The Culture of Small Aggregates of Amphibian Embryonic Cells in vitro

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WITH FOUR PLATES

INTRODUCTION

A common procedure in amphibian embryology has been to remove portions from embryos and culture these under conditions in which the large numbers of cells retain a close-knit association, favourable to the differentiation of primitive organs in the explant. It has not, in general, been the aim to employ the primary explant as a source of a two-dimensional outgrowth of cells on the substrate, as in typical cell culture procedures. Because of their inherent migratory tendencies, however, outgrowths of pigment cells are readily obtained from explants of the amphibian neural crest, and these have stimulated the interest of a number of investigators (see Wilde, 1961). Holtfreter (1938, 1946) and Finnegan (1953) have also observed the migration of cells from explants of Urodele embryos. Several investigators have employed cell cultures as opposed to organ type cultures in induction studies, Niu & Twitty (1953), Niu (1958), Barth & Barth (1959) and Becker, Tiedemann & Tiedemann (1959). In these cases the formation of outgrowths one cell thick from the gastrula ectoderm is favoured by the non-epidermal differentiation of the cells in small groups. The media employed in organ-type cultures have been Holtfreter's physiological salt solution or a variant; cell cultures, however, are improved by the addition of organic supplements. Wilde has used a medium including serum ultra-filtrate as a satisfactory substitute for whole serum, Lytle & Elsdale (in preparation) found that single isolated early blastula cells had an unspecific requirement for protein at low concentration. Barth and Tiedemann supplement a physiological salt solution with serum proteins.

The potential advantages to be sought from cell cultures, as opposed to organ-type cultures, stem from the fact that the cells are individually exposed, and thereby favourably placed for individual observation during their differentiation (Algard, 1953), microsurgery, autoradiography and the application of antisera (Inoue, 1961).

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This paper deals with the culture of disaggregated cells from ectoderm and presumptive mesoderm of amphibian embryos between the mid-blastula and neural tube stages, under conditions that generally favour the differentiation of the cells as monolayers.

MATERIALS AND TECHNIQUES

Embryos of *Rana pipiens* and *Xenopus laevis* were routinely employed, but the following six species were used from time to time as available: *Ambystoma tigrinum, Bufo vulgaris, Discoglossus pictus, Pleurodeles waltlii, Rana temporaria* and *Triturus alpestris*.

The culture assemblies used are shown in Text-fig. 1; the two kinds are each based on a 3 × 1 in. glass slide. One type, 1c, is made by wax-sealing coverslips

Text-fig. 1. (a) Construction of the oil-sealed microchamber based on a 3 × 1 in. microscope slide. The coverslip bearers are made from strips cut from another microscope slide, the central glass block about 1/10 in. square, is cut from a slide of lesser thickness than the bearers. The gap so produced, which houses the culture, may be varied to fine limits without sacrificing the space necessary in order to introduce instruments for the purpose of micromanipulation. These components are bonded together with 'Araldite' epoxy resin adhesive.

(b) An enlarged view of the central area of the assembled chamber. To assemble, cells are placed in a small drop of culture medium in the centre of the coverslip which is then inverted over the central glass block so that the drop makes contact with it. The coverslip may be held in place by two smears of petroleum jelly on the bearers before being waxed into position. Paraffin oil is now run in from the side to surround the microdrop and the glass block.

(c) Construction of the routinely used 'macrochamber' consisting of a slip of glass 3 × 1 in. in size and 1/4 in. thick, with a central hole 1/8 in. diameter. The chamber is completely filled with medium and the coverslips are waxed in position.
over a hole bored through the centre of glass slides of various thicknesses. This type of chamber is filled with culture medium to the exclusion of a gas phase, in the interests of securing the best image quality. The second type, 1a and b, incorporates a paraffin oil seal, permitting both micromanipulation and gas exchange while retaining good optical properties. This chamber is suitable for small volume cultures of a microlitre or less. Both assemblies permit the use of 2 mm. objectives. Cultures were maintained for 7 to 14 days and there were no differences in the behaviour of cells cultured in the two assemblies.

The main source of infections in cell cultures derive from the carry over of cloacal micro-organisms adhering to the embryonic jelly coats. A satisfactory procedure for overcoming this problem has been to immerse the embryos in 70 per cent alcohol for 5 sec. followed by the immediate removal of the jellies in sterile Steinberg's physiological salt solution (PSS) (formula see below), and the passage of the de-jellied embryos through several changes of the same solution. It was found to be important for the success of this procedure to select embryos with unbroken jelly coats, for micro-organisms trapped within the jelly escaped contact with the alcohol. Embryos developed normally following this treatment.

Steinberg's solution for amphibia

The physiological salt solutions used for culturing and disaggregating cells in this work were made to the following formulae attributed to Dr Malcolm S. Steinberg.

Stock solutions made up w/v in distilled water.

A. 17·0 per cent NaCl  
B. 0·5 per cent KCl  
C. 0·8 per cent Ca(NO₃)₂·H₂O  
D. 2·05 per cent MgSO₄·2H₂O

For use 10 ml. of solution A plus 5 ml. of each of solutions B, C and D were made up to 500 ml. with distilled water. 280 mgm. of Tris (Sigma) was added and the pH adjusted to 7·8–8·0 with 1NHCl.

Disaggregating solution

This was made by proceeding as in the previous solution but omitting solutions C and D and adding 60 mgm. of EDTA (Disodium salt of ethylene diamine tetra-acetic acid). Final pH 7·8–8·0 adjusted as above.

Barth's solutions (Barth & Barth, 1959) have also been used occasionally with similar results.

