Differentiation of Cells of the *Rana p. pipiens* Gastrula in Unconditioned Medium

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*WITH THREE PLATES*

**INTRODUCTION**

Instances of differentiation in absence of an inductor have multiplied to such extent that the classical picture of embryonic induction is in process of serious re-examination (Grobstein & Parker, 1958; Nieukoop, 1955; Trinkaus & Groves, 1955). Questioned on the basis of the occurrence of neural differentiation without organizer in explants of presumptive epidermis (Barth, 1941; Holtfreter, 1947, 1955; Itô, 1952), the necessity for a specific inductor has proved difficult to demonstrate in other inductive systems. Grobstein & Parker (1958) have shown that tubule formation in metanephrogenic mesenchyme may occur in abnormal sites *in vivo* without contact with its normal ureteric inductor and even in the anterior chamber of the eye without contact with any other tissue. Niu (1958b) has recently reported a certain percentage of neural differentiation in explants of presumptive epidermis cultured as controls in his salt medium without addition of inductor substances.

Such observations not only emphasize the difficulty of finding an adequate neutral environment for the study of inductors but also suggest the necessity for revision of such concepts as induction and self-differentiation.

The present experiments were undertaken with the objective of working out a simple system for culturing presumptive epidermis in a situation that would permit easy alteration of the culture medium and facilitate observation of the effects on differentiation of specific additions to the medium. The work of Niu (1956) had demonstrated in elegant fashion the advantages of using very small explants which could attach to glass and spread out in a single cell layer eliminating the need for sectioning. The success of Niu’s medium (Niu–Twitty solution) in cultures of *Ambystoma* and *Triturus* explants offered new hope that a similarly modified Holtfreter’s solution might support *in vitro* differentiation of early embryonic cells from *Rana R. p. pipiens* was chosen as the source of presumptive

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epidermis explants because of the relative resistance of this species to neuralization by factors other than the living presumptive chordamesoderm, its normal inductor (Smith & Schechtman, 1954).

The results to be reported in the present paper lend additional support to the idea that presumptive epidermis cells possess a spectrum of differentiation potentials that may be brought to expression without contact with an organizer or even exposure to a medium conditioned by such an organizer.

EXPERIMENTAL PROCEDURE

Operations

Eggs of *R. pipiens* obtained by pituitary injection were used at early gastrula stages (10 plus to 11 minus of Shumway, 1940). Jelly was removed by means of watchmaker's forceps, and the eggs were passed through three changes of sterile 10 per cent. Ringer's solution. All solutions and instruments used thereafter were sterile. The explants were dissected out in solution *X* (see section on solutions) on 2 per cent. washed Bacto-agar. A median strip extending ventrally from animal pole to a line approximately 60° from the animal pole provided presumptive epidermis explants (regions 3 and 4), while the area just above the dorsal lip of the blastopore (region 1) was used as presumptive chordamesoderm (numbers of regions refer to diagrams in Sze, 1953). In the absence of a precise map of the gastrula of *R. pipiens* we took the precaution of observing the development of the donors of the presumptive epidermis. The opening into the blastocoel healed rapidly at room temperatures and normal appearing late neurulae developed within 20 hours. The small wound marking the site of explantation was located ventrally about midway along the antero-posterior axis of the embryo.

Four explants at a time were transferred by means of a Spemann pipette to the versene dissociating medium in a stender dish with agar-coated bottom. The explants, with pigment coat side down, remained in the dissociating medium for an average time of 5 minutes, after which they were returned to solution *X* on agar. The pigment coat layer then could be peeled off easily with glass needle and hair loop, and the underlying layer of presumptive epidermis cells, loosened by the versene treatment, could be teased apart gently into small aggregates of about 125 cells each. Each explant yields approximately 20 aggregates, with an average diameter of 200 μ. These aggregates became spherical and healed within about 30–60 minutes in solution *X*. They then were plated out on to sterile, round coverslips in small stender dishes containing solution *X*. Such cultures were raised at room temperatures ranging from 24° to 27° C. Daily observations could be made with minimal danger of contamination by covering the compound microscope with a shield of transparent plastic sheeting.

