaggregated with EDTA,reaggregated for 2 hours.

Mean percentage labelled cells/square across aggregate sections of various diameters(6-15 samples/aggregate size class)
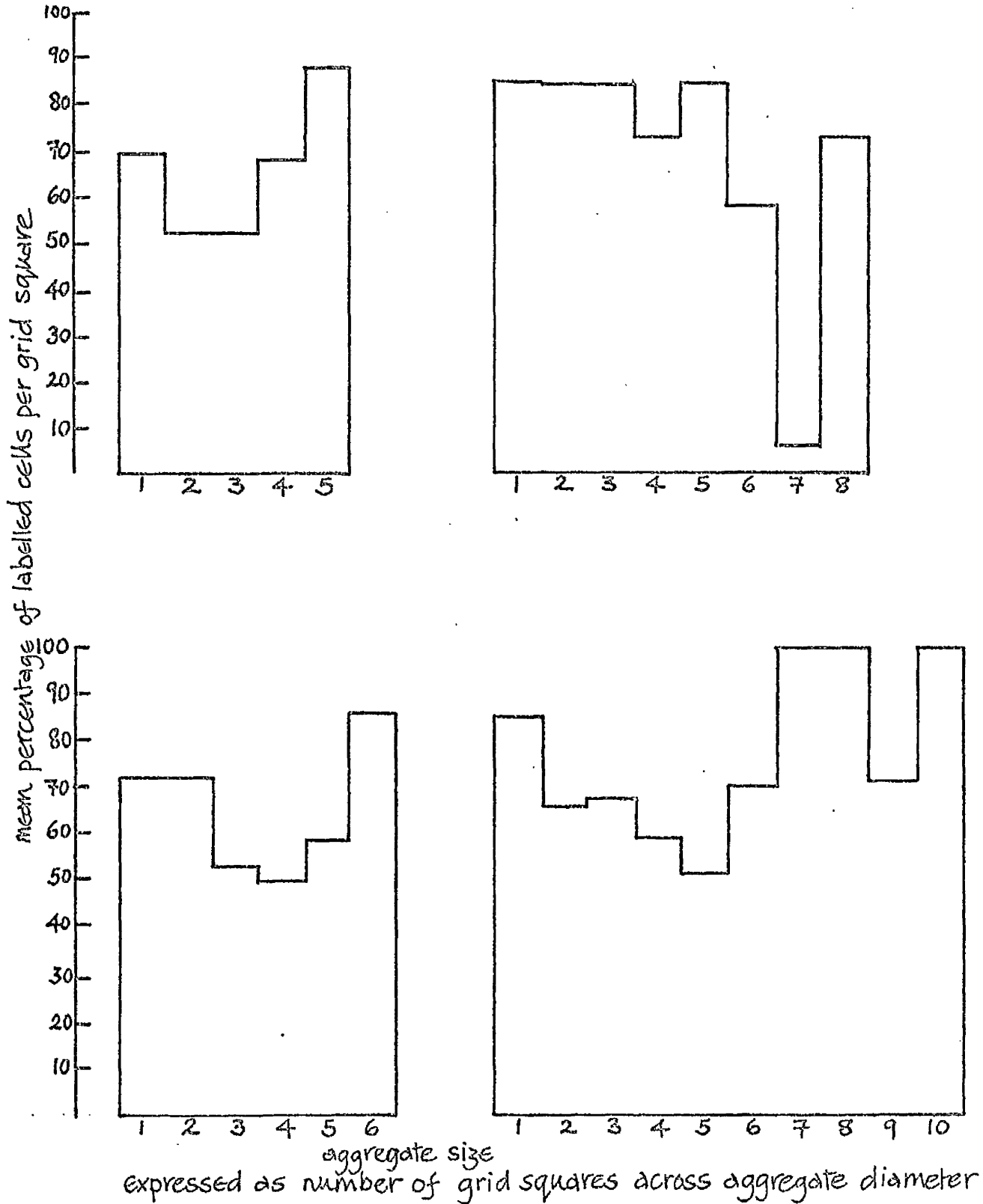
aggregate size
expressed as number of grid squares across aggregate diameter

HISTOGRAM 5

H*LB:cells disaggregated with EDTA,reaggregated for 2 hours.

Mean percentage labelled cells/square across aggregate sections of various diameters(4-6 samples/aggregate size class)

