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ON THE DETERMINATION OF
EARLY CELL DIFFERENTIATION
IN AMPHIBIAN EMBRYOS

by

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CONTENTS

The origin of bilateral symmetry.......................... 1
Function of the dorso-ventral polarity..................... 2
The dorso-ventral activity gradient.......................... 3
Oxygen consumption of isolated blastomeres.............. 4
Inversion of the dorso-ventral polarity................. 5
The mechanism of the dorso-ventral polarity.............. 7
Injection of deoxynucleotides in fertilized eggs...... 9
Effect of deoxynucleosides on cell cultures...... 10
Formation of Ruffini's flask-cells...................... 12
Differentiation of ectodermal cells...................... 13
Spontaneous differentiation........................... 13
Induced differentiation............................... 14
General summary........................................ 16
Acknowledgements........................................ 18
References............................................... 19
THE ORIGIN OF BILATERAL SYMMETRY

Before fertilization the amphibian egg is a spherical body with a graded distribution of yolk from the animal to the vegetal pole. The animal hemisphere has less yolk and is normally characterized by a layer of dark pigment at the surface. These inequalities determine an animal-vegetal polarity, formally represented by an axis connecting the animal and the vegetal pole, which imposes radial symmetry on the egg. This polarity, laid down during oogenesis, is of importance for the future development insofar that the anterior part of the animal will be formed close to the animal pole of the egg and the posterior end in the proximity of the vegetal pole.

The acquisition of bilateral symmetry occurs at the fertilization and is due to the fertilizing sperm, the point of sperm entry and the animal-vegetal polarity together defining a median plane. In many amphibian embryos the bilateral symmetry is visualized a few hours after fertilization by the appearance of the so-called 'grey crescent', which outlines the dorsal side. The grey crescent is formed through contraction of the pigmented cortex towards the animal pole.

It has been demonstrated by artificial fertilization of the frog egg that any plane through the animal-vegetal axis may become the median plane of the organism (Ancel and Vintemberger, 1948). It must therefore be concluded that the
amphibian egg is radially symmetrical before fertilization. Experimentally the dorso-ventral polarity may be established by other means, e.g. mechanical stimulation (pricking) and by physical treatment (illumination, temperature and electrical induction shocks) (cf. Tyler, 1955).

In conclusion it appears that the agents involved in establishing the directional organisation reside in the egg. The animal-vegetal polarity is even determined in the latter, and in the determination of the dorso-ventral polarity the sperm functions merely as a trigger.

FUNCTION OF THE DORSO-VENTRAL POLARITY

In amphibian embryos the first division usually coincides with the plane of bilateral symmetry. The second cleavage, at right angle to the plane of symmetry, divides the embryo into a dorsal and a ventral 'half'. The former, when isolated, will form a normal but dwarfed animal. The latter, which contains little or no part of the grey crescent, forms a defective structure named 'Bauchstück' or 'belly piece', without notochord, nerve system or further organization. When separation is made along the first cleavage furrow, along the plane of symmetry, both blastomeres (right and left) will develop into normal dwarf animals (Spemann, 1903). These experiments convincingly demonstrate that the dorso-ventral polarity is causally related to the future development of a normal animal.
The dorso-ventral polarity early discloses itself in a gradient in cell size, the cells on the dorsal side at any given time being smaller than those on the ventral side. The next manifestation of the polarity is the appearance of pigmented spots around the median in the vegetative area. These spots are formed by a condensation of the faintly pigmented surface by a particular type of cells, first described by Ruffini (1907), who observed that they are flask-shaped, with long necks attached to the surface. Later observations have shown that these necks are microtubuli-containing filopodia (Perry and Waddington, 1966). It thus appears that the dorso-ventral polarity may control the formation of Ruffini's cells, and thus that it is engaged in directing the course of cell differentiation.

The blastopore spreads laterally to form first a crescent and then a circle. The observed temporal gradient in the process of invagination suggests that the dorso-ventral polarity functions in a quantitative rather than a qualitative manner, and it may be envisaged that this effect somehow is correlated with the observed gradient in the process of cell division. The various observations thus indicate the possibility that the dorso-ventral polarity acts by imposing an activity gradient across the embryo, with the dorsal side dominating.

