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In vitro development of isolated ectoderm from axolotl gastrulae

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SUMMARY

The development of ectoderm isolated from the animal pole of axolotl gastrulae is monitored by light microscopy, electron microscopy and analysis of newly synthesized proteins, glycoproteins and glycolipids. When control embryos are undergoing neurulation it is shown that the explants autonomously begin to express epidermal markers and do not express mesodermal markers. However the results suggest that not all the cells become epidermal and electron microscope examination shows that only the outer layer does so, the inner cells remaining undifferentiated.

INTRODUCTION

One of the main advantages of amphibian embryos for studies in experimental embryology is the fact that every cell contains a food reserve of yolk, lipid and glycogen inherited from the oocyte. This means that small regions of tissue from the early embryo can be cultured for several days in simple buffered salt solutions. During this time they can undergo considerable development, in many cases achieving terminal differentiation.

The first systematic study of the behaviour of isolated explants was made by Holtfreter (1938*a*,*b*) on early gastrulae of both newt and frog embryos. He concluded that the entire animal hemisphere would give rise to 'atypical epidermis' although marking studies had shown that in normal development it became neural plate and neural crest in addition to epidermis (Vogt, 1929). This reinforced the conclusion earlier arrived at from the study of exogastrulae (Holtfreter, 1933) that the entire ectoderm will become epidermis in the absence of an inductive stimulus from the archenteron roof.

Since those days the use of isolated gastrula ectoderm as a target tissue for inducing agents has been absolutely crucial for the elucidation of the sequence of inductive interactions. The agents used in experiments may be explants from elsewhere in the embryo, biochemical extracts, other tissues from adult animals or pure chemical substances. One might mention for example the work of Boterenbrood & Nieuwkoop (1973) showing induction of mesoderm by the vegetal hemisphere, that of Born *et al.* (1972) on a purified mesoderm-inducing

factor, the original discovery of neural induction by killed tissue (Bautzman, Holtfreter, Spemann & Mangold, 1932) and the demonstration of the regional specificity of neural induction by Ter Horst (1948).

However all of this work suffers from a basic uncertainty about what the isolated tissue is doing in the period shortly after its treatment. The terminal differentiation which is scored occurs only after several days of culture and in the case of induced structures there is invariably a mixture of cell types present as well as undifferentiated tissue and often some debris. This must inevitably raise the possibility that some of the effects are due not to induction but to selective survival and/or selective differentiation of previously committed cells. Ideally we should like an assay system for inducing factors in which all the cells behaved in the same way and in which the response was measured within hours of the stimulus.

In this paper the behaviour of ectoderm explants is studied by examining the biosynthesis of proteins, glycoproteins and glycolipids. In the accompanying paper it is shown that epidermis, notochord and mesoderm can be identified at the neurula stage by specific biochemical markers. In the present paper it is shown that the ectoderm isolated from the remainder of the embryo expresses epidermal markers on or slightly behind schedule and does not express mesodermal markers. So we can be confident that normal epidermis really is formed in the absence of signals from other parts of the embryo. It seems probable however that not all the cells in the explants are epidermal and electron microscopic examination indicates that at least initially only the outer layer is epidermal while the internal tissue remains undifferentiated.

METHODS

Embryos, dissection and labelling

Axolotl embryos were obtained either by natural or *in vitro* fertilization using animals kept in the laboratory. They were allowed to develop to stage 10, at which time the dorsal blastopore lip is just visible, and a piece of ectoderm around the animal pole was removed using an electrolytically sharpened tungsten needle and a hair loop (Fig. 1). This was cultured in normal amphibian medium at 18 °C (NAM: 110 mM NaCl, 2 mM-KCl, 1 mM-$Ca(NO_3)_2$, 1 mM-$MgSO_4$, 1 mM-$NaHCO_3$, 2 mM-sodium phosphate pH 7·4, 0·1 mM-Na_2 EDTA, 25 μg/ml gentamycin) until controls had reached stage 14 (early neurula). They were then cut open to allow access to the radiolabel and labelled either in 50 μCi[^{35}S]methionine (for proteins), 25 μCi[^{3}H]mannose+25 μCi[^{3}H]galactose (for glycoproteins) or 100 μCi[^{3}H]galactose (for glycolipids) until controls had reached stage 20 which is the time of neural tube closure. They were processed and analysed using methods described in the accompanying paper. Some specimens were labelled for other periods as mentioned in the text. Some were