mean percentage of labelled cells per grid square
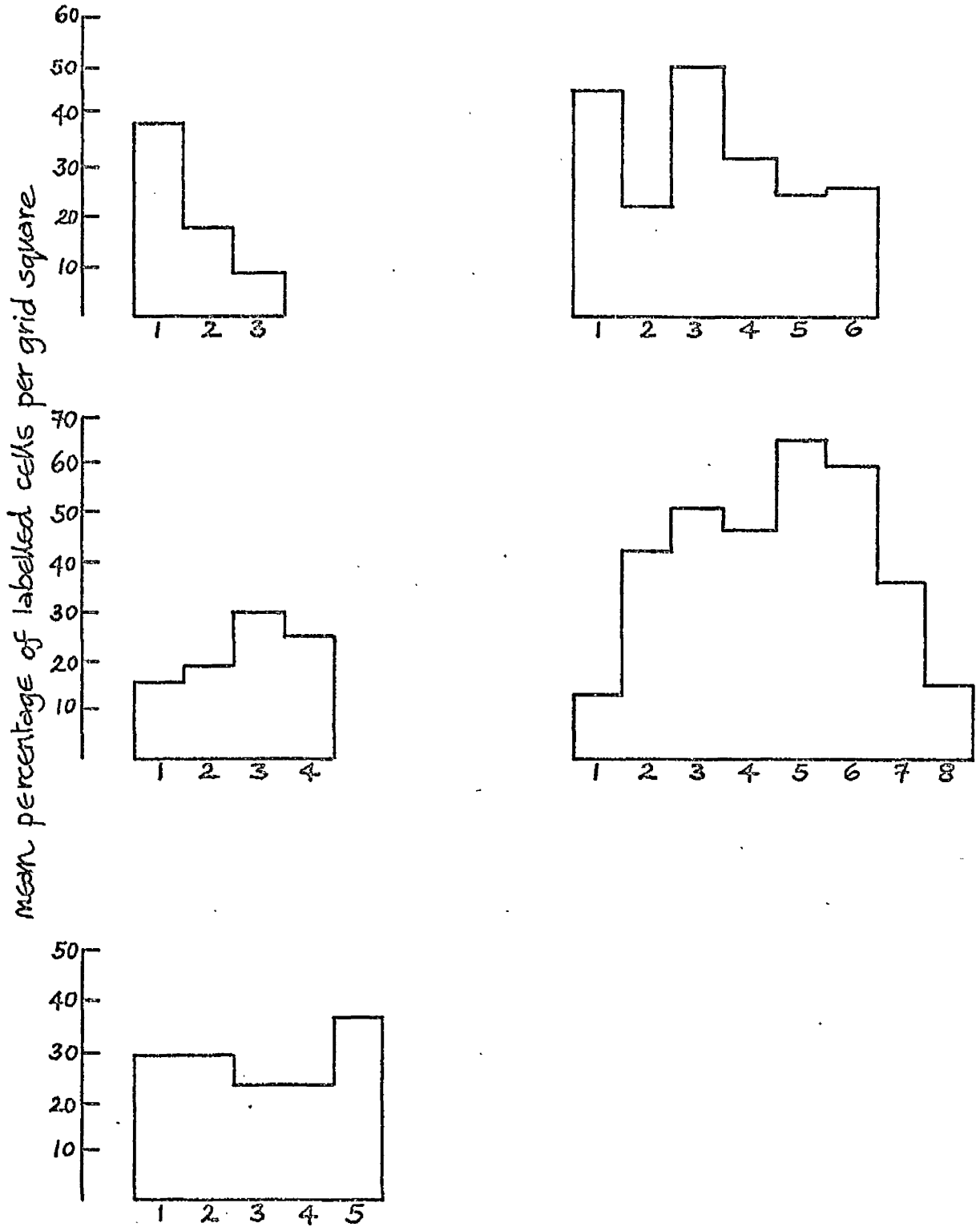
aggregate size
expressed as number of grid squares across aggregate diameter

H*LB

Aggregate size in squares x900	χ^2	df	p	Significant difference +
8	17.52	7	p: 0.02 > p > 0.01	+ at 5% level
6	10.77	5	p: 0.1 > p > 0.05	- at 5% level
5	3.15	4	p: 0.7 > p > 0.5	-
4	3.29	3	p: 0.5 > p > 0.3	-
3	7.83	2	p: 0.02 > p > 0.01	+ at 5% level.

The distribution of cells across these aggregates is not significantly different from random except in the largest and smallest aggregate sizes examined.

What conclusions can be drawn from the histograms and χ^2 tests on the distribution of cells across aggregates formed after 2 hours?

There is evidence from the segregation tests that the cells are grouped according to type. Here the positioning of the segregated cells has been examined in relation to the outside and inside of the aggregate in these reciprocally labelled aggregates formed by EDTA disaggregated cells after 2 hours reaggregation. In LB*H aggregates there is evidence that the two cell types, as recognized by being "labelled" or "unlabelled", are not evenly distributed across the aggregates but a similar general conclusion cannot be drawn from measures in H*LB aggregates. In the cases where the distribution of cell types is not random across the aggregates, it is difficult to assign a position to each cell type relative to the inside and outside of the aggregate (see Histograms 4 and 5). Tentatively limb bud cells may occur more frequently towards the periphery of the aggregates (see Histogram 4 and in Histogram 5 the aggregates of 8 squares width in which the distribution of cells is statistically different from/

from random). This tentative evidence is very interesting as
it suggests that the positioning of cell types in early
aggregates may be the reverse of that found in aggregates
produced after 2 days in gyratory shakers.

A problem of analysing positioning of cells in irreg-
ular aggregates has been mentioned by Adler (1970); in that
the geometric centre of the aggregate cannot be determined.
If the aggregates are spherical and similar in size, random
sectioning provides representative sampling of "levels"
within the aggregate that can be summated from many aggregates.
In other words, results from transects of aggregate sections
of the same width can be added up (as described). Sections
of irregular aggregates cut at random will give representative
sampling of these aggregates for segregation tests if the
grouping of cells in the aggregates is consistent and many
aggregates are sampled. The limitations of these effectively
non-random sections will be operative if transects across
sections of equal square width are summated.

