J. Embryol. exp. Morph. **80**, 289–319 (1984)
Printed in Great Britain © *The Company of Biologists Limited 1984*

Regional biosynthetic markers in the early amphibian embryo

By JONATHAN M. W. SLACK

Imperial Cancer Research Fund, Mill Hill Laboratories, Burtonhole Lane, London NW7 1AD, U.K.

SUMMARY

A search has been made for regional differences in macromolecular synthesis in the axolotl embryo at a stage when the principal regions have become determined but terminal differentiation has not yet begun.

The epidermis of the neurula makes a number of abundant proteins which are not made elsewhere. Some of these are identified by immunoprecipitation as cytokeratins (relative molecular masses (M_r) 62,59,54,51 and 46×10^3). At the same stage a network of tonofilaments becomes visible by electron microscopy and is also confined to the epidermis.

There is no significant incorporation of ^{3}H-sugars into glycoproteins before neurulation. During neurulation specific species begin to be made by the notochord (M_r 265 and 185×10^3), the mesoderm (315×10^3) and the epidermis (170×10^3). The latter species is water soluble, has a native M_r of 470×10^3, is somewhat resistant to trypsin and has a high sugar content. Since these characteristics do not relate to any known glycoprotein it has been given a new name: *epimucin*.

Several neutral glycolipids and gangliosides are present in the early embryo, the most abundant of which is identified as galactocerebroside. Synthesis occurs from fertilization onwards, however even by the neurula stage the qualitative pattern is the same in all parts except for the epidermis which shows two substantial enhancements and one reduction compared to other regions.

The differences reported probably relate to physiological functions rather than to the mechanism of determination itself, so their chief importance is as markers of early embryo regions in experiments on induction.

INTRODUCTION

Regional pattern formation in early development is still understood better for amphibian embryos than for any other animal type (Nakamura & Toivonen 1978, Gerhart 1981, Slack 1983a). We have an general idea of the sequence of fate maps, of the progressive changes of competence of different regions, and of their sequential subdivision in response to inductive signals. However our knowledge remains imprecise enough to allow some credence to be given to occasional critics who deny the reality of embryonic induction altogether (Holtzer 1978, Jacobson 1982).

The main reason for this is the fact that virtually all experimental work has used as its end point the appearance of *terminally differentiated* cells. The intermediate states of commitment which arise in the course of early development

cannot be observed directly and so their existence has been inferred from the types and proportions of differentiated cells which appear later. Even the germ layers which were defined in the nineteenth century on the basis of comparative embryology still cannot be observed directly. So for example the first inductive interaction is supposed to be the subdivision of the animal hemisphere into ectoderm and mesoderm in response to a signal from the vegetal hemisphere (Nieuwkoop 1973, Tiedemann 1976). However nobody has a criterion for identifying 'mesoderm' until characteristic cell types such as notochord, muscle, kidney tubule or erythrocytes have differentiated and this may take anything up to 14 days depending on the species used. So there is plenty of scope for the results to be obscured by selective cell death, selective promotion or inhibition of differentiation, and secondary interactions within the explants or grafts which affect the proportions of the different cell types.

Clearly it is necessary to have objective criteria for identifying regions with specific states of commitment at the time of their formation. Without this our knowledge of the course of development will remain so imprecise that the possibilities for detailed investigation of the mechanism of inductive interactions will remain slim. Light microscopy is not adequate for this purpose because all cells in the early amphibian embryo appear as undifferentiated bags of yolk granules (Fig. 2C–F). As we shall see below the same is largely true of electron microscopy; with the exception of the epidermis there are no qualitative ultrastructural differences allowing the identification of cells once the normal arrangement of embryonic tissue layers has been deranged.

We are therefore led to look for markers at the biochemical level. We might expect to be able to find substances concerned with transient physiological functions known to exist in early embryos such as cell movement, cell adhesion or ion pumping since they would probably be present at fairly high relative abundances. The markers reported on here probably belong to this class. With sufficient resolution we might in addition hope to detect the molecules which code for the different states of commitment themselves, but it is not claimed that this objective has been realized in the present work.

