Patterns of cell division, cell death and chondrogenesis in cultured aggregates of normal and talpid<sup>3</sup> mutant chick limb mesenchyme cells

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**SUMMARY**

Aggregates were prepared from dissociated mesenchyme cells obtained from normal and talpid<sup>3</sup> mutant chick limb buds at stage 26 and were maintained for 4 days in culture. They were shown by autoradiographic techniques to consist initially of populations of uniformly dedifferentiated cells within which chondrogenesis was initiated between 1 and 2 days, leading to the formation of areas of precartilage in the interior of the aggregates.

Measurements of cell population density, cell death and cell division were made in precartilage and non-cartilage regions on sections prepared from normal and mutant aggregates fixed at 1-day intervals and were related to the pattern of chondrogenesis. Non-cartilage areas consisted of cells surrounding the precartilage areas and extended to the surface of the aggregate; these cells showed no special pattern or histochemical reaction. Precartilage areas consisted of one or more 'condensations', comprising cells arranged in concentric rings around a central cell or group of cells, characterized by uptake of [<sup>35</sup>S]sulphate and taking up alcian blue stain in the intercellular matrix. Chondrogenesis was initiated at the condensation foci and spread centrifugally.

Condensations were arranged in a simple pattern, roughly equidistantly from each other and never at the surface of the aggregate. The shape and arrangement of the cells comprising them suggested that they were formed by a process of aggregation towards the condensation foci. The relation of these observations to events in the intact limb bud developing in vivo is discussed.

**INTRODUCTION**

Ede & Agerbak (1968) have used the reaggregation technique of Moscona (1961) to study differences in the adhesive properties of mesenchyme cells taken from limb buds of normal and talpid<sup>3</sup> mutant chick embryos. Those studies were concerned only with differences in the size and shape of the resulting aggregates, but the latter can also be used as small quasi-embryonic model systems in which various aspects of differentiation may be studied at a level of organization which is conveniently intermediate between that of the embryonic limb developing in vivo and of cells dispersed in monolayer culture. Such observations may be

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expected to give information about simple component processes of differentiation and pattern formation which on the one hand are obscured by complex regional differences in the intact developing limb and which, on the other, are not expressed in tissue culture conditions. In the studies reported here observations on mitosis, cell death, cell density, cell orientation and cartilage matrix production in normal chick limb aggregates are described and compared with corresponding observations on aggregates prepared from *talpid* mutant in which the form of the limbs and the pattern of chondrogenesis is greatly altered (Ede & Kelly, 1964; Hinchcliffe & Ede, 1967).

**MATERIALS AND METHODS**

*Talpid* heterozygote fowls were mated and wing buds obtained from the resulting *talpid* (ta3/ta3) and normal (ta3/ta3 and +/+) embryos at 5 days of incubation (stage 26). Aggregates were prepared from the dissociated limb mesenchyme cells, using the techniques described by Ede & Agerbak (1968), and were maintained in the culture medium from 1–4 days in a 5% CO₂ gas incubator at 37.5 °C. Medium-sized aggregates (0.5–1.0 mm) were fixed at daily intervals in Bouin, sectioned at 8 μm and stained variously in haematoxylin and eosin, toluidine blue and alcian blue/chlorantine fast red (AB/CFR).

The total area of whole aggregate sections stained with AB/CFR, and of the precartilage (AB-staining) areas within them, was measured by making camera lucida drawings at ×125 magnification on standard paper, cutting out and weighing the areas representing the differently staining regions, and finally calculating their size by comparison with the weight of a known area drawn at the same magnification.

Estimates of cell density and, by calculation, total cell numbers for each region were obtained by counting all nuclei within sample areas of 784 μm², using an eyepiece graticule at ×950 magnification. With rare exceptions, cells were uninucleate so that each nucleus could be taken as representing a single cell. Abercrombie's correction factor (Abercrombie, 1946) for inclusion of neighbouring sections was used; section thickness was confirmed by focusing through each section and using the gradations on the micrometer fine-focusing screw; nuclear size was estimated from measurements of the longest and shortest diameter of each nucleus in the sample areas.