eg

Serial sections of aggregate A

Serial sections of aggregate B, similar to A' but cut at right angles.
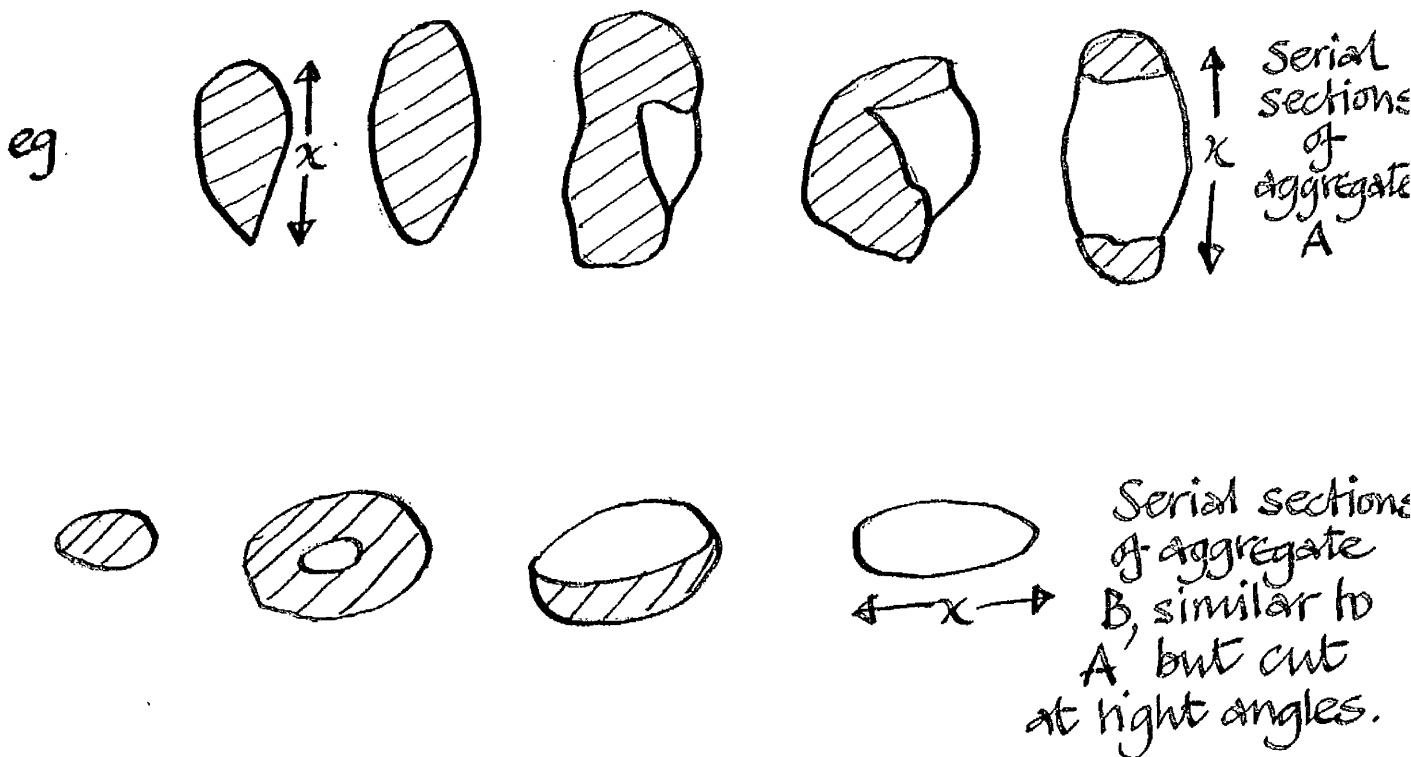
If transects of aggregate sections of size x are
summated any pattern will not be distinguished.

The problem of the scale of pattern seems to be one
which is concerned in the positioning of cells in these
small aggregates.   Segregation, as analysed in TEST I,
refers to groups of seven cells including the target cell.
No definite patterning was determined of these groups at
the higher level relative to the whole aggregate by the
method used but this may be due to the limitations imposed
by the irregularity of these early aggregates. That these
limitations may be operative is suggested by the evidence
that cells are not distributed randomly according to
type across the aggregates. However, the patterning may
not simply relate to the outside and inside of aggregates
and an analysis of the scale of pattern (Yarranton 1970)
might prove informative if small aggregates can be traced
through several sections. Adler (1970) has managed to
examine single early aggregates by employing embedding
and sectioning techniques of electron microscopy.

DISCUSSION

In the investigations presented here the arrangement of 5 day embryonic chick heart and limb bud cells in aggregates formed after a few hours (2 and 4 hours) reaggregation and approximately 2 days reaggregation have been analysed quantitatively. The main findings of this work are:

(1) In aggregates formed after a few hours reaggregation the cell types are markedly segregated. It should be clear from the description of the segregation tests that these take into account differences in the proportions of the two cell types in the aggregates and the results are therefore independent of proportions. In segregation test I the degree of segregation (α) relates directly to the two cell types, heart and limb bud, and corrects for the fact that "labelled" cell suspensions contain a small proportion of cells that have not taken up the label. There is tentative evidence that the positioning of heart cells in the groupoof aggregates analysed (disaggregated with EDTA and reaggregated for 2 hours) is internal.

(2) In aggregates formed after 42 hours reaggregation in reciprocating shakers the cell types are more randomly arranged than in aggregates formed in a few hours.

(3) The degree of segregation of cell types in aggregates formed by cells disaggregated with EDTA after 48 hours reaggregation in gyratory shakers is marked and not significantly different from that in aggregates formed by cells similarly disaggregated in a few hours. Evidence is presented that limb bud cells were positioned internally.

(4) The disaggregation procedures affect the degree of segregation of cell types in aggregates formed after 2, 4/

4 and 42 hours reaggregation.

The finding that in aggregates formed after a few hours reaggregation (this was carried out in couette viscometers) the cell types are markedly segregated contradicts the general view that two cell types initially form aggregates in which the cell types are randomly arranged (Moscona 1965, Burdick and Steinberg 1969, Roth 1968). The evidence for the existence of this first phase of randomly arranged cells in aggregates formed in rotation mediated aggregation systems has been discussed in the introduction. Adler (1970) is the only worker who has produced quantitative evidence on an aspect of "sorting out" that cells in aggregates formed after $1\frac{1}{2}$ hours are not positioned relative to the inside and outside of the aggregate. I have analysed "sorting out" with regard to two aspects, the grouping of cells according to type (segregation) and the positioning of cell types relative to the inside and outside of the aggregate in a few cases. Theoretically, without making any assumptions about how "sorting out" takes place it is clear that given segregation of cell types positioning of the types in a defined manner does not necessarily follow. However, if cell types are positioned in a definite manner relative to the inside and outside of the aggregate the cell types will also tend to be segregated. These points will be discussed further later.

How can the marked degree of segregation of cell types in aggregates formed after a few hours be interpreted?

The possibility that this segregation is due to clumps in the original cell suspensions has been eliminated by the use of cell sieves. Also the tentative evidence that/

that heart is positioned internally in aggregates formed
after 2 hours suggests that the segregation is not due
to the presence of cell clumps in initial cell suspensions.

Moscona (1962, 1965) has suggested that "sorting
out" can be explained on the theory that there are specific
mechanisms by which cells of different types (or species)
adhere (Specific Adhesion Theory). It has already been
pointed out in the introduction that this theory alone
cannot account for the defined positioning of cell types
in aggregates (see Steinberg 1970 for a recent catalogue).
Moscona (1965) estimated that there is a temporal lag
before cells exhibit specific adhesion of about 24 hours.
Roth (1968) in another model system has suggested that
the time lag before onset of specific adhesion is 4 hours,
although the existence of specific adhesion in this
system has been challenged by Curtis (1970).