The results described below concern regional differences in the synthesis of three classes of macromolecule: proteins, glycoproteins and glycolipids. Previous studies on early stages of *Xenopus laevis* have examined only protein synthesis and have emphasized temporal changes rather than regional differences (Brock & Reeves 1978, Bravo & Knowland 1979, Ballantine, Woodland & Sturgess 1979).

The species chosen is the axolotl, partly because the embryo is large enough to permit accurate dissection of the tissue layers and partly because it develops fairly slowly (Table 1). Fast developing embryos tend to be rather mosaic in their properties and to show earlier synthesis of terminal differentiation products relative to morphological stage than slow developing species. for example the synthesis of the muscle specific protein α-actin commences in the fast developing

Xenopus laevis by stage 12 (Sturgess *et al.* 1980) but in the axolotl not until stage 26 (Mohun, Tilly, Mohun & Slack 1980). Since the present study was designed to detect synthesis of substances characteristic of intermediate rather than terminal cell states the slow developing species was preferred.

The results show that the mesoderm is characterized by synthesis of a high relative molecular mass glycoprotein ('S2'), and the notochord by this and two additional glycoproteins ('S2.2' and 'S3.2'). The epidermis is characterized by synthesis of a number of specific cytokeratins, by a major glycoprotein ('epimucin') and by a specific pattern of glycolipid synthesis. No specific marker has been found for the neural plate or for the endoderm, although the latter shows an enhanced synthesis of a high relative molecular mass polydisperse material which is probably proteoglycan. In the accompanying paper the synthesis of these markers is used to monitor the development *in vitro* of ectoderm isolated from the gastrula.

METHODS

Embryos

Axolotl embryos were obtained either by natural matings or by *in vitro* fertilization as described (Mohun *et al.* 1980, Slack 1983b). The relevant stages in early development are listed in Table I, according to Bordzilovskaya & Detlaff (1979). Before labelling the embryos were kept at temperatures between 10° and 22° to vary the rate of development for experimental convenience. Intact embryos develop normally across this range.

In all the experiments reported on here the metabolic labelling was carried out at 18 °C on explants contained in a small volume (0·2–0·4 mls) of normal amphibian medium (NAM: 110 mM-NaCl, 2 mM-KCl, 1 mM-$Ca(NO_3)_2$, 1 mM-$MgSO_4$, 0·1 mM-Na_2EDTA, 1 mM-$NaHCO_3$, 2 mM-Na phosphate pH 7·4, 25 μg/ml gentamycin).

In some early experiments whole embryos were labelled at or before stage 10 by injection of the precursor into the blastocoel (Gurdon 1974). This was found to give underrepresentation of epidermal markers compared with experiments in which the whole embryo was chopped up into random fragments before labelling, and so the latter method was preferred.

Table 1. *Stages of the axolotl mentioned in the text*

Stage	Description	Approx. time from fertilization, 18 °C
7	blastula	18 h
10	early gastrula	40 h
14	early neurula	70 h
20	closed neurula	88 h
30	tailbud	5 days

Dissection

Dissections were performed in NAM containing 0·01–0·02 % trypsin (Sigma type IX), using an electrolytically sharpened tungsten needle and a hair loop. The trypsin allows a clear separation of tissue layers while the presence of the divalent cations in the medium prevents the tissue fragments from disaggregating further into single cells. The fragments were washed in NAM containing 0·02 % soybean trypsin inhibitor (Sigma) for a few minutes and then accumulated in a dish of NAM. All manipulations were performed in 5 cm bacterial Petri dishes covered with 1 % Noble agar (Difco). The labelling was carried out in plastic scintillation vial inserts or in liquid nitrogen storage ampoules. Both of these are reasonably non-adhesive so the explants can easily be recovered. They are also tall and thin so the incubation can be carried out in a small volume and the vial can then be filled once or twice with NAM to rinse off excess radiolabel before processing.