The embryos were dissected in sterile PSS, and the desired regions transferred to the disaggregation medium. Dorsal and dorso-lateral presumptive mesoderm
was removed from gastrulae and the disaggregated cells intimately mixed so that the future chorda and muscle were equally distributed. Similarly, only the dorsal, axial mesoderm from the neurula was cultured. The superficial presumptive epithelial layer (Nieuwkoop & Faber, 1956) of the Anuran embryo is associated, on its outside surface, with a layer of tough pigmented coat-substance (Holtfreter 1943b). Such coated cells have unusual properties which impede the normal culture methods used in this work, and for this reason are normally discarded when embryos are disaggregated. Reference to ectodermal cells in the present paper, therefore, includes only the underlying presumptive sensorial layer (Nieuwkoop & Faber, 1956) of the ectoderm. Presumptive epidermis was removed from gastrulae and the coated cells separated from the inner ectoderm and discarded during disaggregation; the neural epithelium from neurula stages was treated similarly. The disaggregated cells were placed in culture as one to four loose heaps of up to 200 cells per chamber, or seeded sparsely over the floor of the culture vessel. Over 1000 cultures were prepared in the course of the present work. The progress of the cells was observed under the inverted phase contrast microscope, and recorded by 35 mm. still photography and 16 mm. time-lapse cinemicrography.

Two classes of media were employed: Normal Media in which cells generally differentiated according to their presumptive fate and Tissue Extract Media. Normal Media consisted of one of the following additions to Steinberg's physiological salt solution: 0.1 per cent Crystalline Bovine Plasma Albumin (Armour), 0.1 per cent Human Serum Globulin (Bois), 3 per cent Horse Serum (Difco). Further media were obtained by heating the serum medium to 65° or 90°C for 30 min. On the basis of the behaviour of cells, Normal Media can be subdivided into G-type Media consisting of the Globulin and unheated serum solutions, and A-type Media consisting of the Albumin and the heated serum solutions.

The Tissue Extract Media consisted of soluble, granule-free extracts, in Steinberg's solution, of mouse bone marrow and human buffy coat, prepared as follows: Mouse Bone Marrow Extract—Marrow from three adult mouse femurs was homogenized in 1:25 ml. of Steinberg's solution at 0°C for 10 min, and the suspension centrifuged at 35,000 g for 1 hour in the refrigerated centrifuge at 0°C. The clear supernatant was stored at 4°C. Human Buffy Coat Extract—Buffy coat was removed from 40 ml. of human blood, washed with Steinberg's solution, taken up in 2 ml. and frozen and thawed three times, homogenized in the cold for 10 min. and thereafter treated as for marrow. The final pH of all media was between 7.6 and 8.0.

It has been noted that different batches of eggs vary widely in respect of hatchability etc. This variability does not show up in culture as differences in the behaviour of differentiating cells, but as differences in the ease with which cultures from different ovulations may be established. In general, ovulations giving poor hatchability provided cells which differentiated with lower frequency
but which, when they did differentiate, behaved in exactly the same way as cells differentiating with a higher frequency from ovulations of better hatchability. Non-differentiating cells, likewise, appear to exhibit a limited behaviour pattern which is variable to a limited extent, not between ovulations so much as between different conditions of culture. These facts have enabled the following observations to be generalized over the whole of a very large material, except in particular instances where exceptional behaviour has been carefully noted.

OBSERVATIONS AND RESULTS

Disaggregation and reaggregation

Amphibian embryonic cells may be disaggregated by the removal of calcium and magnesium ions (Curtis, 1957, 1962). Blastulae and early gastrulae usually fall apart in calcium and magnesium-free solution (Plate 1, Fig. A), but the presence of chelating agents is required to disaggregate older embryos up to the neural tube stage. The facility with which cells will subsequently reaggregate in calcium containing solutions is related to the ease with which they can be disaggregated. Thus, in the media used in the present study, disaggregated blastula and gastrula cells reaggregate to form balls of tightly coherent cells during their first 1 to 2 hours in culture (Plate 1, Figs. B and C), whereas disaggregated, undifferentiated neurula cells have very limited powers of reaggregation.

Cell behaviour in culture prior to differentiation

Freshly disaggregated cells usually undergo the cyclosis movements described by Holtfreter (1943, 1946), in which a clear cytoplasmic protrusion appears to move around the cell. Following transfer to a non-disaggregating medium, the cells gradually cease these movements over a period of several hours. Some individual cells in cultures from neurulae have been observed to continue these movements for days without subsequent differentiation. Differentiation, the first sign of which among neural or mesodermal cells is the attachment of the cell to the glass by pseudopodia (Plate 1, Fig. E), requires the cessation of cyclosis.

The formation of tubes and vesicles from the surface of cells was observed from time to time (Plate 2, Fig. L). These formations were strikingly similar to the myelin forms produced by suspending lecithin in A-type Medium. The presence of these forms on the surface of undifferentiated cells signifies their lack of a developmental future since they do not divide further or differentiate and generally disrupt in a few hours; their presence in differentiating cell cultures, however, has frequently been noted, and they are particularly abundant in differentiating cultures of neural epithelium.
Undifferentiated cells in contact with the glass occasionally produce cytoplasmic projections (Plate 2, Fig. J). These projections either retract after some hours or become severed from the cell body, thus producing free cytoplasmic fragments (clasmotosis) (Plate 2, Fig. K). These fragments wander around for

**Explanation of Plates**

**Plate 1**

**Fig. A.** Disaggregated uncoated ectoderm cells from the early gastrula of *Rana pipiens*, after 3 hr. in culture. Reaggregation has commenced but is slowed down by the medium which contained too low a protein concentration (1:64 dilution of mouse bone-marrow extract). Under these conditions the cells are slightly flattened and do not exhibit the usual cyklosis movements. × 130.

**Fig. B.** Advanced reaggregation of uncoated ectoderm from the early gastrula of *R. pipiens* in medium (Steinberg's) containing adequate amounts of protein (0.1 per cent plasma albumen). These cells show no tendency to flatten, consequently the perinuclear pigment is not as prominent as in Fig. A. Cells cultured for 3 hr. × 190.