From the results, cells, although adhering to both
like and unlike cells, are grouping according to type and
this is occurring 2 hours after disaggregation. This
suggests a shorter time lag of completely non-specific
adhesion than previously has been postulated on this
hypothesis. In addition as there is tentative evidence
that the cell types are "positioned" in these aggregates
the result could be interpreted on the differential
adhesion or timing hypotheses, which explain positioning
of cells in aggregates.

Steinberg's differential adhesion hypothesis (1964),
(1970) predicts that as soon as there is a choice of
adhesions the cells will "sort out" although he has not
made this point. It would seem therefore that the
finding of segregation of cells according to type in
aggregates/

aggregates formed after short time periods would fit this hypothesis. The internally segregating cell type is the most adhesive (Steinberg 1964) and from the results can be tentatively identified as heart cells after 2 hours disaggregation on this model.

The timing hypothesis of Curtis (1961, 1962) provides an alternative interpretation of the results. Heart cells, tentatively found to be internally positioned, aggregate first, being more adhesive initially. Later limb bud cells become adhesive and reaggregate on to the preformed clusters of heart cells. This would lead to segregation of the cells and "positioning". Reasons for the tentative nature of the evidence for "positioning" of cells in aggregates formed after 2 hours have already been discussed. However, there is an additional complication which is inherent in the interpretation of all the experiments reported here. I have indicated in the introduction that a distinction can be drawn between "sorting out" according to cell type and according to tissue type. Heart and limb bud are composed of several cell types, each of which may vary in adhesiveness, either quantitatively or differentially with time. This could lead to a complicated build-up of aggregates on the timing hypothesis for example.

In the results presented here there is some evidence that heart and limb bud cells in aggregates formed after 2 hours reaggregation are "positioned". Adler (1970) found no quantitative evidence for positioning of cell types from neural tube in aggregates formed after $1\frac{1}{2}$ hours reaggregation. The quantitative methods used here and by Adler have already been compared. The discrepancy in the results may be explained by the different cell types/

types used here and in Adler's experiments.

Comparing aggregates formed after 2 hours with those produced in 4 hours shows in some cases that the degree of segregation of the cells is less marked in the latter case. This argues that the segregation may not be due to "sorting out" during reaggregation on the differential adhesion hypothesis.

The timing hypothesis is attractive because it is possible to interpret the marked degree of segregation of cells in aggregates formed after a few hours without postulating that the cells are motile. However, segregation in early aggregates on Steinberg's hypothesis could be brought about by little gross movement but rather small displacements of cells and small clusters which have been observed in pelleted aggregates (Trinkaus and Lentz 1964).

To trace the time course of segregation during reaggregation from the results reported here is hampered for lack of data on how cells are arranged in aggregates formed during intermediate times of reaggregation between 4 hours and 42 hours. The aggregates produced after 42 hours are the result of complicated interactions between small aggregates. During this time in culture the aggregates become rounded (see also Steinberg 1970) and the cells more closely packed. Often the cells are spread along the edges of the aggregates. The interpretation of results is further complicated in that the aggregates have been prepared from cells reaggregated in three different rotation mediated aggregation systems. There are two alternative ways of looking at the results. It can be assumed that the patterning of cells found in aggregates formed in couette viscometers also occurs in/

in aggregates formed in reciprocating shakers and gyratory shakers after 2 and 4 hours. In other words, that the time course of patterning of cells is represented by the patterning observed in the aggregates analysed, irrespective of the way in which the cells were re-aggregated. Alternatively, the way in which aggregates are built up in the three reaggregation systems is different. There is evidence that this may be so in the case of reciprocating shakers and gyratory shakers, where the arrangement of cells in aggregates formed after approximately 2 days reaggregation is markedly different.

On Steinberg's hypothesis if the cells are segregated and positioned according to cell type in aggregates formed after 2 and 4 hours in reciprocating shakers, fusion and/or lack of motility of cells in aggregates might account for the non-segregation or small degree of segregation of cell types in aggregates formed after 42 hours reaggregation. Fusion of aggregates obviously complicates the situation.

However, in aggregates formed by gyratory shaker technique the cells are markedly segregated according to type and take up defined positions relative to the inside and outside of the aggregates. It should be noted however that there are mistakes in positioning. In this technique the rate of gyration is increased after 17 hours to prevent further fusion of aggregates (Steinberg 1962). For the remainder of the culture period the aggregates may therefore be cultured separately. Thus it would be possible for the cells to "sort out". This suggestion implies that in reciprocating shaker, "sorting out" by the differential adhesion theory cannot "keep pace" with the/

with the fusion of aggregates. The possible effects
of the medium in which the cells were reaggregated in
gyrating shaker flasks and contained embryo extract, will
be discussed later.

In aggregates formed after 48 hours reaggregation
in gyratory shakers the heart cells are positioned
externally. This result agrees with the positioning
reported by Steinberg (1962, 1970) for mixtures of limb
bud precartilage and heart cells in aggregates formed in
gyratory shakers and in hanging drop cultures. It might
be added that as far as I know all reported cases of
"sorting out" of disaggregated cells have been obtained
when the cells were reaggregated in gyratory shakers.
On the differential adhesion hypothesis (Steinberg 1964)
limb bud cells are the most adhesive 48 hours after EDTA
disaggregation. 2 hours after disaggregation it has
been tentatively concluded that heart cells were most
adhesive. This general finding lends support to the
timing hypothesis and is later discussed further. Heart
cells, if originally internal must migrate extensively
to take up an external position later. It could be
argued that the arrangement of cells in aggregates formed
in reciprocating shakers after 42 hours show an inter-
mediate stage, when both cell types are of equal
adhesiveness and the situation is complicated by prolonged
fusion of aggregates.

It is probably more likely however, that the way
in which aggregates are formed is not the same in the
three systems used. In couette viscometers a laminar
shear flow is established (Curtis 1970b) and the shear
rate and other parameters can be calculated (Curtis 1969).
The/

The shear rate is even over the whole container and thus the results obtained in this apparatus reflect the adhesiveness of the cells.   On reciprocating shakers the shear rate varies throughout the flask.   Roth and Weston (1967) found that the number of single cells collected by preformed aggregates varied directly with the size of the aggregate.   Thus it would appear that aggregates in this system can fuse with single cells or small aggregates.   It may be of some significance that aggregates formed after 42 hours in reciprocating shakers show more variable proportions of the cell types than aggregates formed after 48 hours in gyrating shakers, and are also more variable in size.