The standard neurula dissection is shown in Fig. 1. It should be noted that some of the boundaries are natural ones while others are arbitrary (shown by dashed lines). The neural plate is taken from within the neural folds and the epidermis from outside the neural folds. The notochord is clearly demarcated

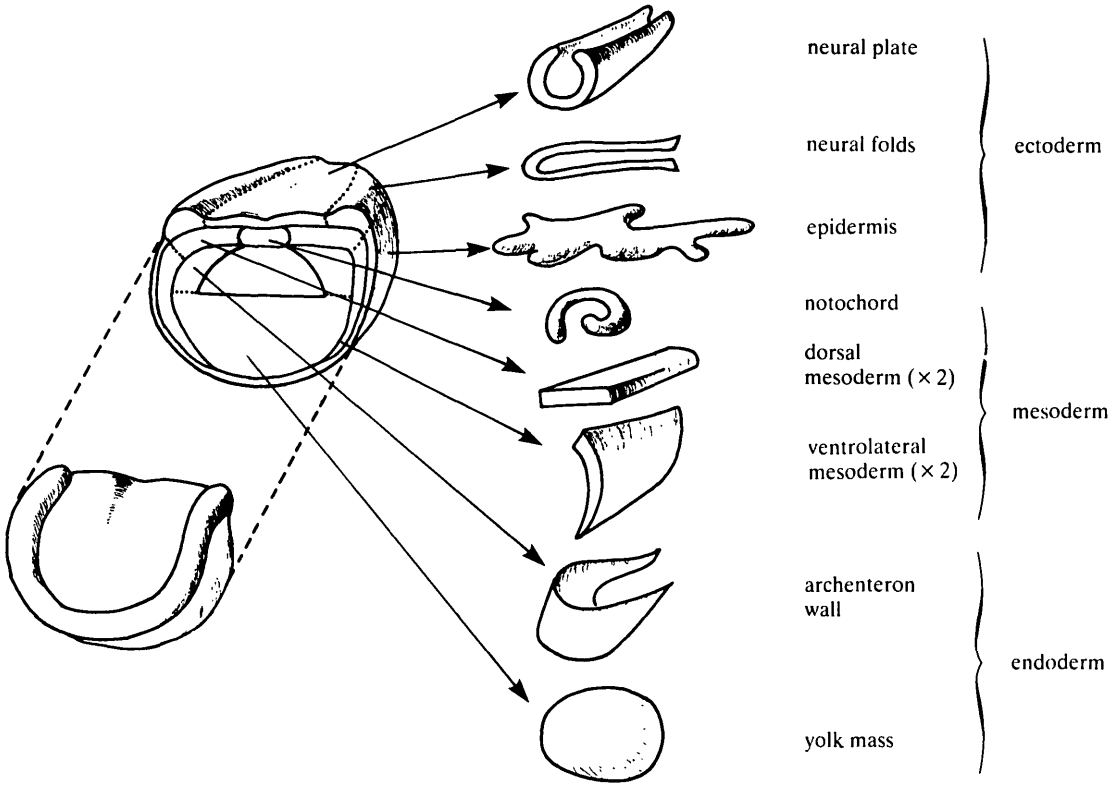


Fig. 1. Dissection of a stage-14 embryo into eight distinct regions.

posteriorly although its anterior extension is somewhat arbitrary. The dorsal mesoderm is that part of the mesodermal mantle which lies beneath the neural plate at this stage, roughly corresponding to the region destined to form the somites. The ventrolateral mesoderm is that part of the mesodermal mantle which, at this stage, lies beneath the epidermis. The archenteron wall consists mainly of the anterior lining of the archenteron cavity, together with the flaps which underly the dorsal mesoderm of the trunk. The region around the blastopore was discarded.