**Fig. C.** Complete reaggregation of uncoated early gastrula ectoderm in Normal Medium (same as in Fig. B) after 24 hr. culture. Such aggregates normally become ciliated with 36 hr. × 190 (*R. pipiens*).

**Fig. D.** Part of an outgrowth of a sheet of cells from a dorsal lip explant from a gastrula of *Triton alpestris* after 5 days in culture. Medium, Barth's saline containing 0.1 per cent bovine gamma globulin. This sheet of cells showed a high mitotic activity (Plate 4, Fig. W) and differentiated into chorda cells on the seventh day. Note the absence of a well defined nucleolar apparatus which is characteristic of cells of this species at this time (compare Fig. E). × 550.

**Fig. E.** Myoblasts differentiating from a disaggregate of presumptive somitic mesoderm isolated from the early neurula of *Rana temporaria*, after 5 days in culture. Note the presence of undifferentiated cells, single nucleolus, and absence of mitochondria from cytoplasmic fringes. Mitochondria become profuse shortly after this stage (compare Plate 3, Figs. M and N). × 500.

**Fig. F.** Myoblasts of *Bufo vulgaris* differentiating and spreading as a sheet after 5 days in culture. (Medium, Barth's saline containing 0.1 per cent gamma globulin.) × 125.

**Plate 2**

**Figs. G TO I.** These figures show the stages in the transformation of a ball of reaggregated uncoated ectoderm, from an early gastrula of *R. pipiens*, into sheets of cells with mesoderm characteristics, in a medium containing a mouse bone-marrow extract (see text). The transformation (induction) is a multi-stage process involving suppression of ciliation. Various cell types including muscle, chorda, various mesenchymal elements, and sometimes nerve cells, are to be found in such cultures. Controls in Normal Media become ciliated balls. (All × 190.)

**Figs. J and K.** Clasmotosis. Uncoated ectoderm cells from an early gastrulae of *R. pipiens*, in a medium containing 0.0016 per cent Albumen. During reaggregation the cells at the surfaces of the groups produce flattened cytoplasmic fans that attach to the glass. At the completion of reaggregation these fans have either retracted, or severed connection with the cells to remain behind as free stellate fragments. This phenomenon has also been observed in cultures containing normal protein concentration.

**Fig. L.** *R. pipiens*. Formation of 'tubes' by a cell from gastrula uncoated ectoderm. This phenomenon is mainly found in association with undifferentiated cells from all germ layers when it indicates a poor prognosis for future differentiation in culture. The 'tubes' are strikingly similar to 'myelin forms' and are thought to indicate a particular instability of the cell membranes. Under standard conditions of culture, cells obtained from different embryos of the same species vary in their predisposition to 'tube' formation. × 250.
PLATE 1

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a time before they die. This behaviour does not limit the subsequent ability of cells to differentiate in culture.

In general it has been found that the older the cells when placed in culture, and the shorter the interval before they would have differentiated in the embryo, the closer the approximation between the time of their differentiation in culture and the differentiation of comparable cells in control embryos. Thus, the differentiation of cells is not significantly delayed by placing them in culture from neural fold embryos, but around one day’s delay is involved when cells from early gastrulae are cultured.

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**EXPLANATION OF PLATES**

**PLATE 3**

**Fig. M.** Myoblasts of *Bufo vulgaris*, isolated from the early neurula, differentiating after 5 days in culture in Barth’s solution containing 0·1 per cent gamma globulin. Note the spherical mitochondria, characteristic of newly germinated cells, in the clear cytoplasmic fringes. x 440.

**Fig. N.** Myoblasts of *X. laevis* at a more advanced stage in differentiation than those of *Bufo* shown in Fig. M. Isolated at the same stage as the *Bufo* cells, the *Xenopus* myoblasts in identical culture conditions achieve the stage shown after only 2 days. x 440.

**Figs. O and P.** Show stages in the differentiation of the Hairy muscle cell from the tail-bud rudiment of *R. pipiens* embryos. In its early development this cell resembles a neuroblast in that its cytoplasm bears on its surface a profusion of fine processes (Fig. M). These persist and the endoplasmic core of the cell becomes striated (Fig. P). Compare with Fig. Q. x 950.

**Fig. Q.** Single striated muscle cell from a 4-day-old culture of *R. pipiens* late gastrula mesoderm in Steinberg’s solution plus 0·1 per cent Albumen. x 500.

**Fig. R.** Part of a sheet of striated muscle cells differentiating in culture from cells of presumptive somite mesoderm of *X. laevis*. Note two prominent nucleoli in the lower cell, and one of the four points of attachment to the substratum two of which form, characteristically, opposite the nucleus in these cells (arrowed). x 940.

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**PLATE 4**

**Fig. S.** Large flat cell from the neural epithelium characteristically found in cultures of cells disaggregated from the neural tube of *R. pipiens*, after 2–3 days. Yolk in these cells tends to become oriented in rows indicating stress lines in the cytoplasm between points of attachment to the glass (arrowed).

**Fig. T.** Cell frequently found in cultures of neural tube disaggregates from *X. laevis* embryos. These cells come to contain large numbers of vacuoles (sv) which replace the yolk (y) as it is utilized. The nature of the substances composing the vacuoles is not known. x 950.

**Fig. U.** Cell from a 3-day culture of *X. laevis* neural tube cells. Cells of this kind exhibit unusual mitochondria, these being frequently long (arrowed) and often branched. Their function is not known. x 950.

**Fig. V.** Fibroblast-like cell of ectodermal origin from a 5-day culture of gastrula ectoderm cells of *Discoglossus pictus*. x 300.