In gyratory produced aggregation however, a velocity gradient is set up across the flask.   Curtis (1970b) has suggested that when cells form aggregates in this system, the aggregates tend to move toward the centre of the flask where the flow rate is decreased. Roth and Weston (1967) also suggest that zoning could occur but that aggregates of large mass move towards the outside of the flask.   From the results I have concluded tentatively that heart cells become adhesive first and aggregate to form small clumps.   In gyratory shakers these clumps will tend to be distributed in a different zone in the flasks from single cells.   The limb bud cells become adhesive later and would tend to reaggregate on themselves.   Eventually the two populations of aggregates will be in the same zone of the flask.   If by this time the relative adhesiveness of the heart and limb bud has reversed it would be predicted on the Steinberg hypothesis that heart aggregates would spread by associative movement/

movement (Abercrombie 1967) over limb bud aggregates.
That aggregates can fuse in this way has been reported
by Steinberg (1970). After 17 hours the rate of gyration
was increased and further fusion of aggregates prevented.
It may be possible however that an increase in gyration
itself may initially cause aggregate fusion if the
aggregates are forced into contact at the centre of the
flask.

The other difference between the experiments
carried out in the gyratory shaker and those in recip-
rocating shaker is the presence of embryo extract in the
medium in the former. All media contain serum, which
Curtis (1967 for review) has suggested may affect the
adhesiveness of cell types differentially (Curtis 1965)
and could provide a basis for his timing hypothesis.
Curtis has also suggested that embryo extract may
similarly affect cells. It is conceivable that in the
presence of embryo extract limb bud cells may become
adhesive before heart cells. In this case, a reversal of
adhesiveness may not occur.

There is some evidence (Curtis 1970) that embryonic
chick liver and neural retina cells do reverse their
adhesiveness in the first five hours after disaggregation
with trypsin. Here the results may suggest a similar
reversal of adhesiveness of embryonic chick heart and limb
bud cells after disaggregation with EDTA.

In the experiments reported here the time course of
segregation is affected by the disaggregation procedure
used to produce the initial cell suspensions. In
aggregates formed after 2 hours by cells disaggregated
with EDTA, the cells are more segregated according to
type than in aggregates formed by cells disaggregated
with/

with the trypsin or the trypsin and EDTA procedures.

Roth and Weston (1967) and Roth (1968) used the trypsin procedure. Roth (1968) reports a temporal lag in the onset of adhesive selectivity of about 4 hours. The results here do show that cells disaggregated by his technique are more randomly arranged in aggregates formed after 2 and 4 hours than those disaggregated with either of the other two techniques. However, it is difficult to draw any conclusions from the results using different disaggregation techniques. It can be argued that cells disaggregated with EDTA are more altered than by any of the other disaggregation procedures, if initial segregation is considered an artefact of the disaggregation procedure; alternatively the reverse could be argued, that EDTA disaggregated cells are least altered by the disaggregation procedure.

The finding that, in aggregates formed after 42 hours in reciprocating shakers the disaggregation procedure affects the degree of segregation of cells is surprising. This suggests that the treatment of cells during disaggregation may have long term effects. An alternative attractive explanation is that different disaggregation procedures release different proportions of cell types from limb buds and hearts. However a similar proportion of "labelled" cells were released from labelled limb buds by each disaggregation procedure, which is a slight indication that this sort of selection may not be occurring in limb bud suspensions. Heart was not similarly investigated. The proportions of the tissue types in aggregates formed after 42 hours re-aggregation from cells disaggregated with the EDTA or with the/

the trypsin procedure were similar to the proportions of
cells initially mixed together. However, in similar
aggregates formed from cells disaggregated with the trypsin
and EDTA procedure there was a smaller proportion of heart
cells than expected from the initial proportions. Edo
(personnal communication) has also found this using the
same disaggregation procedure. It seems possible that
the trypsin and EDTA procedure may "select" a population
of heart cells. However, the proportion of heart cells in
aggregates formed after 48 hours reaggregation in gyratory
shakers by cells disaggregated with EDTA is also lower
than expected from the initial proportions of the tissue
types. This may be due to the way in which aggregates
are built up (see earlier) or to a burst of mitotic
activity in limb bud cells at 2 days in culture (Ede:
personnal communication).

The effect of disaggregating agents on the motility
of cells is interesting. Simms and Stillman (1937) have
found that trypsin treatment stimulates cell emigration
from various isolated adult tissue fragments and
Sigurdson (1942) reported an increase in the rate of
fusion of fragments of embryonic chick heart with trypsin
treatment. In preliminary experiments fusing embryonic
chick heart and liver fragments previously exposed to
EDTA, I have found individual cells recognized by
radioactive labelling of one tissue type in masses of
the other tissue type. It cannot be certain that these
cells have emigrated from the fused tissue mass of this
type. Individual cells at the edge of fragments may
become disaggregated during the shaking. This may
illustrate that the motility of cells may be affected
by disaggregating agents. Weston and Abercrombie (1967)
in/

in fusions of heart and liver pieces untreated with disaggregating agents found little individual movement of cells from one tissue to another.

There is little evidence that cells are able to migrate long distances in aggregates. All previous observations seem to indicate that cells may be displaced by competition between extensible psuedopodia (Trinkaus 1967). The results presented here can be explained without postulating long distance displacements of cells.

It can thus be seen that this work raises more questions than it answers but this quantitative approach should be able to solve the problems raised. Apart from analysing more stages during reaggregation in all three reaggregating systems used, the generality of these findings for other tissue type combinations should be substantiated. The use of cell types from clones for example rather than tissue types might clarify certain points. However, conditions have been partly elucidated which lead to "sorting out" as judged quantitatively. Here the cell types can be shown to segregate according to type and also take up defined positions within the aggregate. This can provide a control system for testing "sorting out" potential of cell types.