Indices for mitoses and dead cells were found by making camera lucida drawings of whole or, if this exceeded the optical field, large parts of aggregate sections at ×500 magnification, marking mitotic and pycnotic nuclei for counting on the drawing, indicating precartilage areas where present, and obtaining the area of the different regions so drawn by the weighing method. The number of cells in each region was obtained as described above and the proportion of mitosing and dead cells calculated. To avoid counting pycnotic fragments as separate nuclei, each small cluster of fragments was counted as
representing a single pycnotic nucleus. The proportion of dead cells is expressed as a percentage of the total cell number and the mitotic index represents the number of cells at any phase of mitosis per 1000 cells.

Autoradiographic techniques were used to label dissociated cells in some cases in order to trace their subsequent fate in aggregates and also to follow the development of precartilage areas as indicated by chondroitin sulphate synthesis.

For labelling, windows were made in the shell over 4-day normal embryos and 50μCi/ml[^3H]thymidine in sterile saline (Amersham Radiochemical Laboratory) injected into the amniotic cavity. Each egg was sealed with Sellotape and incubated for a further 24 h, after which the wing mesenchyme was used in preparing aggregates. The fixed aggregates were sectioned at 8 μm, stained with haematoxylin and eosin or AB/CFR and coated in Ilford L4 emulsion (dilution, 1 emulsion:2dw). Sections were exposed for 14 days at 4 °C and developed in Kodak Dolmi developer.

For following chondroitin sulphate synthesis the culture medium was supplemented with 5 μCi/ml sodium [35S]sulphate (Amersham Radiochemical Laboratory). Sections were stained and coated with emulsion as above, exposed at 4 °C for 28 days and developed in Dolmi developer.

**RESULTS**

**The development of precartilage areas**

Regions showing distinct blue staining with AB/CFR, indicating the presence of mucopolysaccharide in the intercellular matrix, are defined here as ‘pre-cartilage areas’. Initially, though light intercellular staining can be seen throughout 1-day aggregates, there is no sign of the stain being taken up preferentially in any region. At 2 days definite precartilage areas are seen in which the staining is more intense (Fig. 1A, B). Each precartilage area is made up of one or more subregions which we define as ‘condensations’, in which the cells are orientated in concentric rings around a central cell or group of cells (Fig. 1C, D). Mucopolysaccharide staining is most intense at the centre of each condensation and becomes less towards the periphery, and at the centre the cells are rounded while those towards the periphery become increasingly flattened and crescent-shaped. Condensations in *talpid* are less well defined than in normal aggregates, though their general configuration is the same. Whereas in normal condensations the arrangement of the cells in concentric rings is emphasized by the separation of adjoining rings by intercellular matrix, especially towards the centre, the cells of adjacent rings in *talpid* condensations are much less clearly separated.

Percartilage areas are never found at the periphery but always in the interior of aggregates, and they are generally distributed roughly equidistantly from each other. They expand during the period in culture and the larger areas found at 3 and 4 days must have arisen partly by fusion of neighbouring areas. At
Fig. 1. Aggregates at 2–4 days, sectioned at 7 μm and stained with alcian blue/chlorantin fast red (A) or haematoxylin and eosin (B–G). (A) Normal, 2 days, ×125; (B) normal, 2 days, ×125; (C) talpid², 2 days, ×125; (D) normal, 2 days, ×500; (E) talpid², 2 days, ×500; (F) normal, 4 days, ×125; (G) talpid², 4 days, ×125.
Chick-limb mesenchyme in culture

4 days they fill most of the aggregate, and the non-cartilage cells immediately surrounding them are characteristically arranged as a perichondrium of fibroblast-like cells, 2–4 cells thick (Fig. 1E, F). Initially, at 2 days, the total area of precartilage expressed as a percentage of the total aggregate area is less in normal (15·1%, s.d. = ±11·6) than in talpid³ (33·4%, s.d. = ±11·2) aggregates, but its relative expansion is much more rapid so that at 3 days approximately half of the aggregate is taken up by precartilage in both normal (49·5%, s.d. = ±15·0%) and talpid³ (51·9%, s.d. = ±11·6%), while at 4 days 76·9%, s.d. = ±10·8%, of the normal aggregate consists of precartilage and only 55·8%, s.d. = ±7·8%, in talpid³.