**Fig. W.** Dividing cell of *T. alpestris* in a 5-day culture of dorsal lip explants in Barth’s solution containing 0·1 per cent gamma globulin. This culture showed a high mitotic activity on the fifth day and by day seven had differentiated a sheet of chorda cells with characteristic vacuoles. x 125.

**Fig. X.** Myoblast of *X. laevis* fixed in osmic acid from culture to show the osmiophilia of the characteristic large liposome. x 500.
Blastula and gastrula presumptive mesoderm cells in culture

The balls of cells formed by reaggregation may flatten in profile on the second or third days in culture and, over the next day, spread into thin sheets of cohesive cells (Plate 1, Fig. F), commencing the rapid utilization of their yolk and differentiating into chorda or muscle. These times refer to early gastrula cells of *Rana pipiens* in culture; the time course in cultures of other stages of different species varies with the age of the embryo from which the cells are taken, and the inherent developmental rate of the species employed. Differentiation is obtained from up to 30 per cent of aggregates of *Triturus alpestris* and *Discoglossus pictus* cultured in Normal Media. *Xenopus laevis* and *Rana pipiens* aggregates differentiate less frequently, and only about 10 per cent of ovulations of good hatchability provide embryos from which differentiations were obtained. Where a single small culture is established a minimum aggregate size of around thirty cells is normally required for differentiation. In cultures containing more than one aggregate, however, aggregates of smaller numbers of cells have been observed to differentiate in the vicinity of larger differentiating aggregates.

The majority of aggregates, therefore, of up to 150 cells from early embryos, do not differentiate in Normal Media; single cells, or those in aggregates much smaller than thirty cells, invariably fail to differentiate. The behaviour of non-differentiating aggregates is characteristically different in A-type and G-type Media. These aggregates in G-type Media remain unchanged for 1 to 2 weeks and eventually fall apart into cytolysing cells, still packed with yolk. In A-type Media, on the other hand, the aggregates break down on their second or third days in culture into heaps of predominantly healthy, non-coherent cells. This spontaneous process, occurring in a medium in which the cells have previously reaggregated, we term de-adhesion, in order to distinguish it from disaggregation, the loss of cellular cohesion following the removal of calcium and magnesium ions. The de-adherent cells in A-type Media remain healthy for as long as a week, and may occasionally divide; they eventually die without utilizing an appreciable quantity of their yolk, and without differentiating. It is not uncommon for the peripheral cells of an aggregate to de-adhere and the remainder to differentiate.

These differences in the behaviour of non-differentiating cells are not necessarily due to qualitative differences between media. This is illustrated by the results of an experiment in which cells were cultured in an extract from human buffy coat. This extract was used at three different concentrations in Steinberg’s solution: half strength, quarter strength and one-twelfth strength. The intermediate concentration was optimal for the differentiation of *Rana pipiens* aggregates. Aggregates in the strongest solution behaved very similarly to non-differentiating aggregates in G-type Media, and the weakest solution exactly simulated the A-type Medium used as a control, in which no differentiation occurred and all the cells de-adhered. The ovulation used in this experi-
ment, therefore, like the majority of those from *Rana pipiens*, gave aggregates incapable of differentiating in A-type Media. However, a concentration of
eat was found in which all the aggregates differentiated. This suggests that
the concentration of the organic component is an important factor in defining
an optimal medium. This has been confirmed in other experiments using this
extract and another prepared from mouse bone marrow. However, the extract
concentrations optimal for the differentiation of aggregates from different
ovulations were not the same, and this suggests that it may be impossible to
write a formula for a single medium along these lines, in which all *Rana pipiens*
aggregates will differentiate.

Gastrula mesodermal aggregates were made from intimately mixed popula-
tions of disaggregated cells from the dorsal and dorso-lateral gastrula meso-
derm. Due to the mixing, aggregates should have contained presumptive
chorda and presumptive somite cells, yet it was observed that each aggregate
differentiating in Normal Media produced either a chorda sheet or a muscle
sheet, and only very rarely mixed sheets. In one small experiment employing
a single embryo from a *Rana pipiens* ovulation, the mixed presumptive mesoderm
cells were used to set up five aggregates of thirty to fifty cells each in 3 per cent
horse serum, and five aggregates in 3 per cent horse serum heated to 65°C.
Each of the former aggregates produced muscle sheets, and the latter chorda
sheets. A similar result was obtained in another experiment employing non-
disaggregated cells. Since muscle sheets usually predominate in differentiating
cultures in both A-type and G-type Media, the results from these two experi-
ments are not typical. They do, however, suggest that the nature of the organic
component in the medium can have an effect on how presumptive mesoderm
cells differentiate in culture.

Differentiating cultures invariably contained some nerve cells, presumably
due to the inclusion of ectoderm cells; nerve cells were never observed in these
cultures in the absence of differentiating mesoderm cells.

*Late blastula and early gastrula uncoated ectoderm cells in culture*

The tight balls (Plate 1, Fig. C) produced by the initial reaggregation of
ectoderm cells in Normal Media grow surface cilia on their second day in
culture (*Rana pipiens*), and the cells commence the rapid utilization of their
yolk. The aggregates detach from the glass and swim in the medium by a sort
of corkscrew motion. A central fluid-filled cavity formed shortly after ciliation
progressively enlarges, until between the fifth and the ninth day the aggregates
rupture, breaking up into small groups of cells. These continue ciliary activity
for a day or so until their yolk is exhausted.

Like presumptive mesoderm cells, a minimum aggregate size of around
thirty cells is required for differentiation. Non-differentiating aggregates remain
solid, and after a week or two fall apart into cytolysing cells still packed with
yolk. In sharp contrast to presumptive mesoderm cultures, ectodermal aggregates from all ovulations of good hatchability of *Xenopus* and *Rana pipiens* seldom fail to differentiate.