When permanent records of a culture were desired the culture medium was drawn off, and the preparation was washed free of globulin with solution and fixed with aqueous Bouin's solution. Staining with 50 per cent. Harris's haematoxylin and dehydration were carried out with the culture still on a coverslip in
the stender dish. Final dehydration, brief counterstaining with alcoholic eosin, and clearing in xylol were done by lifting out the coverslip with fine forceps and passing it through 20 ml. stender dishes containing the alcohols or xylol. The coverslip bearing the culture was inverted over a slide bearing a small drop of mounting medium.

**Solutions**

1. *Medium for operation and culture*

Preliminary experiments to devise a culture medium for cells of the early *R. pipiens* embryo were based upon modifications of both Holtfreter's solution and Niu-Twitty solution, neither of which proved satisfactory under the conditions of our experiments. The composition of the saline medium used in the present experiments is given in Table 1.

**Table 1**

*Composition of solution X*

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<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td></td>
<td>NaCl (Merck Biol.)</td>
<td>NaHCO₃ 0·200 g.</td>
<td>Na₂HPO₄ 0·300 g.</td>
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<tr>
<td></td>
<td>5·150 g.</td>
<td></td>
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<tr>
<td>KCl</td>
<td>0·075 g.</td>
<td></td>
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</tr>
<tr>
<td>MgSO₄ 7 H₂O</td>
<td>0·204 g.</td>
<td>H₂O to 250 ml.</td>
<td>KH₂PO₄ 0·0375 g.</td>
</tr>
<tr>
<td>Ca (NO₃)₂.H₂O</td>
<td>0·062 g.</td>
<td></td>
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<tr>
<td>CaCl₂ 2 H₂O</td>
<td>0·060 g.</td>
<td></td>
<td>H₂O to 250 ml.</td>
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<td>H₂O to 500 ml.</td>
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Double distilled water prepared in a glass still during the second distillation was used throughout. The stock solutions were kept in the refrigerator. Aliquots consisting of 50 ml. of *A* plus 10 ml. of glass distilled water to correct for evaporation during autoclaving; 25 ml. of *B*; and 25 ml. of *C* were autoclaved for 20 minutes at 15 lb. pressure. Before mixing these three aliquots together, 100 mg. of serum globulin (Bios) were added to the flask containing 25 ml. of sterile *C* or *B*, brought to a boil several times over a flame to sterilize, and cooled before addition to the other components.

The addition of the 0·1 per cent. globulin proved essential. Without globulin the aggregates attached to the coverslip but showed signs of injury (fuzzy cell outlines) within 24 hours and failed to differentiate. Other macromolecular constituents tested in preliminary experiments (albumin, fibrinogen, chick embryo extract, tadpole extracts) gave less or no protection.

The inclusion of globulin in the culture solution has made it possible to obtain cell division and differentiation in volumes of solution ranging from 2 to 16 ml. Preliminary experiments showed that small explants of presumptive epidermis cultured in hanging drops of solution *X* without globulin would differentiate and form nerve fibres. When larger volumes were used (2 or more ml.), the explants failed to differentiate unless globulin was added. With added globulin
presumptive epidermis will form nerve fibres in our largest culture dishes containing 16 ml. of solution.

The pH of solution X varies from 8·1 to 8·8 with the length of time elapsed after autoclaving during which carbon dioxide is driven off and only gradually replaced by atmospheric carbon dioxide. The final pH of solution X in the culture dishes was 8·1.

2. Dissociating medium

During the early phases of this investigation a glycine-KOH buffer at pH 9·8 was compared with versene as a dissociating medium. The alkaline buffer (suggested by the work of Townes & Holtfreter, 1955) gave the same results as did versene for dissociation and later behaviour of the aggregates during culture in solution X. The necessity of adjusting the pH of the glycine buffer introduced danger of bacterial contamination so that versene finally was settled upon as the standard dissociating medium. It was further established that small aggregates of cells obtained by mechanical teasing with a hair loop differentiated upon culture in a manner entirely comparable with that exhibited by aggregates obtained by alkaline or versene dissociation. The chemical dissociation therefore facilitates dissection without affecting the later differentiation capacities of the cells. Composition of the versene medium is set forth in Table 2.