The relation between precartilage areas and condensations is not identical in normal and talpid³ aggregates. The histograms in Fig. 2 represent measurements made on 28 normal and 16 talpid³ aggregate sections collected in 4 experiments at 2 days, with precartilage areas classed according to the number of condensa-

![Graph A](image1.png)

Frequency % (log scale)

No. of condensations per precartilage area

![Graph B](image2.png)

Average size of precartilage area (µm x 10⁷)

No. of condensations per precartilage area

Fig. 2. Relation between condensations and precartilage areas in normal (white strips) and talpid³ (black strips) aggregates at 2 days. (A) Frequency of precartilage areas made up of 1, 2, ..., n condensations; (B) relation between size of precartilage area and number of condensations per precartilage area.
Fig. 3. Autoradiograph sections showing uptake of \[^{35}S\]sulphate (A–C) and distribution of \[^{3}H\]thymidine labelled cells from non-cartilage regions of 5-day limb-bud mesenchyme among unlabelled cells from precartilage regions (D, E) in aggregates at 1–3 days (× 125). (A) Normal, 2 days; (B) normal, 3 days; (C) talpid\(^3\), 3 days; (D) normal, 1 day; (E) normal, 3 days.

Histogram A shows the percentage of times each class was observed and indicates that in both normal and talpid\(^3\) aggregates 50\% of the precartilage areas consisted of a single condensation. But whereas less than 1\% of normal precartilage areas include more than five condensations and none more than seven, a significant percentage of talpid\(^3\) precartilage areas include more than seven condensations and some as many as 15. Histogram B shows the
average size of the precartilage area for each class. The largest areas are found in *talpid*³ aggregates because of the occurrence of areas made up of high numbers of condensations in some cases, but areas made up of the same number of condensations are much larger in normal aggregates. By calculation from these results, the average amount of precartilage produced by a single condensation at 2 days is 0·34 (s.d. = ±0·07) × 10⁴ μm² in *talpid*³ and 0·60 (s.d. = ±0·06) × 10⁴ μm² – about ×2 as much – in normal aggregates.

**Uptake of sodium [³⁵S]sulphate**

The formation and development of the precartilage areas as indicated by chondroitin sulphate synthesis conforms with the histological observations: in 1-day aggregates silver grains are scattered lightly over the whole section and cannot be distinguished from background; at 2 days there is an increased density of grains in the precartilage areas, greatest at the centre (Fig. 3A); at 3 and 4 days the density of grains over the whole of each precartilage area is high. No difference in ³⁵S uptake was detected between normal and *talpid*³ aggregates.

**Origin of cells forming precartilage areas**

Precartilage differentiation has begun in embryonic wing buds at stage 26, so it is important to know whether or not the chondrogenic cells maintain their differentiated state and sort out to form the precartilage areas in the aggregates. For this purpose normal embryos were labelled with [³H]thymidine as described above and only the central mesenchyme, i.e. the chondrogenic portion, of the wing buds was used in the experiment. These cells were dissociated and mixed with an approximately equal number of non-chondrogenic cells from the peripheral region of wing buds from unlabelled normal embryos. Aggregates were fixed at 1, 2 and 3 days, sectioned and processed. There was no evidence of sorting out: at 1 day labelled cells are scattered at random throughout the aggregate (Fig. 3B) and at 2 and 3 days, though there is a slightly more patchy distribution of labelled cells which may be due to differential dilution by cell division, labelled and unlabelled cells are found distributed in roughly equal numbers through both precartilage and non-cartilage areas (Fig. 3C).