With the methods described this dissection can be performed quite cleanly so that for example there are no mesodermal cells adhering to the neural plate or epidermis. Because of the large size of the cells any contamination can easily be seen down the dissecting microscope and scraped off with a hair loop. However it should be borne in mind that several of the boundaries are arbitrary. This means that a qualitative difference in synthesis between two embryo regions may appear as a quantitative difference because the dissection lines do not correspond to the true (and invisible) boundaries. To aid the reader in interpreting the results Table 2 shows the expected overlap between regions which arises from the arbitrary boundaries.

Radiochemicals

All were obtained from the Radiochemical Centre Ltd., Amersham, England.
L-[^{35}S]methionine >800 Ci/mmole
D-[6^{3}H]galactose 20–40 Ci/mmole
D-[2,6^{3}H]mannose 30–60 Ci/mmole
D-[6^{3}H]glucosamine 20–40 Ci/mmole
D-[U^{14}C]glucose >230 mCi/mmole
[^{35}S]sulphate 25–40 Ci/ng
Sodium boro[^{3}H]hydride, solution in 0·5 M-NaOH, 5–20 Ci/mmole

6–12 similar explants, or 1 chopped embryo, were labelled in 0·2–0·4 mls NAM containing 50 μCi total of isotope which had been dried under vacuum and dissolved in NAM. Preliminary experiments showed that incorporation of label

Table 2. *Overlaps arising from dissection boundaries*

A true embryonic region which is more extensive than the explants listed in the first column may extend into those in the second column:

Neural plate	Neural folds
Neural folds	Neural plate, epidermis
Notochord	Archenteron wall
Dorsal mesoderm	Ventrolateral mesoderm
Ventrolateral mesoderm	Dorsal mesoderm
Archenteron wall	Notochord, yolk mass
Yolk mass	Archenteron wall

into TCA insoluble material was approximately linear over the periods used. NaB^3H_4 was used to label glycolipids *in vitro* (see below).

Microscopy

Methods were as described in the accompanying paper.

Protein analysis

This was carried out by two-dimensional gel analysis (O'Farrell 1975) of samples processed essentially by the method of Garrels (1979). Explants labelled with [^{35}S]methionine were rinsed twice in NAM and then homogenized with a Dounce B pestle in 100 μl MN at 0° (MN: 50 μg/ml micrococcal nuclease, Worthington NFCP, in 2 mM-$CaCl_2$, 20 mM-Tris Cl pH 8·8). After one minute, 10 μl of DR solution was added (DR: 1 mg/ml DNAse, Worthington DPFF 0·5 mg/ml RNAse, Worthington RASE, in 0·5 M-Tris Cl pH 7·0, 50 mM-$MgCl_2$), followed by 10 μl 5% SDS, 15% β-mercaptoethanol. The samples were lyophilized and dissolved in 50 μl of two-dimensional sample buffer (2DSB: 9·5 M-urea, 4% NP40, 2% ampholyte, 100 mM DTT). This was microfuged and subjected to two-dimensional gel electrophoresis exactly as described by Slack (1982a). The gels were fixed in 30% methanol, 10% acetic acid and processed for fluorography by the method of Bonner & Laskey (1974). About 100 000 TCA precipitable counts per minute gave a good exposure after one week. The pH gradient was measured by cutting a control focusing gel into 1 cm slices, allowing each to equilibrate with 1 ml of degassed water and measuring the pH with a combination electrode. Relative molecular masses (M_r) were measured by reference to ^{14}C-labelled standards from New England Nuclear: phosphorylase b (97×10^3), BSA (69×10^3), ovalbumin (46×10^3) and carbonic anhydrase (30×10^3), using a calibration plot of log mol wt. vs migration (Hames & Rickwood, 1981).