Induction of early gastrula ectoderm cells in culture has been brought about in two ways; first by culturing mixed aggregates of presumptive mesoderm and ectoderm cells, and second, by growing ectoderm cells in Tissue Extract Media (see Plate 2, Figs. G to I). In both cases the behaviour of the ectoderm cells is different from that associated with epidermal differentiation, and bears striking resemblances to that of presumptive mesoderm cells.

Mixed aggregates behave in one of two ways: in the majority, the cells de-adhere and fail to differentiate, while a minority produce mixed sheets of muscle and nerve cells. The production of cilia is permanently inhibited irrespective of whether the cells subsequently differentiate. The commencement of rapid yolk utilization which is seen in the ciliated control cultures of pure ectoderm as a striking reduction in yolk content, is postponed for 1 or 2 days until the cells start to differentiate; yolk utilization is inhibited in cells that fail to differentiate, and these eventually cytolyse still packed with yolk. Instead of differentiating in balls, the induced cells transform into outgrowths of cells attached to the glass.

Various dilutions of a mouse bone marrow extract were employed as a Tissue Extract Medium. The behaviour of the differentiating ectodermal aggregates resembled that of differentiating presumptive mesodermal cultures. The mesodermalizing potency of mouse bone marrow appears similar to that of normal rat and guinea-pig marrows previously investigated (see Yamada, 1961).

**Neurula cells in culture**

Aggregate size and the variability between ovulations are important factors controlling the ability of blastula and gastrula cells cultured in Normal Media to differentiate. These factors exert little or no influence on the differentiation in culture of cells from neurulae. Disaggregated cells from these older embryos scattered sparsely over the floor of the culture vessel in Normal Media routinely yield 20 to 40 per cent differentiating cells. No differentiation takes place in media lacking calcium and magnesium.

Individual cells in gastrula aggregates transforming into thin sheets of differentiating cells are often too closely packed for detailed observation. Cultures of well-spaced neurula cells, however, are excellent for studying the cytology of differentiating cells.

*The differentiation of gastrula and neurula presumptive nerve cells and mesoderm cells in culture*

Before the commencement of differentiation living embryonic cells anchor to the glass by contact, but appear to possess no distinct regions of their surfaces specialized for attachment.
The event that signifies that the spherical embryonic cell is on the path of morphological differentiation is the pushing out of pseudopodia, or a cytoplasmic fringe attached to the glass. In the case of mesoderm cells and certain cells from the neural plate, the fringes expand or the pseudopodia move apart, so that the cell body becomes greatly flattened in profile and the cell increases its coverage of the glass. This process is sufficiently rapid to mark a well-defined first stage in the process of morphological differentiation. Cells in which the primitive pseudopodia extend directly into nerve axons are exceptions, for the bodies of these cells do not, as a rule, flatten on the glass. The process of attachment followed by the rapid expansion of the cell surface may take place over the space of a few minutes, in the case of certain *Xenopus* cells, or may occupy up to several hours.

The number of pseudopodia, the appearance of the cytoplasmic fringes, the shape of the spread cell and the polarization of its contents, are some of the characteristic features by which a variety of cell types can be identified at this initial stage in their differentiation. Perhaps eventually all or the majority of the cell types differentiating in neurula cell cultures will be distinguishable by these criteria a few hours after they attach to the glass.

The fringes and pseudopodia are initially free of yolk platelets and mitochondria. About the time the cells complete their initial spreading, small spherical mitochondria, often associated in pairs, make their appearance in the clear cytoplasmic areas (Plate 3, Fig. M). At about the same time the small yolk platelets and the pigment granules accumulate in the proximal regions of the fringes and pseudopodia. The larger platelets are packed around, or to one side of, the nucleus, which usually lies roughly in the centre of the cell and becomes exposed to view in the vertical plane as a consequence of the flattening of the cell body (Plate 4, Fig. S).

The *Rana pipiens* cells isolated from the axial regions of the neural plate and early neural fold embryo spread at various times after isolation. This observation has stimulated an analysis of some of the conditioning factors. In general it is observed that when a transverse slice of the axis is disaggregated in Ca and Mg free Steinberg containing Versene, and the cells cultured in Normal Media, the various recognizable types of cells attach and spread in a distinct order. This is, chorda, followed by muscle and nerve, and followed by cells of a fibroblastic type. The time scale for this process for *Rana pipiens* is being documented. At present it appears that all of the chorda and muscle cells spread and begin to differentiate within a few (4–6) hours of each other, chorda being in advance of muscle. Nerve cells which appear later, by which time muscle cells are distinctly recognizable by their definitive shapes, have an extended period of spreading, for individual cells attach and spread over the next few days. Finally, up to 100 hr. after the attachment and spreading of muscle, fibroblastic cells may appear (Plate 4, Fig. V). This picture is complicated by the fact, discovered in the course of examining the system, that slices from the
axis at the level of the first somites yield cells which spread in advance of
similar cells taken from slices obtained from the posterior. Thus it has been
observed that muscle cells from the posterior axial regions are delayed in
spreading by 24 hours as compared with those from anterior regions. These
cells, because of the short period over which they spread when taken from a
confined region, will, in the future, enable one to make a more precise analysis
of the system.

Non-attached cells do not develop the distinctive cytological features of the
differentiated state, and eventually die still packed with yolk. Differentiation
and the commencement of rapid yolk utilization thus depend on the prior
attachment and spreading in all cell types except epidermal cells.

Disaggregated embryonic neurula cells have little tendency to reaggregate.
The tendency to do so, however, is acquired by most cells after attachment,
and may lead to the formation of tissue-like associations among differentiating
cells (Plate 1, fig. F). For example, time-lapse films show that myoblasts move
slowly for some hours immediately following their attachment. Cells that run
into each other are slowed down and tend to associate with their long axes
parallel; in this way muscle bundles are formed, that later, if innervated, twitch
as a unit.