**Cell density**

Cell density here refers to number of cells per unit area, but since we have no accurate measure of cell size this cannot necessarily be equated with packing density, i.e. there may be more or less space between cells at the same density according to whether their cytoplasmic volume is larger or smaller. However, we have no reason to suppose from our histological observations that cytoplasmic volume varied greatly in the course of the experiment and we believe that cell density here probably does reflect packing density. Nuclear size, as measured by averaging the longest and shortest diameters, remained constant at approximately 5·8 μm in all regions of both normal and *talpid*³ aggregates.
Fig. 4. Changes in cell density (A, B), cell death (C, D) and mitosis (E, F) in normal (A, C, E) and *talpid*² (B, D, F) aggregates from 1-4 days. Squares = non-cartilage; triangles = precartilage; circles = average over non-cartilage and precartilage.
Total cell size of the dissociated cells at the beginning of the experiment was approximately 8.7 μm in both normal and talpid3.

Cell density changes are shown in Fig. 4A, B. At 1 day cell density is slightly greater in talpid3 than in normal aggregates. At 2 days this difference in the overall density, obtained at this stage by averaging precartilage and non-cartilage area counts, is more marked and variance tests show it to be clearly significant (P = 0.05).

At 2 days, when the distinction between precartilage and non-cartilage is established, differences appear between these regions. In normal aggregates cell density in the cartilage areas drops steeply to 2 days, levels out to 3 days, then drops steeply again to 4 days; in non-cartilage regions there is also a drop, but not so steep, to 2 days, after which the density remains constant. In talpid3 aggregates cell density in the cartilage areas drops steadily throughout the culture period, finally reaching the same level as in normal precartilage at 4 days; in non-cartilage regions cell density remains approximately constant until 3 days, where there is a slight rise, after which it drops steeply to below the level in normal non-cartilage at 4 days.

Cell death

Viability of normal and talpid3 cells, indicated by eosin staining immediately after dissociation, was approximately 95%. The pattern of cell death found in aggregates is shown in Fig. 4C, D.

Normal aggregates at 1 day include approximately 25% dead cells, but this percentage is sharply reduced in the precartilage areas to about 3% at 3 days and remains at this level at 4 days. In the non-cartilage regions there is a steady but less dramatic reduction to about 11% at 4 days.

Talpid3 aggregates at 1 day include only about half as many, approximately 12%, dead cells. As in normals, the number in the precartilage areas declines rapidly to 3 days, and this reduction continues to complete absence of dead cells at 4 days. Also as in normals, there is a much less dramatic reduction of cell death (except between 1 and 2 days, where it appears to be steeper, but this may not be statistically significant) in the non-cartilage regions, to about 7% at 4 days.

Analysis of variance tests made on the averaged cell death for each day show the daily drop in cell death and the initial difference between normal and talpid3 counts to be highly significant.

Since the anterior and posterior necrotic zones found in normal wing buds are absent from talpid3 embryos (Hinchliffe & Ede, 1967) it was important to establish that the relatively much higher numbers of dead cells found in normal aggregates did not arise by inclusion of dead and dying cells from these regions. For this purpose counts were made on aggregates prepared from normal wing buds from which the necrotic zones had been excised. No significant difference was found between operated and control embryos.
Since precartilage areas occur only in the interior of aggregates it might be proposed that differential counts of cell death might represent differences due directly to the position of cells within the aggregate rather than differences due to their being included in one or other of precartilage or non-cartilage regions, but this is not the case. Fig. 5 is a camera lucida drawing of a typical 2-day normal aggregate section, with the precartilage area outlined and dead cells and mitoses marked in. It shows the relatively low proportion of dead cells in the precartilage area and also indicates that the distribution of dead cells is not related to distance from the periphery or any other feature of the aggregate as a whole.

**Cell division**

The pattern of mitosis in aggregates is shown in Fig. 4E, F, where the mitotic index represents the number of cells in any phase of mitosis per $10^3$ cells.

In normal aggregates there is a peak of mitosis at 2 days, which is much higher in the precartilage areas, followed by low levels of mitosis at 3 and 4 days in both precartilage and non-cartilage regions.