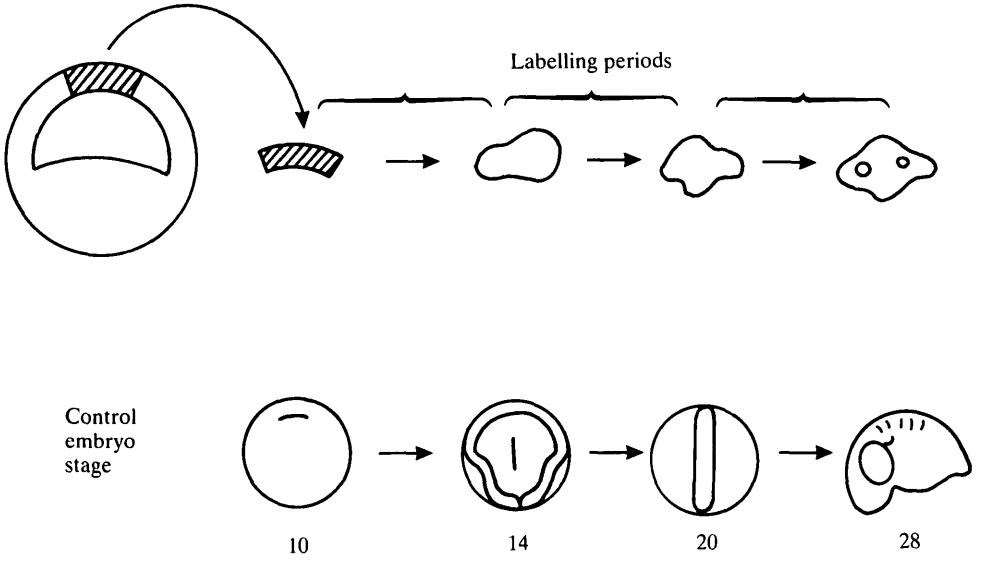

Fig. 1. Experimental design. All three labelling periods were used for glycoproteins but just the middle one (stage 14→20) for proteins and glycolipids.

fixed for light or electron microscopy at times up to 10 days culture. NAM has the same tonicity and Na: K ratio as blastocoelic fluid (Slack, Warner & Warren, 1973) but the divalent cations are present as higher concentrations to prevent disaggregation of explants.

Microscopy

Specimens for light micrqscopy were fixed in Zenkers fluid with 5 % acetic acid, washed in tap water, demercurified in 1 % iodine in 35 % alcohol stained in borax carmine in 35 % alcohol, dehydrated via n-butanol and embedded in 56 °C m.p. wax. Sections were cut at 6–8 μm and counterstained with 0·01 % naphthalene black in saturated picric acid.

Specimens for electron microscopy were fixed for 1–2 hours at 4 °C in 2 % glutaraldehyde in 0·2 M-sodium phosphate pH 7·4. After several washes in buffer they were postfixed in 1 % OsO_4 in veronal acetate buffer pH 7·3. They were then dehydrated in graded ethanols, soaked in epoxypropane and embedded in Araldite epoxy resin. Sections were cut on a Reichert ultramicrotome, grid stained with lead citrate followed by uranyl acetate and examined in a Siemens Emiskop 1 electron microscope.

RESULTS

Microscopy

Immediately after explantation the ectoderm fragments consist of a layer of cells about three to four deep (Fig. 2A). These round up over a few hours and