The time from attachment to complete morphological differentiation varies
with the cell type and the species, from 1½ to 5 days. For example, *Xenopus*
muscle cells acquire striations (Plate 3, fig. R) after around 36 hr., *Rana pipiens*
muscle cells (Plate 3, fig. P) after 2 to 3 days.

Description of cell types

Muscle cells

Attachment is by means of two localized pseudopodia on opposite sides of
the cell. These move apart during the spreading period, and the cell body is
drawn into a spindle shape. The nucleus usually lies in the centre of the cell
and divides the yolk into two parts. Somewhat later in development two cell
types can be distinguished. The first shows a smooth outline interrupted by
four or six points of attachment to the glass; there are one, sometimes two, of
these attachments at each pole of the cell, and one on either side of the nucleus
(Plate 3, fig. R). The second type occurs in cultures of neurula cells from the
future tail region. This cell is also spindle shaped, but has an abundance of fine
cytoplasmic 'hairs' along its surface (Plate 3, figs. O and P) and these assist
the polar pseudopodia in attaching the cell to the glass. These cells, termed
'hairy muscle cells' showed a tendency to become binucleate, whereas the other
variety were invariably uninucleate over the period of observation of up to
15 days. Both types develop cross-striated fibres, but cultures usually contain
a proportion of cells that acquire no striations. The majority of mitochondria
in muscle cells are filamentous forms.
The neural epithelium series

Axon producing cells. The cells attach by means of two or more localized pseudopodia, one or more of which transform directly into the growth cones of neurites. The number of processes, their individual sizes and activities and the number of fine cytoplasmic hairs, are all variable characteristics of these cells. The cell body is not usually flattened in profile, but remains roughly spherical, although a minority of cells bodies flatten to expose the nucleus. The axon producing cells are probably divisible into a number of cell types.

Non-axon producing cells. These cells differentiate in profusion from cells derived from all regions of the neural plate. They form a rather uniform cell type with several unusual features (Plate 4, fig. S). Attachment is by means of a cytoplasmic fringe completely surrounding the cell. Spreading is very extensive, and these cells come to present by far the largest area to the glass of any so far encountered. These are not, however, inordinately bulky cells, for they retract to a moderate size when the cytoplasm is withdrawn prior to their frequent divisions. The unusually thin cytoplasmic fringes of these cells often exhibit striae, probably indicating the lines of stress existing between points of adhesion to the glass. Some of the smaller yolk platelets and the mitochondria often become aligned in rows in association with these striae, presenting a strikingly ordered appearance. The cell is usually distinctly polarized with the nucleus lying on one side, backed by the main mass of the yolk; it is not unusual to observe a second accumulation of the smaller platelets separated from the main mass by a region of clear cytoplasm. These cells undergo repeated divisions in vitro, and bi- and tri-nucleate cells are common in older cultures. Axon-producing cells show a peculiar affinity for the cytoplasmic fringes of these cells, frequently spreading over the surface or passing beneath.

Chorda cells

The future chorda cell attaches to the glass by means of a continuous, peripheral cytoplasmic fringe. The cell body flattens to expose the nucleus as the fringe expands. The flattened cell is egg shaped and polarized and tends to associate in sheets (Plate 1, fig. D). The nucleus lies nearer the pointed end of the cell, the yolk mass nearer the opposite end. The chorda vacuoles develop around the nucleus and often abut on the nuclear membrane. Young chorda cells divide (Plate 4, fig. W) in culture up to the time they start to form vacuoles.

Primitive blood cells

These are identified in culture by their resemblance to cells obtained from the circulation of hatching tadpoles. The cells are small and spherical with a curious tuft of cytoplasm projecting from the surface, giving them the appearance of commas with very small tails. The cells migrate considerable distances over the glass and show no tendency to associate.
Ciliated cells

Epidermal cells. These are the first cells to differentiate in the embryo and in culture. Unlike all other cell types so far observed they do not attach to the glass, but differentiate in coherent balls. The differentiated cells are spherical with a single tuft of several cilia confined to a small region of the surface.

Epithelial cells. These cells have only been observed in a single series of cultures in which presumptive mesoderm cells from *Rana pipiens* were grown in an extract of human buffy coat. They differed from ciliated epidermal cells in three ways: first, they differentiated in culture several days later than epidermal cells would have done, second, the cell body spreads on the substratum (sheets of muscle in this case), and third, the more numerous cilia are distributed over the larger part of the cell surfaces.

Pigment cells

These differentiate in cultures of neural crest cells. The large, roughly star-shaped cells are extremely mobile, and the pigment granules can be seen in time-lapse films to be in constant motion. The morphology and behaviour of these cells in culture has been described by Algard (1953). Associated with these cells are others of a similar form and behaviour but completely lacking in pigment.

The connective tissue series

These cells attach and spread on the glass after the chorda cells, the myoblasts and many of the nerve cells. Included here are the fibroblasts (Plate 4, fig. V), so called because they resemble the fibroblasts of mammalian or avian cell cultures; also, the chorda sheath-cells which secrete collagen fibrils on to the glass, and certain other cells that accumulate granules and vacuoles (Plate 4, fig. T). In addition, there are nondescript cells remarkable only for their wide spectrum of mitochondrial morphologies (Plate 4, fig. U), suggesting that a number of cell types have yet to be recognized.

DISCUSSION

There are marked specific differences in the ease with which neural and mesodermal cells from gastrula embryos will differentiate in vitro: About one third of *Discoglossus pictus* and *Triturus alpestris* aggregates differentiate in Normal Media, whereas only about one in ten of those from *Xenopus laevis* and *Rana pipiens* do so. The variation in the embryos of the latter two species is preponderantly between ovulations. Curtis (1957) has also found, in the case of *Xenopus* embryos, that there is a variation between ovulations, in his case, in the total amount of calcium the embryos contain. It is possible that these two types of variation may be related in some way.