<p>| TABLE 2 |</p>
<table>
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<th>Composition of dissociating medium</th>
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<tr>
<td><strong>A</strong></td>
</tr>
<tr>
<td>NaCl (Merck Biol.) 6,800 mg.</td>
</tr>
<tr>
<td>KCl 100 mg.</td>
</tr>
<tr>
<td>Versene* 744 mg.</td>
</tr>
<tr>
<td>H₂O to 500 ml.</td>
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* Disodium ethylenediamine tetraacetate (Fisher).

The medium for dissociation thus consists of versene in double 'A' Niu-Twitty solution from which calcium and magnesium have been omitted. Its pH is 7·6. Glass distilled water was used. Aliquots consisting of 50 ml. of A, 25 ml. of B, and 25 ml. of C were autoclaved separately and combined under sterile conditions before use.

OBSERVATIONS AND RESULTS

I. Differentiation of standard cell types from the gastrula of R. pipiens

In order to identify cell types under experimental conditions it is obviously necessary to know what given cell types look like under the standard conditions used for culture.
Identification of cell types

Preparations of explants from the dorsal and lateral lips of the blastopore of early gastrulae as well as explants from the chordamesoderm and neural plate of older embryos were cultured in order to provide a basis for the identification of cell types.

Explants of the lateral lip of early stage 11 gastrulae attach to the coverslips and differentiate into nerve-cells with nerve fibres, muscle which shows irregular twitching, pigment cells, and 'giant' cells in 3 days at room temperatures. The cultures show progressive differentiation and maintain themselves for from 10 to 20 days.

Nerve-cell differentiation is shown in Plate 1, fig. 1 in a 4-day culture. The individual fibres originate as outgrowths of neuroblasts as described by Harrison (1910). Many nerve fibres may migrate out together, forming a nerve-trunk from which groups of fibres branch. The fibres often terminate in peripheral cells but sometimes migrate in a straight line over the coverslip without making attachment.

Muscle-cell differentiation is illustrated in Plate 1, fig. 2 in a 5-day preparation. Although this particular cell does show cross-striations, often functional muscle-cells show no cross-striation. The form of muscle-cells varies a great deal, but the cell usually can be recognized by its large size, elongate shape, and the presence of internal fibres after fixation and staining.

In 60 per cent. of the cultures single muscle-cells or groups of muscle-cells were observed to twitch at intervals. A period of several contractions was followed by a period of quiescence for several minutes after which twitching was resumed. Muscle-twitching continued for 6 days at room temperatures, after which time the muscle remained quiescent. The muscle then responded to electrical stimulation for two additional days.

Although 100 per cent. of the cultures contained muscle-cells, only 60 per cent. exhibited muscle-contraction. In the latter group nerve-fibres from an adjacent mass of nerve-cells terminated in the muscle-cells. The non-functional muscle-cell did not receive nerve fibres. These muscle-cells did, however, contract when stimulated electrically.

The above observations suggest that the nerve-cell bodies discharge at irregular intervals and send impulses along the fibre to muscle which responds by twitching. Further evidence of nerve action was obtained when simultaneous muscle-twitching was observed in two or more masses of muscle-cells with a common nerve supply. When nerve fibres migrate and make contact with muscle-cells some of the fibres may continue to migrate and innervate a second group of muscle-cells. When this occurs the two groups of muscle-cells twitch at the same time and remain quiescent for the same period. As many as three separate masses of muscle-cells connected only by nerve fibres have been observed to undergo simultaneous muscle-twitching. When the nerve fibres are scraped from
the coverslip with watchmaker's forceps the denervated muscle-mass stops twitching, while the innervated mass continues to contract. A single nerve fibre is sufficient to conduct impulses to a single muscle-cell. Plate 1, fig. 3 shows a single muscle-cell with a single nerve fibre. This cell was the terminal muscle-cell of a chain of three groups of cells showing simultaneous twitching.