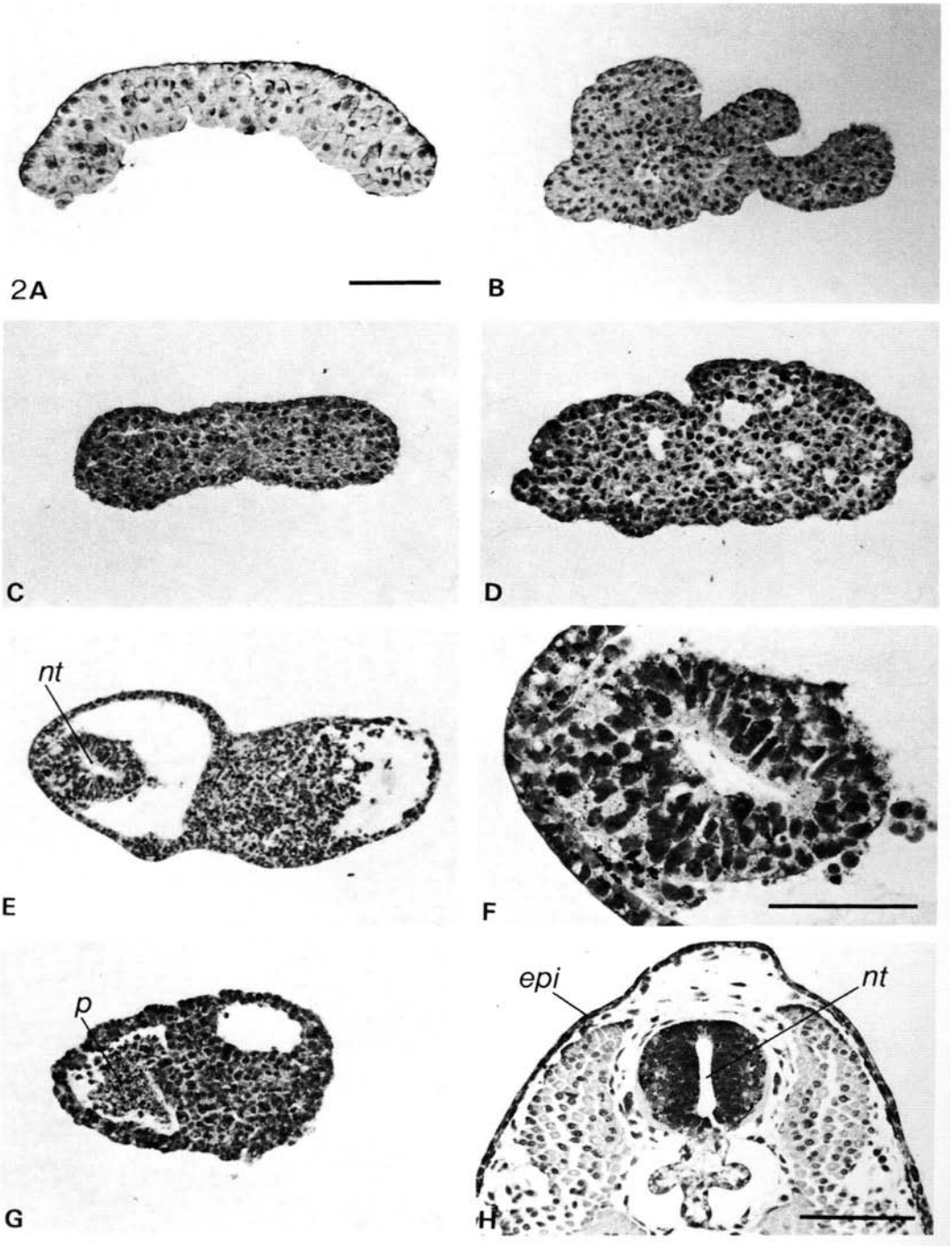

Fig. 2. (A) Ectoderm explant immediately after removal from embryo. Scale bar 0·2 mm, B–E and G same magnification. (B) Ectoderm explant stage 14. (C) Stage 20. (D) Stage 28. (E) A long-term (10-day) culture. nt. neural-tube-like structure. (F) the same. Scale bar 0·1 mm. (G) A long-term culture including a pycnotic region: p. (H) a control embryo after 10 days. epi: epidermis nt: neural tube. Scale bar 0·2 mm.