The aim behind the use of Normal Media was to obtain a medium in which
cells differentiate solely according to their presumptive fate, and this has been achieved. Ectoderm cells readily react in culture to the inducing properties of mouse bone marrow extracts, and they can be persuaded to differentiate into neural and mesodermal cells by simple variations in the ionic components of the medium, see Yamada (1961) and Barth et al. (1960), and even in hanging drops of Niu and Twitty saline (Niu, 1958). However, neural and mesodermal differentiation of gastrula ectoderm was never observed in Normal Media. This is in agreement with Barth et al.'s findings in so far as these investigators report that low bicarbonate (absent in Normal Media) and unheated globulin (present in some Normal Media) favour epidermal differentiation.

The observation that epidermal balls accumulate fluid in a central vacuole, the expansion of which eventually disrupts the aggregate, fits well with Tuft's (1957) ideas about fluid balance in the embryo. He has suggested that a net flux of water enters the embryo through the ectoderm. The behaviour of epidermal balls seems to demonstrate the consequences of this activity in an in vitro system working in the absence of an osmotic gradient.

The event that marks the first visible sign of differentiation in neural and mesodermal cells, is the attachment of the cells to the glass by cytoplasmic fringes, and localized pseudopodia. Attachment is followed by the spreading and elongation of these protrusions, a process at first rapid but slowing down after some hours when the cell has achieved an approximation to its definitive form. The rapidity with which the attachments are made, and with which they expand, causes the cell to undergo a striking change in form over the course of a few hours, or even minutes in the case of certain *Xenopus* cells. Because of their position in the life history of the cell, we refer to these events as germination. Germination precedes the visible indications of cytodifferentiation such as chorda vacuoles, and striated muscle fibres, by a specific and constant time interval; it has never been observed for a cell to attach and spread when already in possession of these structures. Following germination there is a fairly rapid utilization of yolk that does not occur in cells that remain embryonic. Mitochondria are sparse or absent in newly germinated cells, and when present they are small spheres; during the following day they increase rapidly in numbers and usually diversify into filamentous forms (see also Karasaki, 1959). Thus, germination appears to indicate a time at which potentialities within the cell are mobilized in the interests of cytodifferentiation. All the evidence supports the assumption that germination is a necessary prior condition for cytodifferentiation. However, it cannot be categorically stated at the present time that this is so in all cases, for it is possible that some non-germinated cells may undergo a limited differentiation which would not be revealed by examination under the phase-contrast microscope.

There is a definite temporal order in which cells of different fate germinate. Within cells of a single type there are differences in the time of germination, depending on the point of origin of the cells within the axial field.
presumptive anterior trunk somite cells in culture germinate a day in advance of presumptive myoblasts from the posterior region of the *Rana pipiens* neurula. Because neurula cells cultured singly without close neighbours behave in the above manner, the mechanism controlling the time of germination probably resides within these cells, rather than in a field imposing control from without. In the case of gastrula cells little can be said, except that ectoderm cells suffer a change in their timing control with induction (see below).

When disaggregated gastrula and neurula cells are placed in Normal Media as a sparse scattering of single cells on the floor of the culture vessel, up to a third of the neurula cells differentiate but none of the gastrula cells do so. This implies that cells change, during their natural evolution in the embryo as it develops from the gastrula to the neurula, to render a fair proportion of them able to differentiate under the above conditions of culture. As gastrula cells invariably fail to differentiate in these cultures, it seems that they are unable to pass through the natural evolution of cells in the embryo, and become arrested gastrula cells. Disaggregated cells from neurulae normally germinate after a few hours in culture, but in a medium lacking calcium and magnesium germination is permanently inhibited. These latter, therefore, remain arrested neurula cells. Both arrested gastrula and neurula cells produced in these ways are initially healthy cells that may remain viable for some days after arrest and may even divide, but eventually cytolysed still packed with yolk. The end of gastrulation and the end of neurulation mark, therefore, periods in the life of the embryo during which neural and mesodermal cells undergo fairly abrupt changes. The later of the two periods of change is visibly indicated by the germination of the cells, while no striking morphological changes in the cells coincide with the earlier change. Both these periods of change are, in addition, periods during which cells are changing their adhesive properties. Gastrula cells disaggregate in the absence of calcium and magnesium, and reaggregate when these ions are restored. The disaggregation of neurulae requires exposure to EDTA, after which return to calcium-containing solutions leads to little reaggregation. Post-neurula cells cannot be disaggregated with EDTA, for which purpose proteolytic enzymes must be employed. This change between neurula and post-neurula cells is further illustrated by the observation that disaggregated neurula cells in culture reacquire the ability to form mutual adhesions after germination.

The observations suggesting the dependance of cytodifferentiation, and certain accompanying phenomena, upon the prior germination of the cells, support the idea that germination marks a profound and rather abrupt step in the development of the mesoderm cell, which all must take during their evolution towards the differentiated state. The observations on cultured gastrula and neurula cells, including the phenomenon of de-adhesion, suggest that a prior step, taken around the end of gastrulation, is equally vital and necessary in the normal evolution of neural and mesodermal cells. The nature
of this change is obscure but, like germination, it is coincident with a period of changing adhesive properties of the cells, and serves to illustrate the close relationship between morphogenetic processes and cell surface phenomena.

Small aggregates of gastrula ectoderm cells in Normal Media routinely produce ciliated balls, whereas the majority of similar aggregates of presumptive mesoderm cells do not differentiate. Ciliated cells appear in ectoderm cultures about a day before mesoderm cells in parallel cultures germinate. These observations show that the evolution towards the epidermal differentiation in culture is different from the development of neural and mesodermal cells, and does not involve the two steps discussed earlier. Differentiating ciliated cells do not attach and spread on the glass, but although they do not germinate in the manner of neural and mesodermal cells, they do undergo a comparable change by which the potentialities of the cells are rapidly mobilized in the interests of cytodifferentiation. The rapid utilization of yolk commences at around the time the first cilia are evident, and Karasaki (1959) reports a build up of mitochondria \textit{in vivo} at a corresponding time.