In talpid³ aggregates the initial level of mitosis at 1 day is similar to normals. There is a peak in the precartilage areas at 2 days, but much lower than that found in normals, followed by low levels at 3 and 4 days. In the non-cartilage regions there is no peak at 2 days, but thereafter there is a slow rise, with the highest level at 4 days, rather than a decline to lower levels.

**DISCUSSION**

*Sequence of events in aggregates*

Each aggregate consists initially of a population of uniformly dedifferentiated cells: labelled cells from the precartilage region of the limb bud reaggregated
with unlabelled cells from the non-cartilage region are subsequently found randomly distributed between precartilage and non-cartilage in the cultured aggregates, indicating that dedifferentiation has occurred during cell dissociation and re-association, followed by a new differentiation within the aggregates.

No visible cell differentiation occurs during the first day, but initiation of chondrogenesis must occur during the course of the second, since at 2 days $^{35}$S-labelling and alcian blue staining indicate secretion of precartilage mucopolysaccharide in localized areas, defined as condensations, in which the cells are arranged in concentric rings around a central cell group where staining is most intense. The decreasing gradient of stain intensity towards the periphery suggests that initiation of chondrogenesis must have occurred at the condensation centre.

Condensations expand by inclusion of more and more of the undifferentiated cells at their periphery and by 2 days several neighbouring condensations have often become merged to form areas of precartilage with multiple condensations. Precartilage areas in turn expand as a result of this process and also by the cells of the condensations being forced apart by secretion of cartilage matrix into the extracellular spaces between them. In later stages a well-defined perichondrium is formed from the mesenchyme cells surrounding each precartilage area, which must to some degree resist further expansion.

**Cell density**

When dissociated cells are reaggregated they adhere very closely together but in the course of time they become more like the mesenchyme from which they were derived, with cytoplasmic processes maintaining contact between cells which are now separated by intercellular spaces. It is this process which must account for the steep drop in overall cell density in normal aggregates between 1 and 2 days. In the condensations the cells are further separated by the secretion of mucopolysaccharide matrix, which accounts for the steep drop in cell density in the cartilage areas of normal aggregates (though the levelling off between 2 and 3 days is puzzling).

In *talpid3* aggregates there is no decrease in cell density in the non-cartilage regions, and this may be related to the observation by Ede & Agerbak (1968), confirmed experimentally by Ede, Flint & Curtis (unpublished), that *talpid3* cells are more adhesive to each other than normal cells. In the precartilage areas of aggregates Ede & Agerbak found that the mutant cells are united to each other by more extensive regions of cytoplasm. Here we observe that at 2 days the rings of precartilage cells in the condensations are not clearly separated and that the condensations do not expand so much as in normal aggregates; it appears that the close adhesion of *talpid3* cells to each other inhibits the intercellular deposition of matrix, possibly by feedback suppression.
Cell death

The aggregates at 1 day include a high proportion of dead cells which will almost all have been produced by injury during the dissociation process and subsequently by conditions in culture. The count of dead cells declines in succeeding days, indicating that dead cells are eliminated in some way, but it is unlikely that no subsequent cell death occurs. Dead cells are removed, probably as seems most likely from work by Fallon & Saunders (1968) and Ballard & Holt (1968) on the intact chick and rat limb bud – by macrophages differentiated from neighbouring mesenchymal cells. The proportion of dead cells at any stage depends on the efficiency of their removal and the rate at which cells become necrotic.

In normal aggregates the decline in cell death is greater in precartilage than in non-cartilage areas. Since precartilage areas never occur at the periphery of aggregates it might be argued that this represents a differential distribution relative to the surface of the aggregate, but in fact there is no gradient of cell death from the surface to the centre and in any case precartilage areas rarely occur exactly centrally. The difference is therefore due not to the position of the precartilage but to some special property of the chondrogenically differentiated cells which leads either to more efficient mopping up of dead cells by macrophages or to occurrence of less cell death among them.