Immunoprecipitation

Immunoprecipitation of cytokeratins was carried out using a rabbit antibody to human skin keratin kindly donated by Dr J. G. Rheinwald (Harvard Medical School). Explants of epidermis labelled with [^{35}S]methionine were homogenized in 25 μl MN and 5 μl DR. 0·18 ml of NET including 0·5% SDS was added (NET: 0·15 M-NaCl, 1% NP-40, 50 mM-Tris Cl pH 8·0, 5 mM-EDTA, 0·01% NaN_3), the mixture was rehomogenized and centrifuged in the Beckman microfuge for 4 minutes. 100 μl of supernatant were mixed with 0·5 ml NET and 10 μl of antiserum and left for 30 minutes at room temperature. Then 50 μl of 50% protein A-agarose (Sigma) in NET was added and the mixture was gently rocked at 4 °C for 90 minutes. The agarose beads were washed twice in NET and then shaken with 50 μl 2DSB to elute the bound immune complexes. Control precipitations were carried out without antibody. About 1% of the TCA precipitable radioactivity in a labelled epidermal extract was specifically precipitated by the anti-keratin.

Glycoprotein analysis

The tissue explants were labelled with sugars, subjected to a simple fractionation into yolk, soluble and particulate fractions, and analysed on one-dimensional SDS gradient gels.

The explants were rinsed twice in NAM then homogenized at 0° with the Dounce B pestle in 50 μl MN solution containing 0·2 M-sucrose. After a few seconds 10 μl of DR solution was added, and after about a minute, 0·45 ml HM solution (HM: 0·2 M-sucrose, 1 mM-$MgCl_2$, 10 mM-Tris Cl pH 8·0, 1 mM-PMSF, 5 mM-iodoacetamide). This was rehomogenized vigorously. Large yolk granules were spun down with a short, very slow spin (45 seconds at mark 1, Gallenkamp bench centrifuge) and the supernatant was removed to a polycarbonate ultracentrifuge tube. The yolk pellet was washed with a further 0·5 mls HM and this supernatant added to the first. The pooled supernatants were centrifuged at 40 000 rpm (125 000 *g*) for 30 minutes in the Ti50 rotor of a Beckman ultracentrifuge. Two volumes of ethanol at −20° were added to the supernatant and the resulting precipitate ('soluble fraction') was spun down and dissolved in 50 μl of one-dimensional sample buffer for SDS gel electrophoresis (1DSB: 62·5 mM-Tris Cl pH 6·8, 10 % glycerol, 2 % SDS, 5 % mercaptoethanol, 0·05 % bromophenol blue). The ultracentrifuge pellets ('particulate fraction') were also dissolved in 50 μl of 1DSB. In a typical fractionation of a whole embryo, 18 % of TCA precipitated counts were in the soluble fraction, 65 % in the particulate fraction and 17 % in the yolk, the latter having the same composition on gels as the particulate fraction.

These samples were analysed on linear 4–8 % polyacrylamide gradient gels. No stacking gel was used, instead a small amount of 4 % gel mixture was polymerized around the slot-forming comb above the gradient gel. As usual the gel buffer was 0·375 M-Tris Cl pH 8·8 and the tank buffer 25 mM-Tris–0·19 M-glycine pH 8·3, both with 0·1 % SDS. In some experiments equal counts were run in each track, in others equal amounts of protein.

Relative molecular masses were determined by plotting log standard M_r against log acrylamide concn. (Hames & Rickwood, 1981). The standards were unlabelled myosin (205×10^3), β-galactosidase (116×10^3), phosphorylase b (97×10^3) and BSA (66×10^3) all from Sigma. For some experiments the high molecular weight kit from Pharmacia was used along with non-reduced γ-globulin (150×10^3). After mild denaturation the kit gives bands at 330×10^3 (thyroglobulin subunit) and 220×10^3 (half ferritin). Both groups of standards gave similar results.

The gels were run until the tracking dye neared the bottom and were then fixed in 30 % methanol, 10 % acetic acid containing 0·05 % Coomassie Blue for 1 hour. They were destained overnight in the same fixative and fluorographed by the method of Bonner & Laskey (1974). The relative molecular mass markers remained visible after PPO impregnation and drying and were marked with

radioactive ink. 5–10 000 TCA insolubl c.p.m./track was found to give a satisfactory exposure after 4 weeks.