THE DORSO-VENTRAL ACTIVITY GRADIENT

A great number of experiments have attempted to establish
whether a dorso-ventral activity differential is linked to a corresponding differential in energy consumption. These observations generally confirm the assumption that the dorsal region is metabolically more active than the ventral region (cf. Needham, 1942; Brachet, 1960).

**Oxygen consumption of isolated blastomeres**

The present experiments were carried out on eggs from *Xenopus laevis* (I). In these the first division plane usually coincides with the plane of bilateral symmetry, while the second divides the embryo into two smaller dorsal and two larger ventral blastomeres. From dry weight determinations 60% of the total mass was found to be confined to the ventral blastomeres.

At the 4-cell stage was isolated two (right, left, dorsal or ventral) blastomeres and the respiration of normal embryos and the various half embryos was determined during development. The respiration measurements were made by means of the electromagnetic diver respirometer (Løvtrup, 1973).

It was found that during the early stages of development no measurable difference was observed between the four half embryos. This means that on dry weight basis the oxygen consumption is approximately 50% higher in the dorsal half embryos than in the ventral ones at this early stage. The existence of a dorso-ventral activity differential, as reflected by the oxygen consumption, has thus been corrobo-
rated. In the normal embryos, and in the developing half embryos the respiration increases gradually and substantially from the onset of gastrulation; the values for the half-embryos being at every stage half of that of the normal embryo. In the ventral cell aggregate only a slight increase in oxygen consumption occurred.

*Inversion of the dorso-ventral polarity*

Is there any connection with the higher activity at the dorsal side and the fact that cell differentiation begins in this spatial region of the embryonic surface? The association of the dorso-ventral polarity with metabolic events, and the possible quantitative nature of this interrelation has been analysed through the speeding up of cellular metabolism. In a series of experiments, in which a lateral temperature gradient was applied to a fertilized egg, it was possible to shift the plane of bilateral symmetry towards the heated side (Glade et al., 1967). Experimental modification of the dorso-ventral polarity has also been achieved by unilateral restriction of the oxygen supply. Under these conditions it was found that the dorsal side always develops towards the oxygenated side (Løvtrup and Pigon, 1958); thus, if the ventral side is exposed to oxygen, an inversion of the dorso-ventral side takes place.

In the experiments to be described here, one normal embryo of *Xenopus laevis* was confined to the upper end of a diver respiration chamber to ensure unilateral supply of
oxygen (II). The embryos developed thus confined for 40 hours, during which time the oxygen consumption was recorded with the automatic electromagnetic diver.

In agreement with the previous results the blastopore and the tail always formed at the aerobic side. The results of the manometric determinations show that the oxygen consumption is distinctly higher in the controls, normally respiring embryos, than in the confined ones. The rate of consumption was also found to be higher with the dorsal side exposed to the air bubble than with the original ventral side outwards. This supports the previously registered differential in energy consumption, the dorsal side respiring at a higher rate than the ventral one. However, gastrulation occurs later in the inverted embryos, and there is no detectable difference in total oxygen consumption during gastrulation for the non-inverted and the inverted embryos. The same holds for the period of cleavage till the onset of gastrulation.

It thus appears that the normal function of the dorso-ventral polarity is to ensure a higher activity at the dorsal side, with the effect that gastrulation is initiated there. The crucial experiment for testing this proposition was to place an isolated ventral half under conditions of restricted oxygen supply. By imposing a gradient in oxygen consumption and, presumably, an activity gradient upon the isolated ventral half embryos, it was possible to induce the formation of a blastopore lip and the posterior part of the body. As mentioned above there is always a complete suppression of development with ambient supply of oxygen.
The various observations discussed here suggest that a gradient in metabolic activity is specifically involved in the establishment of bilateral symmetry in amphibians, and that the dorso-ventral polarity functions in a quantitative rather than a qualitative manner. If this is true then it follows that the metabolic events in the early embryo somehow favour the cell differentiation leading to the formation of Ruffini's cells.