At 1 day the number of all dead cells is much lower in talpid than in normal aggregates; by 4 days there are no dead cells at all in talpid precartilage areas and though the decline is not so steep in non-cartilage areas the number of dead cells here is still significantly less than in normal aggregates. Either macrophage activity is more efficient in talpid than in normal aggregates or talpid cells are more viable in these conditions. Superior viability seems the more likely alternative: J. M. Cairns (1967, personal communication) has shown that stripping the apical ectodermal ridge from the normal chick limb bud leads rapidly to cell death in the underlying mesenchyme, but that this is delayed by about 5 h when the same operation is done in the talpid mutant. It seems that talpid mutant cells are more resistant to injury and death in the intact limb bud (absence of necrotic zones), in the operated limb bud (removal of apical ectodermal ridge) and in the suboptimal conditions in cultured aggregates.

Cell division

At 1 day the proportion of dividing cells is similar in normal and talpid aggregates and there follows a peak of mitosis in both at 2 days. In talpid aggregates this peak is smaller and occurs only in the precartilage areas; in normal aggregates the precartilage peak is higher, but there is also a smaller one in the non-cartilage. The peak is probably in part a post-trypsinization effect of the dissociation technique, similar to that described by Burger (1970) in cultures of mouse fibroblasts and by Sefton & Rubin (1970) in cultures of chick embryo
fibroblasts after addition of trypsin and other proteolytic enzymes. Since, as appears above, talpid³ cells are more resistant to injury by trypsin, this would account in large part for the difference in overall mitotic indices in normal and talpid³ aggregates. The difference which remains in both between precartilage and non-cartilage areas must be otherwise accounted for and recalls the work of Holtzer (1968), Abbott & Holtzer (1968) and Lasher & Cahn (1969) on chondrogenesis in vitro, where a burst of mitosis is integrally associated with cartilage formation and suggests simultaneous synthesis of DNA and chondroitin sulphate.

Thus the mitotic peaks at day 2 in the precartilage areas are probably due to a combination of two factors: (1) a post-trypsinization effect, (2) the initiation of chondrogenesis. The much lower overall level of mitosis in talpid³ aggregate is, according to this hypothesis, due to the cells reacting less strongly than normals to the trypsin-dissociation process, whereas the difference between levels in precartilage and non-cartilage in both is probably due entirely to the mitotic burst associated with cartilage initiation. No meaningful statistical index of significance can be given for these low frequencies, but the difference is of the same order of magnitude in both normal and talpid³ aggregates.

From 2 to 4 days there is a steep drop in mitotic indices in all except talpid³ non-cartilage areas. The fall in the precartilage areas is almost certainly due to the cessation of mitosis after differentiation which J. M. Cairns (1968, personal communication) and Janners & Searls (1970) have shown occurs in the cartilage of the intact limb bud. In normal aggregates the fall which occurs in non-cartilage areas may result from deterioration of the medium, which, as the studies of cell death show, has a less deleterious effect on talpid³ cells. The rise which occurs in talpid³ non-cartilage regions is still not explained but Ede & Flint (unpublished) have also observed it in talpid³ limb buds in vivo.

Pattern, form and spacing of condensations

Starting from a uniform population of limb mesenchyme cells, there develops within each aggregate a pattern of precartilage areas formed by condensations which are roughly equidistantly spaced and never present at the surface. Condensations begin at foci consisting of a single cell or a small group of cells and expand centrifugally, so that the origin of this pattern is to be looked for in the factors determining the initial distribution of foci rather than the specification of each cell as chondrogenic or non-chondrogenic according to its position within the aggregate. The problem resembles that posed by the initiation of aggregation and non-random spacing of centres in slime moulds, for which a solution based on a mathematical analysis of the breakdown of stability in a homogeneous cell population has been proposed by Keller & Segel (1970).