BIBLIOGRAPHY

ABERCROMBIE, M. 1946. Estimation of nuclear population from microtome sections.
Anat.Rec., 94, 239.

ABERCROMBIE, M. 1967. Contact Inhibition: The phenomenon and its biological implications.
National Cancer Monograph 26, 249-273.

ABERCROMBIE, M. and
HEAYSMAN, J.E.M.1953. Observations on the social behaviour of cells in tissue culture - I. Speed of movement of chick heart fibroblasts in relation to their mutual contacts.
Exp. Cell Res., 5, 111,-31.

ABERCROMBIE, M. and
HEAYSMAN,J.E.M. 1954. Observations on the social behaviour of cells in tissue culture - II. "Monolayering" of fibroblasts.
Exp.Cell Res., 13, 276-91.

ADLER, R. 1970. Changes in Reaggregation Pattern during neural tube differentiation.
Dev.Biol., 21, 403-423.

ANDERSON, N.G. 1953. The mass isolation of whole cells from rat liver.
Science, 117, 627-8.

AUERBACH, R. and
GROBSTEIN, C. 1958. Inductive interaction of embryonic tissue after dissociation and reaggregation.
Exp.Cell Res., 15, 384-397.

BAILEY, N.T.J. 1964. Statistical methods in biology. pp ix + 200.
The English Universities Press Ltd., London.

BASERGA, R. and
MALAMUD, D. 1969. Modern Methods in Experimental Pathology.
Autoradiography: Techniques and Application. pp. 265.
Hoeber Medical Division, Harper and Row, Publishers, Inc.

BLUMENSON, L.E. 1967. Dynamic adhesion and separation of cells in vitro. I. Mathematical analysis of the experimental system.
J.Cellular Physiol. 70, 7-22.

BONNER, J. T. 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mould. *Dictyostelium discoideum*. J.Exp.Zool., 106, 1-26.

BRESCH, D. 1955. Recherches preliminaires sur des associations d'organes embryonnaires de Poulet en culture *in vitro*. Bull.Biologique, 89, 179-88.

BULMER, D. 1959. Dimedone as an aldehyde blocking agent to facilitate the histo-chemical demonstration of glycogen. Stain Technol., 34, 95-98.

BURDICK, M.L. and STEINBERG, M.S. 1969. Embryonic cell adhesiveness: do species differences exist among warm-blooded vertebrates? Proc.Nat.Acad.Sci., Wash., 63, 1169-1175.

CAHN, R.D. and CAHN, M.B. 1966. Heritability of cellular differentiation: Clonal growth and expression of differentiation in retinal pigment cells *in vitro*. Proc.Nat.Acad.Sci., U.S., 55, 106-113.

CARTER, S.B. 1965. Principles of cell motility: the direction of cell movement and cancer invasion. Nature, Lond., 206, 1183-7.

CLARK, P.J. and EVANS, F.C. 1955. On some aspects of spatial pattern in biological populations. Science 121, 397-398.

COCHRAN, W.G. 1954. Some methods for strengthening the common χ^2 tests. Biometrics, 4, 417-451.

CURTIS, A.S.G. 1960. Area and Volume Measurements by random sampling methods. Med.Biol.Illustr., X, 261-266.

CURTIS, A.S.G. 1961. Timing mechanisms in the specific adhesion of cells. Exp.Cell Res.Suppl., 8, 107-22.

CURTIS, A.S.G.   1962.   Cell contact and adhesion.
                            Biol.Rev., 37, 82-129.

CURTIS, A.S.G.   1963.   The effect of pH and temperature
                            on cell reaggregation.
                            Nature, Lond., 200, 1235-6.

CURTIS, A.S.G.   1965.   Some interactions between cell
                            and medium in culture.
                            Arch.Biol.Liege, 76, 209-15.

CURTIS, A.S.G.   1967.   "The Cell Surface", pp. X + 405.
                            London: Logos Academic Press.

CURTIS, A.S.G.   1969.   The measurement of cell
                            adhesiveness by an absolute
                            method.
                            J.Embryol.Exp.Morph., 22, 305-25.

CURTIS, A.S.G.   1970.   On the occurrence of specific
                            adhesion between cells.
                            J.Embryol.Exp.Morph., 23, 253-272.

CURTIS, A.S.G.   1970b.   Problems and some solutions in
                            the study of cellular aggregation.
                            Symp.Zool.Soc.Lond. 25, 335-352.

CURTIS, A.S.G. and
  GREAVES, M.F.   1965.   The inhibition of cell aggregation
                            by a pure serum protein.
                            J.Embryol.Exp.Morph., 13, 309-26.

De HAAN, R.L.   1964.   Cell interactions and oriented
                            movements during development.
                            J.Exp.Zool., 157, 127-38.

EASTY, G.C. and
  MUTOLO, V.   1960.   The nature of the intercellular
                            material of adult mammalian
                            tissues.
                            Exp.Cell Res., 21, 374-85.

FISHER, R.A., and
  YATES, F.   1963.   Statistical Tables for biological,
                            agricultural and medical research.
                            X + 146.
                            Oliver and Boyd.

GALTSOFF, P.S.   1925.   Regeneration after dissociation.
                            (An experimental study on sponges)
                            - I. Behaviour of dissociated cells
                            of Microciona prolifera under
                            normal and altered conditions.
                            J.Exp.Zool., 42, 183-222.

GERISCH, G.   1960.   Zellfunktionen und zellfunktions-
wechsel in der entnicklung von
Dictyostelium discoideum – I.
Arch.entnicklung, 152, 632-54.

HORDER, A.   1958.
    Editor   The Ilford Manual of Photography.
Ilford Ltd., Essex.

HUMPHREYS, T. 1963.   Chemical dissolution and in vitro
reconstruction of sponge cell
adhesions – I.   Isolation and
functional demonstration of the
components involved.
Devel.Biol., 8, 27-47.

HUXLEY, J.   1921.   Differences in viability in different
types of regenerates from dissociated
sponges, with a note on the entry
of somatic cells by spermatozoa.
Biol.Bull., 40, 127-9.

LATHJA, L.G. and
    OLIVER, R.   1959.   The application of autoradiography
in the study of nucleic acid
metabolism.
Lab.Invest., 8, 214-224.