Induction involves a diversion of the ectoderm cells into a pathway apparently identical with that undergone by presumptive mesoderm cells. This involves, first, the inhibition of the mobilization of the cell for epidermal differentiation, second, the passage of the cells through the two obligatory steps at which cultured cells may arrest in their evolution to neural and mesodermal cells.

\textbf{SUMMARY}

1. Methods are described for the short term culture of disaggregated amphibian embryonic cells in a medium consisting of about 0.1 per cent protein in Steinberg’s physiological salt solution. In a medium containing whole serum or serum protein cells differentiate according to their presumptive fate in the embryo. The use of a particle-free extract of mouse bone marrow causes ectoderm cells to develop as mesoderm.

2. Gastrula ectoderm cells will differentiate ciliated epidermis providing they are permitted to reaggregate into balls of about thirty cells and upwards. Gastrula mesoderm cells and induced ectoderm cells likewise do not differentiate unless aggregated, but the majority of aggregates fail to differentiate. This seems to be because these cells have exacting requirements for precise concentrations of the protein component in the medium. There is some variation between species in this respect, and requirements vary between ovulations in the case of \textit{Rana pipiens} and \textit{Xenopus laevis}.

3. Ciliated epidermal cells differentiate in cohesive balls that detach from the glass. All other cell types differentiate as thin outgrowths of cells attached to the glass by cytoplasmic fringes or pseudopodia.

4. The first visible event in the differentiation of non-epidermal cells is their attachment to the glass by pseudopodia or cytoplasmic fringes, and the rapid
expansion of these contributes to the deformation of the cell from the spherical embryonic form to that approximating to the form of the differentiated cell.

5. This rapid change in form and activity is termed germination. It appears to be a necessary preliminary for cytodifferentiation, the rapid utilization of yolk and the build up of large mitochondrial populations. Germination precedes the formation of striated muscle fibres, vacuoles in chordal cells and other indications of cytodifferentiation by a time interval that is characteristic for each cell type within a species.

6. The time and order in which cells differentiate in culture is related to the behaviour of comparable cells in the embryo. It is concluded that, by the neurula stage, the control of the time of germination is vested in the individual cells.

7. It is concluded that the development of neural and mesodermal cells involves two necessary periods of decisive change, one at the end of gastrulation unconnected with any gross morphological changes in the cells, the second, closely following neural closure and involving the in vivo counterpart of germination in culture, by which the potentialities of the cells are mobilized in the interests of cytodifferentiation. Both these important epigenetic events coincide with changes in the adhesive properties of cells.

RéSUMÉ

Culture in vitro de petits agrégats de cellules embryonnaires d'amphibiens.

1. On décrit des méthodes de culture à court terme de cellules embryonnaires désagrégées d'amphibiens, dans un milieu consistant en solution physiologique de Steinberg additionnée d'environ 0.1% de protéines. Dans un milieu contenant le sérum complet ou des protéines sériques, les cellules se différencient selon leur destinée présomptive dans l'embryon; l'utilisation d'un extrait aparticulaire de moelle osseuse de souris provoque le développement en mésoderme des cellules ectodermiques.

2. Les cellules ectodermiques de la gastrula différencient un épiderme cilié à condition d'avoir pu se réagréger en sphérules d'environ trente cellules ou davantage. De même, les cellules mésodermiques et les cellules ectodermiques induites de la gastrula ne se différencient pas si elles ne se sont pas réagrégées, mais cependant la majorité des agrégats ne se différencient pas. Il semble que ceci soit dû au fait que ces cellules ont des exigences strictes vis-à-vis d'une concentration précise du composant protéique du milieu. Il existe une certaine variabilité à cet égard entre les espèces étudiées, et les exigences varient selon les pontes dans le cas de Rana pipiens et Xenopus laevis.

3. Les cellules épidermiques ciliées se différencient en sphérules cohérentes qui se détachent du verre. Tous les autres types cellulaires se différencient en croissant sous forme de minces nappes cellulaires adhèrent au verre à l'aide de franges cytoplasmiques ou de pseudopodes.
4. Le premier phénomène visible dans la différenciation des cellules non-épidermiques est leur adhésion au verre à l’aide de pseudopodes ou de franges cytoplasmiques, dont l’expansion rapide contribue à la déformation rapide de la cellule, de la forme embryonnaire sphérique à celle ressemblant à la forme de la cellule différenciée.

5. Ce changement rapide de forme et d’activité est nommé ‘germination’. Il paraît être un prélude nécessaire à la cytodifférenciation, à l’utilisation rapide du vitellus, et à la formation de populations mitochondriales importantes. La germination précède la formation des fibres musculaires striées, des vacuoles dans les cellules chordales, et des autres indices de cytodifférenciation, selon un laps de temps caractéristique de chaque type cellulaire dans une espèce donnée.

6. Le moment où, et l’ordre dans lequel se différencient les cellules, sont en rapport avec le comportement des cellules comparables dans l’embryon. On conclut que, au stade neurula, le contrôle temporel de la germination est dévolu aux cellules prises individuellement.

7. On conclut que le développement des cellules neurales et mésodermiques comporte nécessairement deux périodes de modifications décisives, l’une à la fin de la gastrulation, sans liaison avec de fortes modifications morphologiques des cellules, l’autre étroitement consécutive à la fermeture du névraxe et impliquant la contrepartie in vivo de la germination observée en culture, par laquelle les potentialités des cellules sont mobilisées au service de la cytodifférenciation. Ces importants phénomènes épigénétiques coïncident tous deux avec des modifications de l’adhésivité cellulaire.

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