Some of the cells from lateral lip explants behave in a different fashion from presumptive nerve- and muscle-cells. While the latter form masses, other cells spread out as individual cells and assume shapes varying about the stellate form. On the third day very distinctive slate-grey pigment granules form a pigmented ring near the nucleus. After 9 days of culture these cells assume the shape of pigment cells. The description of differentiation of pigment cells will be given in more detail in the section dealing with the effects of lithium chloride.

Another type of cell has been termed a 'giant' cell. It is characterized by its large size and large nucleus as compared with a neuroblast. The term giant cell is inappropriate since these cells are simply so flattened against the coverslip that the area of the cell and nucleus is greater than in a spherical cell. We have not been able to ascertain the fate of these cells with any certainty. However, on the 6th-7th days a new formation of cells appears. Elongate cells line up in parallel fashion to form a sheet one cell in thickness. The sheet increases in surface area with time, and it may be that the giant cells contribute to it. Mitotic figures are present in the stained sheet of cells. Plate 1, fig. 4 illustrates a portion of a sheet of elongate cells after staining. The cell type is termed X in this paper, since we have not obtained any recognizable cell type from such cells.

Although dorsal and lateral lip explants from the early gastrula do contain presumptive notochord cells, we have found only occasional differentiation of notochord. Within the mass of cells which forms nerve fibres small clumps of larger cells with very clear cytoplasm may be diagnosed as notochord cells.

Our study of the stained preparations is by no means exhaustive and possibly we have overlooked other cell types present. Our main objective was to identify a few common cell types for comparison with our experiments with the presumptive epidermis.

Cellular differentiation from presumptive epidermis

Explants of the presumptive epidermis, prepared as described in the section on methods, attach to coverslips and cell migration is under way by the second day at room temperatures. Cells leave the main central mass and spread out in all directions as individual cells and also as a sheet. Since the cells are thin, the surface areas of the nucleus and cytoplasm are large and the cells are termed 'giant' cells. Patches of the sheet are composed of ciliated cells and in some instances the entire mass may fail to attach and instead develop cilia at the surface. These swim about for several days. From attached masses nerve fibres develop on the fourth day. They migrate over the surface of the sheet of giant cells and on to the glass surface where they may continue to migrate or make
attachment to individual cells in the periphery. The central mass of cells containing neuroblasts continues to send out nerve fibres, and by the 6th day (Plate 2, fig. 5) many individual nerve fibres are present, as well as bundles of fibres. The fibres branch and also form networks. Many fibres terminate in peripheral cells.

On the seventh day elongate cells with elongate nuclei appear and the cells line up in parallel fashion. The sheet of unoriented cells with circular or ellipsoidal nuclei forms wedge-shaped patterns of oriented cells at the periphery (Plate 2, fig. 6). The parallel alignment of cells then spreads throughout the entire sheet. The appearance of the cell which we call \( X \) is shown in Plate 1, fig. 4 after 3 weeks at room temperature.

While the sheet of cells is undergoing the transformation described above, the nerve fibres become detached from the sheet and later from the glass surface and finally the central mass containing cell-bodies loosens and floats off. As a result, only the oriented sheet of cells and a few detached cells are left on the coverslip after about 10 days of culture. No significant changes can be seen between 10 days and 3 weeks of culture.

Study of the living cultures and of the stained preparations have given no evidence of muscle, pigment, or notochord cells from presumptive epidermis.

The fate of small explants from the outer pigmented layer of epidermis was followed for several days in culture. The cells become ciliated and long streamers of mucus are exuded from the explant. Generally the explant does not attach to the coverslip. While the inner layer requires globulin for survival, the outer layer lives well in solution \( X \) without globulin. Similarly, the wounds in the early stage 11 donors of the presumptive epidermis heal well and gastrulation and neurulation occur in solution \( X \) without globulin. Globulin therefore appears to be necessary only for survival of cells from the inner layer of the gastrula.

II. The effect of lithium upon differentiation of presumptive epidermis

Lithium chloride was added to solution \( B \) and boiled briefly to sterilize before mixing with \( A \) and \( C \) containing 100 mg. of globulin. The final concentration of lithium chloride was 0·1 M in the standard solution \( X \) with globulin and the pH of the mixture was 8·5. Following operation in the standard solution \( X \), aggregates were immersed in the lithium solution for intervals varying from 1 to 5 hours after which they were plated out on to coverslips in the standard solution \( X \) for culture. Occasional experiments were made using long duration (18–19 hours) of lithium treatment at low concentration (0·005 M LiCl). The results did not differ from those obtained with the short lithium treatment.