Condensations in talpid³ aggregates are only half the size of normal condensations at 2 days, yet because many more of them are initiated they produce a greater area of cartilage relative to total aggregate area and larger individual
areas of cartilage than in normals. This increased capacity to initiate condensation centres in *talpid* may be related to the greater overall cell density found in the mutant aggregates at this stage and is comparable to the situation in monolayers of dissociated mouse-limb mesenchyme cells, where Umansky (1966) found that the number of differentiating cartilage nodules was directly related to the cell population density. There is another possible explanation: that the aggregates examined have been formed by fusion of smaller aggregates in which chondrogenic determination has already occurred and that the larger number of foci in *talpid* is simply a reflexion of the production of smaller and more numerous aggregates in *talpid* than in normal reaggregation (Ede & Agerbak, 1968); but our observations on the relation of the form of the 2-day aggregates to the distribution of cartilage areas within them do not generally support this alternative.

The characteristic concentric arrangement and crescent-shaped appearance of the cells in a condensation, with decreased cell thickness and increasing radius of curvature towards the periphery, is difficult to explain except by the following hypothesis: the cell or small group of cells at the condensation centre attracts peripheral neighbouring cells to crowd in on it, so that they tend to wrap themselves around it; this ring of cells attracts still more peripheral cells which become rather more flattened in crowding in on the central mass because the radius of curvature is now greater; and so on. That this pattern is in fact produced by cells crowding in on a central cell is shown by the cell pattern in aggregation centres of *Dictyostelium minutum* (Gerisch, 1968), a slime mould in which the myxamoebae move in to the centre from all directions rather than in streams as in the better known *D. discoideum*.

Nearer our case, Holtfreter (1968) has described how, in the amphibian *Ambystoma maculatum*, cartilaginous nodules which in his illustrations clearly exhibit this pattern of cells are built up by aggregation on one or a few centres when the behaviour of mesectodermal cells in dispersed monolayer culture is altered by the addition of a cartilage inducer, the pharyngeal ectoderm. He believes that a primary ‘heterogenetic’ induction by this tissue produces a primary cartilage rudiment and that this is succeeded by a secondary ‘homoio-genetic’ induction exerted by the rudiment itself, which expands by assimilating peripheral mesectoderm cells to itself by first attracting them to it and then causing them to differentiate chondrogenically. In the chick-limb cartilage is formed apparently without a heterogenetic inducer, but the period of heterogenetic induction in our aggregate system would correspond to the period of condensation initiation. Thereafter our hypothesis for the expansion of the condensations is exactly the dual process of attraction and homoioigenetic induction which Holtfreter proposes for his system. We note that not only condensation initiation but also chondrogenic transformation would in this case be related to cell packing density. The histological picture and our cell counts might appear to contradict this account: cell density is actually less in cartilage areas and
sections (e.g. Fig. 1C) show that cells are further apart at the centre of the condensation than at the periphery; but this follows from the fact that the chief consequence of differentiation is the secretion of intercellular matrix which forces the cells apart, so that there will be a sequence of cell aggregation by active movement followed by cell dispersion caused by matrix secretion.

Relation to precartilage condensations in the intact limb bud

How far are the roughly spherical condensations found in aggregates, non-randomly but irregularly spaced, comparable to the cylindrical precartilage condensations, spaced according to an inherited pattern, which prefigure the skeletal elements in the developing limb bud, and might the same developmental mechanisms which we have suggested operate in chondrogenesis in culture apply also in the intact embryo? In the chick limb bud the condensations in T.S. certainly show a clearly concentric arrangement (Ede, 1971) though the variation in cell shape from centre to periphery does not appear clearly as it does in the smaller condensations of the aggregates; however, the pattern we find in aggregates has been shown by Anikin (1929, see Fig. 6) to exist with diagrammatic clarity in the small cartilage rudiments of developing digits of amphibians; he describes only the shapes of the nuclei, but it seems most likely that these reflect the form of the cell as a whole. The answer will be important for the
problem of skeletal pattern formation – whether it is a question of each cell being specified as falling within a potentially chondrogenic or non-chondrogenic region, or whether it is a question of the central foci only being specified, together with a constraint on the ultimate expansion of each condensation – and to obtain it will require a very detailed histological and cytological study.

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REFERENCES


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