LeBLOND, C.P.,
    KOPRIWA, B.M. and   in "Histochemistry and Cytochemistry"
    MESSIER, B.   1963.   ed. Wegmann, R.
Pergamon, London.

LEVINE, S.   1960.   Effect of Manipulation on P^{32} loss
from tissue culture cells.
Exp.Cell Res., 19, 220-227.

LILIEN, J.E.   1968.   Specific enhancement of cell
aggregation in vitro.
Dev.Biol., 17, 657-678.

LUCEY, E.C.A. and
    CURTIS, A.S.G.1959.   Time-lapse film study of cell
reaggregation.
Med.Biol.Illus., 9, 86-93.

MARRABLE, A.W. 1962.   The counting of cells and nuclei
in microtome sections.
Quart.J.Micr.Sci., 103, 331-47.

MINTZ, M.   1964.   Formation of genetically mosaic
mouse embryos and early development
of "Lethal"-(t^{12}/t^{12})- "Normal"
Mosaics.
J.Exp.Zool., 157, 273-291.

MOORE, A.R.   1952. An animalizing effect of trypsin
                and its inhibition by lithium in
                developing eggs of _Strongylocentrotus_
                dtoobacluensis.
                J.exp. Zool., 121, 99-104.

MOSCONA, A.   1952. Cell suspensions from organ
                rudiments of chick embryos.
                Exp.Cell Res., 3, 535-539.

MOSCONA, A.   1956. Development of heterotypic combina-
                tions of dissociated embryonic
                chick cells.
                Proc.Soc.exp.Biol., N.Y., 92,
                410-16.

MOSCONA, A.   1957. The development _in vitro_ of chimeric
                aggregates of dissociated embryonic
                chick and mouse cells.
                Proc.Nat.Acad.Sci., Wash., 43,
                184-94.

MOSCONA, A.   1960. Patterns and mechanisms of tissue
                reconstruction from dissociated cells,
                From
                      "Developing Cell Systems and
                      their Control", edited D.Rudnick.
                Ronald Press, New York.

MOSCONA, A.   1961. Rotation-mediated histogenetic
                aggregation of dissociated cells.
                Exp.Cell Res., 22, 455-75.

MOSCONA, A.A.   1962. Analysis of cell recombinations in
                experimental synthesis of tissues
                in vitro.
                J.Cell Comp.Physiol., 60, Suppl.1,
                65-80.

MOSCONA, A.A. 1963a. Inhibition by trypsin inhibitors of
                dissociation of embryonic tissue by
                trypsin.
                Nature, Lond., 199, 379-80.

MOSCONA, A.A. 1963b. Studies on cell aggregation: demon-
                stration of materials with a
                selective cell-binding activity.
                Proc.Nat.Acad.Sci.Wash., 49, 742-7.

MOSCONA, A.   1965. Methods III B. Preparation of cell
                        suspensions p. 49-60,
                        and
                        "Recombination of dissociated cells
                        and the development of cell
                        aggregates." p. 489-531,
                        From "Cells and Tissues in culture"
                        Volume I. edited E.N. Willmer.
                        Academic Press.

MOSCONA A.A.,   1968. Cell aggregation: properties of
                        specific cell-ligands and their
                        role in the formation of multi-
                        cellular systems.
                        Dev.Biol., 18, 250-277.

MOSCONA, A.A. and
  MOSCONA, M.H. 1967.   Comparison of aggregation of
                        embryonic cells dissociated with
                        trypsin or versene.
                        Exp.Cell Res., 45, 239-43.

MOSCONA, M.H. and
  MOSCONA, A.A. 1966.   Inhibition of cell aggregation
                        in vitro by puromycin.
                        Exp. Cell Res., 41, 703-6.

MOSKOWITZ, M.   1963. Aggregation of cultured mammalian
                        cells.
                        Nature, Lond., 200, 854-6.

OKADA, T.S.   1965. Immunohistological studies on the
                        reconstitution of nephric tubules
                        from dissociated cells.
                        J.Embryol.Exp.Morph., 13, 299-307.

PANTIN, C.F.A. 1964.   "Notes on microscopical technique
                        for Zoologists" pp X + 77.
                        Cambridge University Press.

PAUL, J.   1965. Cell and Tissue Culture.
                        pp XI + 371.
                        E. & S. Livingstone Ltd.,
                        Edinburgh and London.

PEARSON, E.S. and   Biometrika Tables for statisticians.
  HARTLEY, H.O. 1966.   Volume I. xvi + 264.
  (Editors).   Cambridge University Press.

PETHICA, B.A.   1961. The physical chemistry of cell
                        adhesion.
                        Exp.Cell Res., Suppl., 8, 123-40.

PIELOU, E.C. 1961. Segregation and symmetry in two-species populations as studied by nearest neighbour relations. J.Ecol., _49_, 255-269.

PIELOU, E.C. 1962. Runs of one species with respect to another in transects through plant populations. Biometrics, _18_, 579-593.

RINALDINI, L.M. 1958. The isolation of living cells from animal tissues. Int.Rev.Cytol., _7_, 587-647.

RINGER, S. 1880. Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. J.Physiol., _3_, 380-393.

ROGERS, A.W. 1967. Techniques of Autoradiography pp. 338. Elsevier Publishing Company.

ROSENBERG, M.D. 1960. Microexudates from cells grown in tissue culture. Biophys.J. _I_, 137-159.

ROTH, S.A. 1968. Studies on Intercellular adhesive selectivity. Dev.Biol., _18_, 602-631.

ROTH, S.A. and WESTON J.A. 1967. The measurement of intercellular adhesion. Proc.Nat.Acad.Sci., Wash., _58_, 974-980.

ROUX W. 1894 Uber den "Cytotropismus" der Furchungszellen des grasfrosches. (_Rana fusca_). Arch.f.Entwickmech., _1_, 43-68.

SEARLS, R.L. 1967. The role of cell migration in the development of embryonic chick limb bud. J.Exp. Zool. _166_, 39-50.

SHAFFER, B.M. 1957. Aspects of aggregation in cellular slime moulds - I. Orientation and chemotaxis. Amer.Nat., _91_, 19-35.