A difference in behaviour of aggregates treated with lithium from those not so treated becomes apparent as early as the second day. The aggregates spread out completely on the coverslips as flat sheets of cells; only rarely does a small clump remain. On the third day, by which time control whole embryos kept at the same temperature have reached hatching (S 20), the aggregates have become massive sheets of about 100 flat, semi-detached giant cells each. By the fourth
D I F F E R E N T I AT I O N O F G A S T R U L A C E L L S I N V I T R O

day an accumulation of pigment in each cell, that is sometimes apparent even on the third day, is a typical feature of these lithium-treated cells. Near the nucleus an accumulation of pigment granules forms a slate-coloured ring in the cytoplasm (Plate 2, fig. 7). This type of cell was never observed in standard presumptive epidermis cultures, although pigment-ring cells were common in cultures of presumptive chordamesoderm in solution X.

From the fifth day on there occurs a progressive transformation of the pigment-ring cells into a variety of cell shapes. These changes occur first at the edges of the large sheets of ring cells and by 14–16 days only a few ring cells remain, the rest having assumed some of the following shapes. Dendritic and spindle-shaped cells are found (Plate 2, fig. 8 and Plate 3, fig. 9). In other cells the pigment rings disperse to give uniform pigmentation in large cells with amoeboid processes (Plate 3, fig. 10). In some instances these were seen to become vacuolated and to transform into highly dendritic melanophores (Plate 3, fig. 11).

The effect of lithium is a highly consistent one. When, as described above, the aggregates attach and spread out into a single layer of large cells they undergo 90–100 per cent. transformation into the pigment-ring cells. When an occasional small clump of cells remains at the centre of such a sheet of cells, nerve fibres extending out from the clump are a not infrequent character of later differentiation. The fate of the pigment-ring cells during the second week of culture is varied, as described above, but none of the cell types thus evoked are characteristic of presumptive epidermis cultured in solution X.

A suggestive although preliminary result was obtained when the presumptive epidermis explants were treated from 3 to 5 hours with 0·1 M LiCl in solution X in which water had been substituted for bicarbonate and carbon dioxide had been bubbled through to a pH of 5·35 before the beginning of the treatment. In addition to the massive transformation into ring cells and their differentiation into the usual spindle-shaped, mesenchyme-like, or dendritic cells typical of lithium cultures, two new types of cells appeared in some of the cultures. Notochord, diagnosed on the basis of its tubular structure and vacuolated cells, was found in some cultures (Plate 3, fig. 12), and in others a cell type characterized by possession of emerald green pigment vacuoles appeared during the second week of culture.

D I S C U S S I O N

The results of the foregoing experiments contribute to the recent revival of the issue of differentiation potentialities of normal presumptive epidermis cells. Cultured in a medium of pure salts, the small explants from the inner layer of presumptive epidermis showed injury and cytolysis and failed to differentiate. Upon the addition of serum globulin what appears to be a protective action of the protein was found, and nerve-cells with nerve fibres differentiated from presumptive epidermis.

Grobstein & Parker (1958) have outlined most succintly the puzzling prob-
lem of the non-specific effects upon self-differentiation of in vivo sites. The present results could well be interpreted as a protective or 'sparking' action by the globulin, permitting expression of a wider range of inherent differentiation potentialities. Bautzmann (1929) and Holtfreter (1929) demonstrated neuralization of ectoderm in explants in the cavity of the eye and in the coelomic fluid respectively.

The necessity for 'conditioning' a medium containing serum for culture of cells is universally recognized (e.g. Earle, Bryant, & Schilling, 1954). Most workers provide for such conditioning by using a low ratio of medium to cell number. It is significant that the present experiments demonstrate that upon addition of globulin, the ratio of volume of medium to cells no longer is a critical factor.