Table 1. *Long-term cultures of ectodermal explants*

	Ectoderm Series 1	Ectoderm Series 2	Epidermis
Epidermis (external)	12	15	9
Small cells (internal)	12	15	9
Neuroepithelium	5	0	0
Internal cavity	12	11	9
Pycnotic cells	2	8	1
Total explants:	12	15	9

Table 2. *Appearance of ultrastructural markers*

	Desmosomes	Tonofilaments	Cilia	Mucus granules
Ectoderm cultures:				
Stage 10	−	−	−	−
14	+	−	−	−
20	+	+	+	−
26	+	+	+	−
35	+	+	+	+
39	+	+	+	+
Normal epidermis:				
Stage 14	+	+	+	−
20	+	+	+	−
26	+	+	+	−
32	+	+	+	+

by stage 14 (here as elsewhere the stage is that reached by control embryos) consist of a solid ball of cells (Fig. 2B). The cells are smaller and more numerous than those at stage 10 showing that cleavage divisions are continuing. At stage 20 they look similar although the outer layer of cells is possibly more cuboidal in form than the inner cells (Fig. 2C & 3A). By stage 28 (about 3 days culture) a cavity is beginning to appear (Fig. 2D) and a few pycnotic cells become visible in some cases. Two separate series of explants were cultured for 10 days (control stage 39) for comparison with other workers' results. One of these showed significant autoneuralization (Fig. 2E,F compare with H) while the other showed no neuralization but significant clumps of pycnotic cells (Fig. 2G, Table 1). A series of epidermal explants taken from stage-14 neurulae showed no neuralization and only one case with a pycnotic region.

Electron microscopic studies were made on cultured explants of ectoderm and compared to epidermis from intact embryos of different stages (Table 2). It may be seen that the specific epidermal markers of tonofilament bundles, ciliated cells and, later, mucus granules appear in the isolated ectoderm (Fig.

3C–F). Desmosomes also appear but these are not normally exclusive to the epidermis being found in the neural plate as well. The early markers (tonofilaments and cilia) appear slightly later in the ectoderm cultures than in normal epidermis. Also they are found only in the outer cell layer of the explants. The internal cells at stage 20 appear loose packed and undifferentiated (Fig. 3B).

Biochemical studies

The results presented here depend on markers described in the preceding paper. Proteins from ectoderm explants labelled with [^{35}S]methionine from stage 14→20 were analysed on two-dimensional gels. A typical result is shown in Fig. 4. Seventeen spots or groups of isoelectric variants are shown which are characteristic of neurulae rather than gastrulae, showing that the tissue has matured at approximately the same rate as normal embryos. These spots include all of the cytokeratins which are specific to the epidermis and also three spots which are specific to the epidermis but are not cytokeratins and eight spots which are enchanced in epidermis as well as other neurula tissues. It does not