Trypsin and collagenase digestions

A particulate preparation from whole embryos was homogenized in 0·1 M-NaCl, 50 mM-Tris Cl pH 8·0, 10 mM-$CaCl_2$. One embryo's worth was incubated with 10 μg trypsin (Sigma type IX), or with 25 units collagenase (Form III, Advance Biofactors) plus N-ethyl maleimide to 5 mM, for 30 minutes at 37 °. The reactions were stopped by adding an equal volume of double strength 1DSB. This was boiled for 1 minute, microfuged and analysed by gel electrophoresis. The trypsin treatment completely removed all Coomassie Blue staining material.

Native gradient gel electrophoresis

Epimucin was extracted from ^{3}H-sugar-labelled epidermis by nucleasing as described above, then homogenizing in 100 μl of NB (NB: 90 mM-Tris, 80 mM-boric acid, 2·7 mM-Na_2EDTA, 1 mM-PMSF, 5 mM-iodoacetamide) and microfuging. Glycerol was added to increase the density and the sample was loaded onto a 4-16 % polyacrylamide gel made in the same buffer (minus protease inhibitors) and previously prerun at 70V for 20 minutes. The gels were run overnight at 160 V (total 2500 V/h) which is sufficient to move all components to the acrylamide concentration at which they will migrate no further. The M_r markers used were the Pharmacia high molecular weight kit which contains thyroglobulin (669×10^3), ferritin (440×10^3), catalase (232×10^3), LDH (140×10^3) and BSA (67×10^3). The gels were stained and fluorographed in the usual way and the relative molecular mass of the radioactive band was estimated from a plot of log standard M_r vs log mobility.

Affinity chromatography

After nucleasing, [^{35}S]methionine-labelled epidermis explants were homogenized in 0·4 mls of 10 mM-sodium phosphate pH 7·4 plus protease inhibitors. The large yolk granules were spun off and the supernatant was rehomogenized after the addition of sodium deoxycholate to 1 %. This was microfuged and passed through an 0·2 ml column of lentil lectin-Sepharose (Pharmacia) and washed with 2·5 mls of 10 mM-sodium phosphate pH 7·4, 1 % sodium deoxycholate. The column was eluted with 1 ml of 2 % α-methyl-mannoside in the same buffer. The eluate was precipitated with 2 volumes of ethanol at −20 °, dissolved in 1DSB and analysed by SDS gradient gel electrophoresis.

Sugar analysis

Epimucin bands were cut out from the gels with the aid of the fluorograms. The pieces were swollen in distilled water and the backing paper was removed. They

were then cut up into 1 mm cubes and hydrolysed in 2 M-HCl for 3 h at 100 °C or 0·1 M-HCl for 1 h at 100° (mild hydrolysis for fucose). The hydrolysates were removed, microfuged and the supernatants dried down three times in a stream of nitrogen at 80 °C. They were lyophilized overnight in the presence of NaOH pellets. They were then dissolved in a small volume of water, applied to Merck Silica Gel G TLC plates and analysed by chromatography in two runs of chloroform: methanol: water 55: 45: 10. A number of sugar standards were run in parallel and were visualized with the naphthoresorcinol reagent. The radioactive bands were visualized by fluorography using the method of Bonner & Stedman (1978).

Glycolipid analysis

Animal glycolipids are all glycosphingolipids and are compounds of ceramide with one or more sugar residues, ceramide being a compound of the long chain base sphingosine with a fatty acid (Hakomori 1981). Glycolipids are soluble in organic solvents and are stable to mild alkaline hydrolysis. In this paper CMH, CDH, CTH, C4H will indicate respectively ceramide with 1, 2, 3 or 4 sugars attached. Each of these substances gives two neighbouring bands on thin-layer chromatography because of the presence of forms containing a hydroxylated fatty acid residue. Gangliosides are glycolipids containing one or more sialic acid residues. The standards used in the present work were GM1, GD1a, GD1b and GT1 whose detailed formulae are given by IUPAC-IUB (1978).