Qualitative differences between conditioned media were indicated by Niu's (1956) work. While Niu obtained neural differentiation from presumptive epidermis cultured in medium conditioned for 7–10 days by inductor tissues, he obtained an estimated 73 per cent. myoblast differentiation in medium conditioned for 12 days. The present results show that conditioned medium is not necessary for neural differentiation, but we have as yet observed no differentiation of myoblasts from presumptive epidermis, despite the fact that our medium supports the differentiation of functional striated muscle from lateral lips of the blastopore. We feel that the question is still open as to whether different substances or different concentrations of the same substance are required for myoblast differentiation. A recent assay (Fraser, 1957) of the stimulatory effect of various fractions of egg albumin on somite differentiation in the early explanted chick embryo suggests rather complex though still fairly non-specific requirements. For example, alanine and a sulphur-containing protein are among the requirements for somite differentiation.

Another indication that globulin is not entirely adequate to support differentiation under the present conditions of culture comes from the low incidence of notochord in our cultures. This apparent deficiency, however, might be due to the difficulty of diagnosing notochord cells before they become oriented into their characteristic tubular arrangement (Mookerjee, Deuchar, & Waddington, 1953).

The mass transformation of cell type obtained with lithium treatment has two important aspects. First, the lithium effect is a consistent one affecting at least 90 per cent. of the cells under optimal conditions. Secondly, the new cell types produced by lithium differentiate long after the lithium is removed, showing that a permanent transformation has occurred. A disappointing feature of certain other transformed cells has been their reversion to original cell type upon removal of the transforming agent.

The appearance of cells after brief lithium treatment bears a striking resemblance to that pictured and described by Wilde (1955b) for ventral ectoderm and neural crest cultures of Ambystoma to which phenyl alanine or tyrosine has
been added. Wilde noted the appearance of pigment granules first near the nucleus of phenyl alanine-induced melanophores (cf. our pigment-ring cells), confirming Niu's (1954) earlier study.

The mutual repulsion, i.e. spreading and migration, of the lithium-treated presumptive epidermis cells resembles that demonstrated by Twitty (1953) and Twitty & Niu (1954) for pro-pigment cells in vitro. The need for oxygen in melanin synthesis (Twitty & Niu, 1954) would be well fulfilled in our experiments where cells are spread out in a thin layer and exposed to a relatively large volume of culture medium. The vacuolization of giant cells during transformation into melanophores pictured by Niu (1954) was observed in our lithium cultures (Plate 3, fig. 11). In our experiments a release of melanogenic chemicals from disintegrating melanophores (Niu, 1954) cannot be involved in the transformation.

As to the biochemical basis for the lithium effect on differentiation of presumptive epidermis, a search of the literature on lithium is of little assistance. Long used by embryologists as an experimental tool (see references to literature in Dent & Sheppard, 1957), the lithium ion has received little attention from biochemists. Some information has come from the work of Ranzi and his colleagues on the effect of lithium on protein structure (Ranzi et al., 1957, cite references to their earlier work). Further information presented by Gustafson (1950) implicates lithium as an inhibitor of respiratory enzymes and protein synthesis. That lithium in the amphibian egg is bound by material other than yolk was demonstrated by autoradiography by Dent & Sheppard (1957), an observation in agreement with the earlier work of Ficq (1954) who found a concentration of lithium associated with melanin granules. The observation in the present experiments that an exposure of as short duration as one hour sufficed to alter the direction of differentiation might indicate that such binding of the ion by some cell structure is a comparatively rapid event.

Although the fact that presumptive epidermis can be neuralized by abnormal treatment of a continuing character was demonstrated by Waddington, Needham, & Brachet (1936), 'shock' treatments of presumptive epidermis that result in neuralization first were described by Holtfreter (1947). Yamada's (1950) extensive studies using ammonia shock treatments furthered the concept that presumptive epidermis could be 'dorsalized' by such non-specific means. Pasteels's (1953) activation of presumptive epidermis by brief centrifugation at low speeds may belong in the same category, since explants of presumptive epidermis made immediately after centrifugation developed neural structures. Flickinger (1957), using carbon dioxide treatment at pH 3.7 for 10–60 minutes, also reported neuralization of presumptive epidermis. The present experiments amplify the findings of these investigators in that the presumptive epidermis is shown capable of neuralization without 'shock' treatment when protected by globulin in the culture medium.