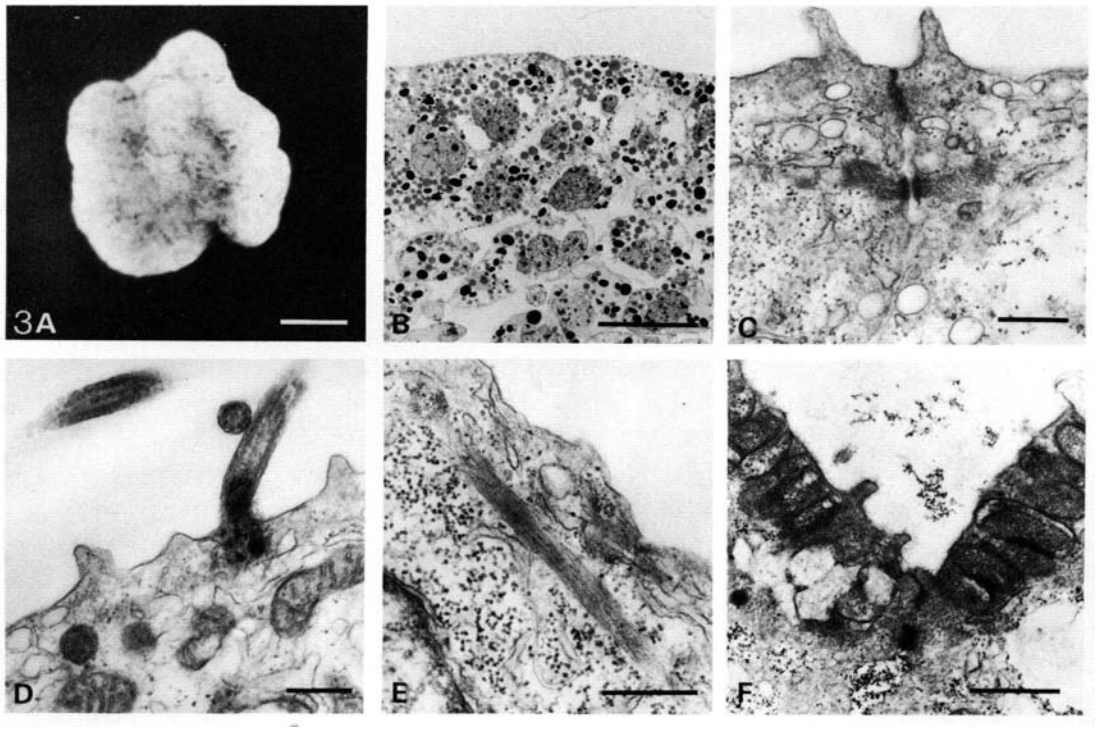

Fig. 3. (A–D) Ectoderm explants at stage 20. (A) Viewed in incident light. Scale bar 0·25 mm. (B) Resin embedded specimen showing epithelial nature of outer cells and loose-packed nature of inner cells. Scale bar 40 μm. (C) Desmosome with tonofilament bundles. Scale bar 0·5 μm. (D) Cilia. Scale bar 0·5 μm. (E) Ectoderm explant stage 26. Tonofilament bundle. Scale bar 0·25 μm. (F) Ectoderm explant stage 35. Mucus granules. Scale bar 1 μm.

include a pair of spots, numbered 18 in the accompanying paper, which is specific to the notochord.

Glycoproteins were labelled with ^{3}H-sugars and visualized by one dimensional SDS gradient gel electrophoresis. The ectoderm was labelled over three time periods, stage 10→12, 14→20 and 20→28 (Fig. 1) and the glycoproteins found in soluble and particulate fractions are shown in Fig. 5. As in intact embryos glycoprotein synthesis commences during neurulation. In the soluble fraction epimucin appears at this stage but there is little high relative molecular mass polydisperse material. In the particulate fraction epimucin is also prominent, accompanied by the glycoproteins S3 and S5, particularly in the last time interval which corresponds to the stage of head extension (stage 20→28).

The synthesis of epimucin is absolutely characteristic of epidermis, and the absence of S2, S2.2 and S3.2 indicates that there is little or no mesoderm or notochord in the explants.

Glycolipids were extracted from explants labelled from stage 14→20 with [^{3}H]galactose, subjected to alkaline hydrolysis and analysed by thin-layer chromatography together with control explants from neurulae. The results are shown in Fig. 6. It can be seen that the pattern resembles that of the non-epidermis tracks rather than that of the epidermis. In the accompanying paper it is shown that all of the tissues in the early embryo except epidermis have this pattern. It suggests therefore that the explants contain much tissue which is not epidermis, presumably that lying inside the outer cell layer.

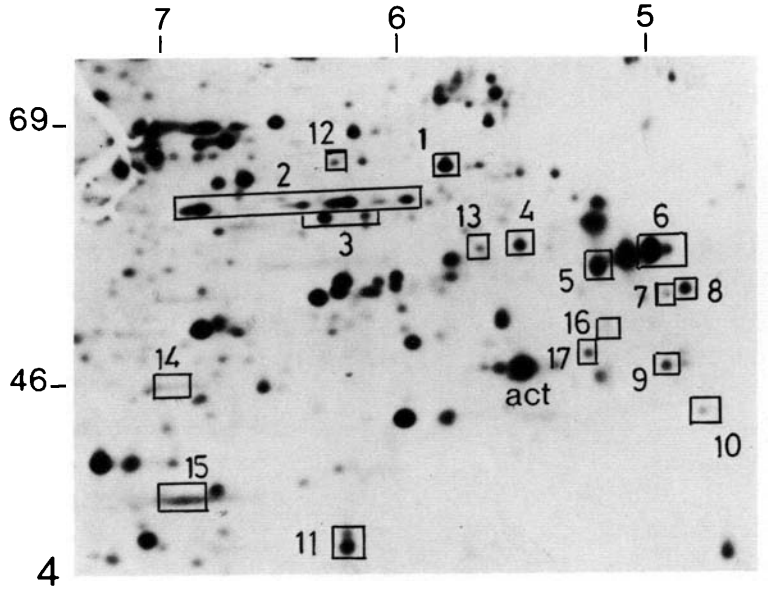

Fig. 4. Two-dimensional gel of newly synthesized proteins made by ectoderm explants labelled from stage 14→20 with [^{35}S]methionine. Actin: act. pH values are shown at the top, and relative molecular mass markers (×10^{3}) on the left side.