It has been suggested by Holtfreter (1943) that these cells actively pull in the surface, thereby being responsible for, or at least involved in, the process of invagination. Ruffini's cells occur spontaneously only in the vegetal hemisphere, in the dorsal as well as in the ventral region. The latter is evidenced by the fact that in isolated ventral cell aggregates pigmented spots are scattered in the light vegetal area. It thus seems as if the differentiation of Ruffini's cells is controlled by the animal-vegetal polarity, while the formation of the dorso-ventral polarity is to impose a temporal gradient on this process.

THE MECHANISM OF THE DORSO-VENTRAL POLARITY

After fertilization, the egg undergoes a number (7-8) of 'synchronous' cell divisions. These cell divisions are distinguished by the fact that the G₁ and the G₂ phases are absent (Graham and Morgan, 1966) and that the S and M phases are of approximately equal length, the MI thus being about 50. In the late blastula (stage 7), a gradual decrease occurs in the MI, and in the gastrula the value becomes
less than 10 (Chulitskaia, 1970). At this stage $G_1$ and $G_2$
are present and constitute the major part of the cell cycle. Since
the dorso-ventral polarity is associated with a differential in cell size, the possibility exists that an explanation of the mechanism of the dorso-ventral polarity may be related to the latter, and that the mechanism responsible for the 'synchronous' cell divisions may somehow be involved.

According to analyses made on amphibian embryos (Hoff-Jørgensen and Zeuthen, 1952; Løvtrup, 1955, 1966) the amount of deoxyriboside-containing material remains constant during the early period of development, in spite of the mitotic activity going on, suggesting that DNA synthesis occurs on the basis of this material. The quantity of deoxyriboside-containing material lies in the range of 0.05-1 µg per egg, with a slight net increase beginning in the mid-blastula stage. This stage also marks the end of the rapid 'synchronous' cell divisions (Chulitskaia, 1970). It may be envisaged that the decrease in the rate of cell divisions reflects the exhaustion of preformed reserves. As regards the nature of the latter it was found that the egg of *Xenopus laevis* contains substantial amounts of the four deoxyribonucleoside triphosphates in approximately equimolar concentrations (Woodland and Pestell, 1972).

Since the end of 'synchrony' coincides approximately with the beginning of cell differentiation (Dettlaff, 1964) the possibility exists that the presence of deoxynucleotides prevents cell differentiation, and that the latter process cannot begin before these reserves are exhausted.
In order to test this theory one may modify the amount of cytoplasmic material by injections of deoxynucleotides into the embryo.

Injection of deoxynucleotides in fertilized eggs

30 nl of a solution of deoxyribonucleotides was injected into fertilized eggs of *Xenopus laevis* (III). The amount of deoxyribonucleoside triphosphates injected corresponded to 4, 16 and 64 times the normal content of the egg (32 pmoles).

As expected, mitotic counts showed the MI of the controls to decrease distinctly during gastrulation. In contrast, the injected embryos (64 x the normal content), of an age corresponding to stage 11, have a value of the MI close to those of stage 9 controls. We may thus conclude that the injected deoxyribonucleotides have prolonged the phase of high mitotic activity. It was furthermore found that in a varying number of instances the development and thus cell differentiation, was completely suppressed in the experimental embryos. This effect is concentration-dependent and has the greatest inhibitory effect when all four nucleotides are injected. Any mixture of these nucleotides is of equal influence, thus none of the nucleotides is of particular importance for the development arrest. These experiments thus support the contention that deoxynucleotides present in the cytoplasm inhibit the onset of cell differentiation.

In order to examine the effect of injected deoxyribonucleotides on RNA synthesis, controls and experimental
embryos were incubated in NaH\(^{14}\)CO\(_3\) (5 \muCi/ml) for ten hours at room temperature. The incorporation into the RNA of the injected embryos was significantly lower than in the controls. This effect of injected nucleotides may account for the developmental block previously described.

The proposition that the cytoplasmic deoxynucleotides suppress cell differentiation, and hence morphogenesis, thus appears to have an experimental support. Since, accordingly, the blastomeres must exhaust their store of deoxyribonucleotides before they can transform or differentiate, we may, with reference to classical embryology, say that the embryonic cell acquires competence only when the state of exhaustion has been reached; before that they may be said to be 'precompetent'. The effect of deoxyribonucleotides does not exclude, of course, that other substrates are involved in this control function.