SHEFFIELD, J.B. and MOSCONA, A.A. 1969. Early stages in the reaggregation of embryonic chick neural-retina cells.
Exp.Cell Res., 57, 462-466.

SIGURDSON, B. 1942. A new method for demonstrating cyto-antibodies in vitro.
Proc.Soc.Exptl.Biol. and Med. 50, 974-980.

SIMMS, H.S. and STILLMAN, N.P. 1937. Substances affecting adult tissue in vitro. I. The stimulating action of trypsin on fresh adult tissue.
J.Gen.Physiol. 20, 603-619.

SIMNETT, J.D. 1968. The measurement of mitotic incidence and radioautographic labelling index from tissue sections: some mathematical considerations.
J.Roy.Mic.Soc. 88, 371-382.

STEEDMAN, H.F. 1960. Section cutting in microscopy pp.172.
Oxford, Blackwell.

STEFANELLI, A. and ZACCHEI, A.M. 1958. Sulle modalita di aggregazione di cellule embrionali di pollo disgregate con tripsina.
Acta Embryol.Morph.Exp., 2, 1-12.

STEFANELLI, A., ZACCHEI, A.M., and CECCHERINI, V. 1961. Ricostituzioni retiniche in vitro dopo disgregazione dell'abbozzo oculare di embrione di pollo.
Acta.Embry.Morph.Exp. 4, 47-55.

STEINBERG, M.S. 1958. On the chemical bonds between animal cells: a mechanism for type-specific association.
Amer.Nat., 92, 65-82.

STEINBERG, M.S. 1962. On the mechanism of tissue reconstruction by dissociated cells - I. Population kinetics, differential adhesiveness and the absence of directed migration.
Proc.Nat.Acad.Sci., Wash., 48, 1577-82.

STEINBERG, M.S. 1963. "ECM"; its nature, origin and function in cell aggregation.
Exp.Cell Res., 30, 257-79.

STEINBERG, M.S. 1964. The problem of adhesive selectivity in cellular interactions. From "Cellular membranes in development", edited M.Locke. Academic Press, New York, 321-66.

STEINBERG, M.S. 1970. Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. J.Exp.Zool. 173, 395-434.

STILLSTRÖM, J. 1963. Grain Count corrections in autoradiography. Internat.J.Appl.Radiat.Isotopes, 14, 113-118.

TOWNES, P.L. 1953. Effects of proteolytic enzymes on the fertilization membrane and layers of the amphibian embryo. Exp.Cell Res., 4, 96-101.

TOWNES, P.L. and HOLTFRETER, J. 1955. Directed movements and selective adhesion of embryonic amphibian cells. J.Exp.Zool., 128, 53-120.

TRINKAUS, J.P. 1961. Affinity relationships in heterotypic cell aggregates. Colloques.Internat.C.N.R.S., 101, 209-25.

TRINKAUS, J.P. 1963. Behaviour of dissociated retinal pigment cells in heterotypic cell aggregates. Ann.N.Y.Acad.Sci., 100, 413-34.

TRINKAUS, J.P. 1966. Morphogenetic cell movements in "Major Problems of Developmental Biology" edited M. Locke. The 25th Symposium of the Society for Developmental Biology. New York, Academic Press, p.125-176.

TRINKAUS, J.P. 1969. Cells into Organs: The forces that shape the embryo. XVI + 237p. Prentice-Hall, New Jersey.

TRINKAUS, J.P. and GROSS, M.C. 1961. The use of tritiated thymidine for marking migratory cells. Exp.Cell Res., 24, 52-7.

TRINKAUS, J.P. and
LENTZ, J.P.   1964.   Direct observation of type-specific
                           segregation in mixed cell aggreg-
                           ates.
                           Devel.Biol., 2, 115-36.

TRINKAUS, J.P. and
MONAHAN   1969   reported in
                           Trinkaus (1969) Cells into Organs.

WEISS, P. and TAYLOR,   Reconstitution of complete organs
A.C.   1960.   from single cell suspensions of
                           chick embryos in advanced stages of
                           differentiation.
                           Proc.Nat.Acad.Sci., Wash., 46,
                           1177-85.

WESTON, J.   1967.   Cell Marking in "Methods in
                           Developmental Biology" edited by
                           F. Wilt and N.K.Wessells.
                           N.Y., Crowell.

WESTON, J.A. and   Cell mobility in fused homo- and
ABERCROMBIE, M. 1967.   heteronomic tissue fragments.
                           J.Exp.Zool., 164, 317-324.

WHITTAKER, J.R. 1963.   Changes in melanogenesis during
                           the dedifferentiation of chick
                           retinal pigment cells in culture.
                           Dev.Biol., 8, 99-127.

WILDE, C.E., Jr. 1959.   Differentiation in response to
                           the biochemical environment. In
                           "Cell, Organism and Milieu"
                           ed. D. Rudnick. Ronald Press, New
                           York.

WILLMER, E.N.   1945.   Growth and form in tissue cultures.
                           From "Essays on Growth and Form"
                           edited W. Le Gros Clark.
                           Oxford University Press, New York.

WILLMER, E.N.   1960   "Cytology and Evolution".
                           Academic Press, New York.

WILSON, E.V.   1907.   On some phenomena of coalescence
                           and regeneration in sponges.
                           J.Exp.Zool., 5, 245-58.

YARRANTON, G.A. 1970.   Pattern Analysis by regression.
                           Ecology 50, 390-395.

ZWILLING, E.   1954.   Dissociation of chick embryo cells
                           by means of a chelating compound.
                           Science, 120, 219.

ZWILLING, E.   1959. A modified chorioallantoic
                          grafting procedure.
                          Trans. Bull. 6, 115-116.

ZWILLING, E.   1963. Survival and non-sorting of nodal
                          cells following dissociation and
                          reaggregation of definitive streak
                          chick embryos.
                          Devel.Biol., 7, 642-52.

ZWILLING, E.   1968. Morphogenetic Phases in development
                          184-207
                          in "The Emergence of Order in
                          developing Systems". Ed. M. Locke.
                          27th Symposium for the Society for
                          Developmental Biology.