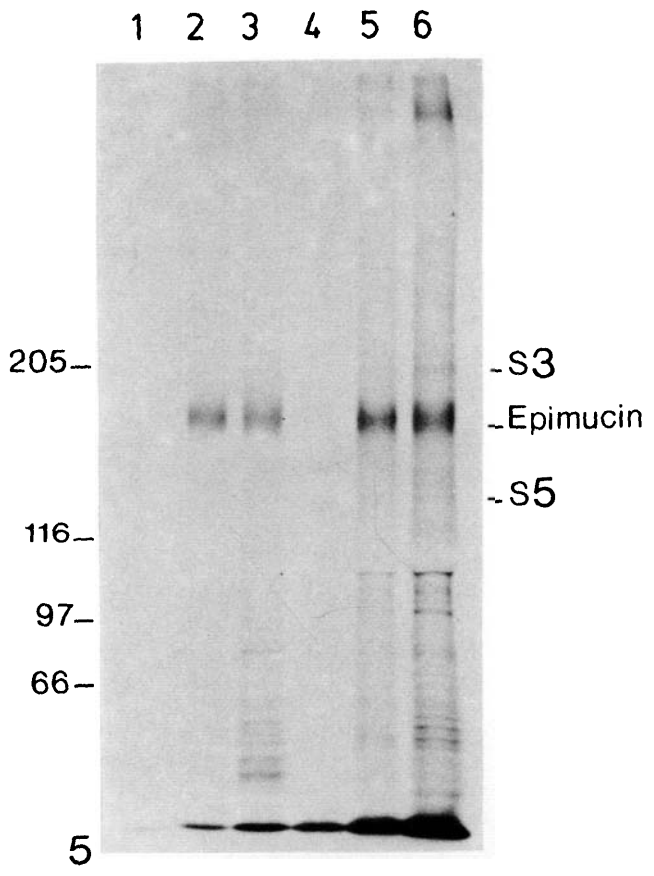

Fig. 5. Synthesis of glycoproteins by ectoderm explants. Tracks 1–3 soluble fractions labelled stages 10→12, 14→20 and 20→28. Tracks 4–6 particulate fractions from same specimens.

Each track contains material from initial supernatants containing 80 μg protein.

DISCUSSION

The EM study and the protein and glycoprotein analyses of the cultures shows that tissue identical to normal epidermis appears either on schedule or retarded only by a few hours. The EM results suggest that this is present as an outer layer surrounding an undifferentiated population of internal cells.

Both the protein and more especially the glycoproteins analysis suggest that no mesoderm or notochord is present since the characteristic S2.2 and S3.2 notochord markers are absent and the bands S2 and S6 which are enhanced in mesoderm and notochord respectively are not enhanced here. The low level of sugar labelled high relative molecular mass polydisperse material also suggests that endoderm is not present. However the glycolipid anlysis shows that much of the tissue is not epidermis.

So if the tissue inside the explants is not epidermis then what is it? There seem to be three possibilities: it might be residual totipotent undifferentiated ectoderm,

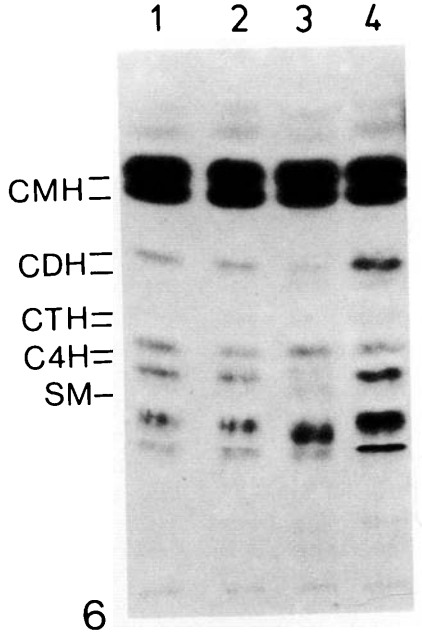

Fig. 6. Thin-layer chromatogram of [^{3}H]galactose-labelled alkali stable lipids. Left to right: ectoderm explant, neural plate, epidermis, archenteron roof, all labelled stage 14 → 20.