**Effect of deoxynucleosides on cell cultures**

The observed effect of deoxynucleotides has been corroborated through observations on cultivated cells (IV). The cells were isolated from around the animal pole or from the vegetal hemisphere in the blastula of the axolotl, *Ambystoma mexicanum*.

Under normal conditions, the animal cell aggregates round up and become ciliated on the third day of culture, indicating a spontaneous transformation to ciliated cells. Vegetal cell aggregates give rise to fibroblast-like cells
on the third day. If these cells are Ruffini's cells then our observations on cultured cells directly corroborate the contention that the cell differentiation leading to Ruffini's cells is the responsibility of the animal-vegetal polarity. Both kinds of spontaneous cell differentiation are suppressed when deoxynucleosides are added to the medium in a concentration of 10 mM, the cells going on dividing at least twice more than the control cells, as evidenced by the reduced size of the deoxynucleoside-treated cells. Autoradiographic estimations of the incorporation of uridine in the nuclei also show that deoxynucleosides suppress the synthesis of RNA.

Addition of cytosinarabinoside, which prevents \textit{de novo} synthesis of DNA (Pizer and Cohen, 1950), has no effect on the differentiation pattern. The differentiated ciliocytes and Ruffini's cells appear at the normal time, but they are four times larger than the controls. These experiments suggest that after the exhaustion of their deoxyribonucleotide reserves, the cells normally divide twice before they undergo differentiation. If \textit{de novo} synthesis of DNA is prevented, however, they may differentiate, in agreement with the exhaustion theory advocated above.

From the fact that the differentiated control cells and cytosinarabinoside cells appeared at the same time, it may be inferred that also other factors are involved in the control of spontaneous embryonic differentiation.
FORMATION OF RUFINI'S FLASK-CELLS

It has long been known that gastrulation in amphibian embryos, and thus the formation of Ruffini's cells, is completely suppressed by anaerobiosis. It therefore may be assumed that the formation of Ruffini's cells, in some way or other, is dependent upon the occurrence of certain oxidative processes. We have tried to approach this problem through observing the effect of various inhibitors on cultured cells (V). It may be anticipated that inhibitors of RNA synthesis are also inhibitors of the differentiation of vegetal cells into Ruffini's cells. This was experimentally confirmed for actinomycin, cordycepin and deoxynucleosides. KCN, dinitrophenol and malonate, supposed to interfere with the oxidative metabolism, had the same effect. Lactate was also found to suppress differentiation, but the effect was different from that of the other agents, for lactate must be present before the second day of culture; the other inhibitors were active also when added on the second day. An explanation of this difference may possibly be found in the fact that, as shown in autoradiographic studies, lactate suppresses RNA synthesis, but much less efficiently than the other inhibitors of RNA synthesis.

None of the experiments reported above gives any clue to the mechanism of the animal-vegetal polarity. The fact that lactate inhibits cell differentiation may explain the effect of anaerobiosis, but for various reasons it can be excluded that lactate is involved directly in the control of cell differentiation in the normal embryo.
DIFFERENTIATION OF ECTODERMAL CELLS

As previously mentioned, in a balanced salt solution cells isolated from the animal pole at blastula stage differentiate into ciliated explants, while they never spontaneously give rise to fibroblast-like cells.

Spontaneous differentiation

By means of vital staining and other techniques it has been shown that the part of surface ectoderm located outside the neural folds may be regarded as the prospective epidermis (Holtfreter, 1936). Ciliated cells frequently appear in the outer layer of the embryo but not in the underlying cell layer, which are exposed to the internal medium of the blastocoel. By culturing explants from the animal region of the blastula in different tonicities (VI), it was possible to show that the transformation to ciliated cells is suppressed by hypertonicity, but favoured in a hypotonic medium. This cell differentiation, as might be expected, is dependent upon the synthesis of RNA, as shown by its suppression by actinomycin. It also requires the occurrence of some process of sulphatation, probably of some glycosaminoglycan, since it is inhibited by selenate (Wilson and Bandurski, 1958). These findings indicate that contact with a hypotonic external medium is necessary for the differentiation of ciliated epidermal cells.
Induced differentiation

The isolated ectoderm of the blastula thus has a limited range of differentiation when left to itself. However, by adding LiCl to the growth medium it is possible to obtain fibroblast-like cells from animal region explants (Barth and Barth, 1959). Exactly the same cell type appears if animal cells are mixed with a few vegetal cells (V). Apparently, the latter through homotypic induction can impress their differentiation pattern on the animal cells.