Explants labelled with D[6^{3}H]galactose were rinsed twice in NAM and then homogenized with a Dounce B pestle in 0·15 mls chloroform+0·3 mls methanol (all solvents redistilled). The homogenate was centrifuged for 5 minutes at low speed (Gallenkamp bench centrifuge, mark 1) and the supernatant removed. The pellet was rehomogenized with 0·3 mls chloroform+0·15 mls methanol, recentrifuged and the supernatant pooled with the first. The pooled supernatants were evaporated to dryness at 45° under a stream of nitrogen and dissolved in 0·45 mls 0·1 M-KOH in 1: 2 chloroform: methanol. This was incubated at 37° for 4 h to allow complete deacylation of phospholipids and triglyceride (Narasimhan, Hay, Greaves & Murray 1976). The mixture was neutralized with 0·1 M-HCl and adjusted by addition of chloroform and water to the composition: chloroform: methanol: water 1: 1: 3. This is a two-phase mixture which separates lipids in the lower phase from water-soluble substances in the upper phase. It differs from the Folch partition (Folch, Lees & Sloane-Stanley, 1957) in that gangliosides as well as neutral glycolipids enter the lower phase, as judged by trials with mixtures of pure compounds. The emulsion was resolved by a one-minute spin in the microfuge and the upper phase was removed and discarded. The lower phase was washed once or twice with a volume of fresh upper phase equal to the first and was then evaporated under nitrogen and dissolved in 100 μl of chloroform: methanol 2: 1. Total lipid extracts were prepared in the same way with the omission of the drying and alkaline hydrolysis steps.

Lipid preparations were fractionated by one- or two-dimensional thin-layer chromatography on Merck HPTLC Silica Gel G plates using solvent mixtures as given in the figure legends. Neutral and phospholipid standards were obtained from Sigma and glycolipid standards from Supelco. Digalactosyl ceramide was a gift from Dr R. Gigg (NIMR, London). After chromatography the plates were dried and then exposed to iodine vapour to visualize the standards and the major components in the samples. The positions of these were recorded on tracing paper and the iodine was allowed to evaporate from the plates in the 37 °C room. The plates were then processed for fluorography by the method of Bonner & Stedman (1978) which involves impregnation with 0·4 % PPO in 2-methyl naphthalene.

Large scale lipid extractions

Unlabelled lipid extracts from whole embryos were prepared as follows. The embryos (around 500) were homogenized in 5 volumes of chloroform: methanol 1: 2 followed by 5 volumes of chloroform: methanol 2: 1. The extract was adjusted to the Folch partition ratios with chloroform and 0·58 % NaCl and shaken vigorously. The lower phase was washed twice with fresh upper phase and then evaporated in a rotary evaporator and subjected to the alkaline hydrolysis procedure described above (10 mls total). It was then repartitioned and washed once with upper phase, reevaporated and dissolved in a small volume of chloroform. This was run onto a 10 ml column of silicic acid (Sigma SIL-LC) which was eluted with 100 ml chloroform followed by 100 ml methanol. These eluates were evaporated, dissolved in a small volume of chloroform, and stored at −20 °C. The chloroform eluate contains the fatty acid methyl esters arising from the hydrolysis plus cholesterol. The methanol eluate consists mainly of sphingomyelin plus other alkali resistant phospholipids and should also contain all the neutral glycolipids. The glycolipids were visualised by labelling with galactose oxidase and sodium boro[^{3}H]hydride (Suzuki & Suzuki 1972). After a pre-reduction with excess unlabelled $NaBH_4$, 2 mg of lipid were dissolved in 0·2 mls tetrahydrofuran (THF: redistilled over $NaBH_4$) plus 0·2 mls 0·1 M-potassium phosphate pH 7·0. 10 units of galactose oxidase (Sigma) were added and the mixture was incubated at 37 ° for 1 hour. The reaction was terminated by adding 50 μl of 0·1 M-KCN and the THF was evaporated in a stream of nitrogen. The lipid was recovered with the 1: 1: 3 partition described above and was redissolved in 0·2 mls THF. 200 μCi of NaB^3H_4 were added and the mixture left for 1 hour at room temperature. Excess borohydride was destroyed by the addition of 20 μl 1 M-acetic acid in a radioactive fume hood and the lipid was recovered by the 1: 1: 3 partition and analysed by thin-layer chromatography.