it might be neuroepithelium or it might be epidermis which is unable to synthesize its specific markers because of its internal position. The last possibility is perhaps unlikely in view of the fact that some neural development can be obtained in certain cases on long term culture (Fig. 2E, F and Barth & Barth (1974), Holtfreter (1944)). A definitive answer will depend on further experiments in which the internal tissue is extracted and placed in environments which actively promote either epidermal or neuroepithelial development. But such experiments cannot be done properly until we have found a specific positive marker for the neuroepithelium. Existing marker such as tetanus toxin or intermediate filament proteins (Mirsky *et al.*, 1978; Raff *et al.*, 1979) are for differentiated neurons or glia rather than for neuroepithelium. Naturally the search for an early neuroepithelial marker will be continued with vigour.

Since no mesodermal markers are synthesized by animal pole explants the experiments which show mesoderm induction by vegetal pole tissue (Boterenbrood & Nieuwkoop, 1973) or by purified factors (Born *et al.*, 1972) probably do represent a genuine alteration of the cells' developmental pathway rather than an artifact of selective cell death. The mechanism of this process can now be investigated in more detail using the formation of mesodermal markers by explanted ectoderm since this is a more objective and rapid criterion for the response than the histology used by previous workers.

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REFERENCES

BARTH, L. G. & BARTH, L. J. (1974) Ionic regulation of embryonic induction and cell differentiation. *Devl Biol.* **39**, 1–22.

BAUTZMANN, H., HOLTFRETER, J., SPEMANN, H. & MANGOLD, O. (1932) Versuche zur analyse der Induktionsmittel in der Embryonalentwicklung. *Naturwissenschaften* **20**, 971–974.

BORN, J., GEITHE, H. P., TIEDEMANN, H., TIEDEMANN, H. & KOCHER-BECKER, U. (1972) Isolation of a vegetalizing inducing factor. *Hoppe Seylers Z. Physiol. Chem.* **353**, 1075–1084.

BOTERENBROOD, E. C. & NIEUWKOOP, P. D. (1973) The formation of the mesoderm in urodelean amphibians. V. Its regional induction by the endoderm. *Wilhelm Roux Arch. EntwMech. Org.* **173**, 319–332.

HOLTFRETER, J. (1933) Organisierungsstufen nach regionaler Kombination von Entomesoderm mit Ektoderm. *Biologisches Zentralblatt* **53**, 404–431.

HOLTFRETER, J. (1938*a*) Differenzierungspotenzen isolierter Teile der Urodelengastrula. *Wilhelm Roux Arch. EntwMech. Org.* **138**, 522–656.

HOLTFRETER, J. (1938*b*). Differenzierungspotenzen isolierter Teile der Anurengastrula. *Wilhelm Roux Arch. EntwMech. Org.* **138**, 657–738.

HOLTFRETER, J. (1944) Neural differentiation of ectoderm through exposure to saline solution. *J. exp. zool.* **95**, 307–340.

MIRSKY, R., WENDON, L. M. B., BLACK,P., STOLKIN, C. & BRAY, D. (1978) Tetanus toxin: a cell surface marker for neurones in culture. *Brain Res.* **148**, 251–259.

RAFF, M. C., FIELDS, K. L., HAKOMORI, S. I., MIRSKY, R., PRUSS, R. M. & WINTER, J. (1979) Cell type specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. *Brain Res.* **174**, 283–308.

SLACK, C., WARNER, A. E. & WARREN, R. L. (1973) The distribution of sodium and potassium in amphibian embryos during early development. *J. Physiol.* **232**, 297–312.

TER HORST, J. (1948) Differenzierungs und Induktions-leistungen verscheidener Abschitte der Medullarplatte und der Uradarmdaches von Triton im Kombinat. *Wilhelm Roux Arch. EntwMech. Org.* **143**, 275–303.

VOGT, W. (1929) Gestaltungsanalyse am Amphibienkeim mit ortlicher Vitalfarbung. II. Gastrulation und Mesodermildung bei Urodelen und anuren. *Wilhelm Roux Arch. EntwMech. Org.* **120**, 384–706.

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