In some interesting recent experiments by Karasaki (1957) it was demonstrated
that disaggregation alone by such agents as Ca-free Holtfreter's is not a sufficient condition for dorsalization, a conclusion supported by our own control experiments using mechanical dissociation to obtain small explants. Karasaki suggests that the neuralization brought about by 'shock' treatments (acids and alkalis) may be mediated by changes in the physical structure of the endoplasm manifested by active protoplasmic movements. Lithium in Karasaki's experiments had a suppressive influence upon dorsalization, which he associates with the increase in viscosity of proteins effected by lithium.

In the present experiments also lithium had a suppressive influence upon the differentiation of nerve-cells from presumptive epidermis. Suppression of this capacity for neural differentiation having been brought about by lithium, the presumptive epidermis cells then exhibit a further differentiation potentiality; they can form melanophores and mesenchyme.

SUMMARY

1. A method has been devised for the culture of gastrular cells of R. pipiens.
2. Histological and functional differentiation of nerve, muscle, and pigment cells is described as well as the differentiation of ciliated, mucus-secreting, and notochord cells.
3. The inner layer of the presumptive epidermis differentiates into nerve-cells, ciliated cells, and 'giant' cells, in addition to an unidentified cell type $X$.
4. Brief lithium treatment of presumptive epidermis results in a permanent transformation into pigment cells and mesenchyme.

ACKNOWLEDGEMENT

We owe the suggestion of the versene mixture employed for dissociation to the late Dr. Richard Stearns.

REFERENCES

DIFFERENTIATION OF GASTRULA CELLS IN VITRO


EXPLANATION OF PLATES

Plate 1

Fig. 1. A 4-day culture of the lateral lip of the blastopore of Stage 11 showing 'giant' cells and nerve fibres. High power.

Fig. 2. A 5-day culture of the lateral lip of the blastopore of Stage 11 showing a striated muscle-cell. This cell exhibited muscle-twitching at intervals. High power.

Fig. 3. Muscle-cell from a 6-day culture of dorsal lip of blastopore of Stage 11. The cell possessed four extensions containing fibrils and contracted simultaneously with two nearby groups of muscle-cells. The only nervous connexion to the single muscle-cell is shown in the lower left-hand corner. High power.

Fig. 4. A 3-week preparation of presumptive epidermis of Stage 11 showing the elongate cells with elongate nuclei. Similar preparations are obtained from dorsal lip explants. High power.

Plate 2

Fig. 5. A 6-day culture of presumptive epidermis of Stage 11 with maximal proliferation of nerve fibres. High power.

Fig. 6. Earliest stage in the formation of elongate cells with elongate nuclei. Presumptive epidermis of Stage 11 at 7 days. Nerve fibres still present. High power.

Fig. 7. Portion of a sheet of presumptive epidermis cells 6 days after a 2-hour treatment with 0-1 M LiCl administered to explants made from Stage 11. Cytoplasm does not stain; pigment-rings and kidney-shaped nuclei are visible. Low power.

Fig. 8. Mesenchyme-like cells in a 6-day culture of presumptive epidermis in solution containing 0-005 M LiCl. Low power.

Plate 3

Fig. 9. High-power view of some cells at edge of sheet of 'pigment-ring cells' after 10-day culture. Stage 11 presumptive epidermis, source of the cells, was treated with 0-005 M LiCl in solution X for 18-5 hours.

Fig. 10. Early stage in formation of melanophores from presumptive epidermis 15 days after an 18-5-hour treatment with 0-005 M LiCl of explants from Stage 11. High power.

Fig. 11. Vacuolization of pigmented cells in process of becoming highly dendritic melanophore. Stage 11 presumptive epidermis was treated in 0-1 M LiCl for 4-5 hours. High power.

Fig. 12. Notochord from presumptive epidermis treated at Stage 11 with LiCl and CO₂ for 5 hours. Culture was fixed on eighth day. High power.

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