The organizing properties of the dorsal lip in the amphibian embryo has been demonstrated in several ways (cf. Holtfreter and Hamburger, 1955). For many years it has also been presumed that the determination of the presumptive neural fold and the neuralization of animal cells is mediated by some agent emanating from the dorsal lip. The inducing action has been demonstrated by the fact that the graft from the dorsal lip can induce the formation of secondary embryos in regions with different prospective fates (Spemann, 1938).

Many experiments have also been devoted to the search for the chemical agent of the primary induction. Various substances have been found to be active, but all this work is hampered by the fact that unspecific agents like Li$^{2+}$ are effective. Therefore one can never be sure that a given substance is specific or not. Under these circumstances it would seem necessary to require of a presumed inductor that it is known to be present in the inducing cells.
It has been suggested that heparan sulphate is a general constituent of the fibroblast cells (Conrad and Hart, 1975). It has also been shown by Kosher and Searls (1973) that there is an intense incorporation of sulphate in the cells around the blastopore and that the sulphated glycosaminoglycan in question probably is heparan sulphate (cf. also Höglund and Lövtrup, 1976). It therefore seems likely that the fibroblast-like Ruffini's cells, responsible for invagination, contain heparan sulphate. That sulphatation is necessary for the appearance of Ruffini's cells is demonstrated by our finding that the inhibitor selenate completely suppresses their formation and also the process of gastrulation in intact embryos (V).

We have tested the possibility that heparan sulphate is the agent of the primary induction by adding it to the growth medium of cultured cells from the animal region (VII). In the concentration of 0.01 μg/ml the animal cells differentiated as usual into ciliated aggregates. The concentration of 10 μg/ml suppressed aggregate formation and cell differentiation. In the intermediate concentrations, 1 μg/ml and 0.1 μg/ml, outgrowth of fibroblast-like cells occurred followed by the subsequent appearance of various ectodermal differentiation patterns, as mesenchyme cells, nerve cells, pigment cells, etc.). Obviously heparan sulphate is effective in very low concentrations, it is therefore possible that it may be the agent normally involved in the primary neural induction.
Through measurements of respiration of isolated blastomeres and in local regions of amphibian embryos, it is shown that the dorso-ventral polarity is associated with a differential in oxygen consumption; the dorsal side being the most active. It is concluded that the gradient in energy consumption is related to a gradient in activity and furthermore that this is instrumental in embryogenesis by imposing a temporal gradient on the process of cell differentiation. The determination of early cell differentiation and its association with the animal-vegetal axis is evident by the fact that the cell differentiation necessary for the onset of gastrulation occurs spontaneously only in vegetal cells. The function of the dorso-ventral polarity is to ensure that this cell differentiation begins at a spatially confined region.

The quantitative nature of the effect of the dorso-ventral polarity, and its association with metabolic activity, is demonstrated by the finding that upon unilateral restriction of oxygen supply, gastrulation always begins at the aerobic side.

It is furthermore proposed that the deoxyriboside triphosphates present in the fertilized egg may control the early development, in that these substrates promote cell divisions but inhibit the onset of cell differentiation. Since the exhaustion is correlated to the cell size and since the dorso-ventral polarity imposes a differential with respect to this parameter, it follows that the syn-
thesis of RNA and hence also cell differentiation, may begin earlier at the dorsal than at the ventral side.

The spontaneous differentiation of Ruffini's cells, involved in the initiation of gastrulation, is suppressed by lactate as well as various inhibitors of RNA synthesis. The effect of lactate differs from that of the other agents insofar as suppression is ineffective after a certain period of time.

The differentiation of Ruffini's cells from isolated vegetal explants occurs spontaneously. Similar fibroblast-like cells may be formed in explants from the animal region provided they are induced by vegetal cells or by heparan sulphate. It is suggested that heparan sulphate may serve as a natural neural inductor in the amphibian embryo.
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