Gangliosides

Unlabelled gangliosides were prepared from the pooled upper phases from the original Folch extraction. This was evaporated at 40 °C in a rotary evaporator, dissolved in about 10 mls distilled water and dialysed against two or three

changes of 1·5 litres water in the cold room. The contents of the bag were evaporated in the rotary evaporator and dissolved in a small volume of chloroform: methanol: water 30: 60: 8. This was applied to a 1 ml column of DEAE-Sephadex (acetate form) made up in the same solvent mixture (see Mormoi, Ando & Magai 1976) and washed with 5 ml of the same mixture followed by 2 ml of methanol. Gangliosides were eluted with 5 ml of 0·3 M-ammonium acetate in methanol and this was evaporated, dissolved in water and dialysed. About 0·46 μg/embryo were recovered. When assayed by the procedure of Jourdian, Dean & Roseman (1971) this proved to be about one third ganglioside. The preparations were labelled by the method of Veh, Corfield, Sander & Schauer (1977) which exploits the sensitivity of the neuraminic acid side chain to mild periodate oxidation. 100 μg were dissolved in 0·1 ml 0·1 M-sodium acetate pH 5·5, 0·15 M-NaCl. 10 μl 20 mM-$NaIO_4$ was added and the mixture was left for 10 minutes at 4 °C. Then 10 μl 10 % glycerol was added to terminate the reaction and the mixture was dialysed against 50 mM-sodium phosphate pH 7·4, 0·15 M-NaCl for 2 h using a BRL microdialyser. 200 μCi of NaB^3H_4 were added and allowed to react for 1 h at room temperature. The mixture was then dialysed against water overnight at 4 °C, lyophilized, dissolved in 100 μl chloroform methanol 2: 1, and analysed by thin-layer chromatography.

RESULTS

Appearance of early embryo regions by light and electron microscopy

Normal stage-14 and stage-20 embryos are shown in Fig. 2. It is apparent that various parts such as neural tube, notochord and so on can be recognized by virtue of their relation to the whole, but that the actual cells all look the same. In the light microscope the cytoplasm appears in all cases to consist simply of densely packed yolk granules.

Explanted notochord, neural tube, somite mesoderm and epidermis from stage-20 embryos were examined by electron microscopy. As described by previous workers the cells are large and always contain abundant yolk granules, lipid droplets, glycogen and melanosomes. In between these particles is the functional cytoplasm containing mitochondria, smooth membranes and ribosomes. The epidermis is distinct from the other tissues in that it contains a network of tonofilament bundles terminating at desmosomes (Fig. 3A,B). There are also occasional ciliated cells in this layer (Fig. 3C,D).

As far as the other types of tissue are concerned the absence of qualitative differences is striking. There are probably some quantitative differences insofar as the Golgi apparatus seems better developed in the notochord and larger masses of ribosomes are found in the neural tube than elsewhere. However it would not be possible to identify a cell definitely by its ultrastructural appearance unless it displayed the specific epidermal features of cilia or tonofilament bundles (desmosomes are found also in the neural tube). These conclusions are broadly

in line with previous ultrastructural studies on amphibian embryos (eg, Schroeder 1970, Burnside 1971) and so will not be further elaborated. A more detailed account of epidermal development is given in the accompanying paper.

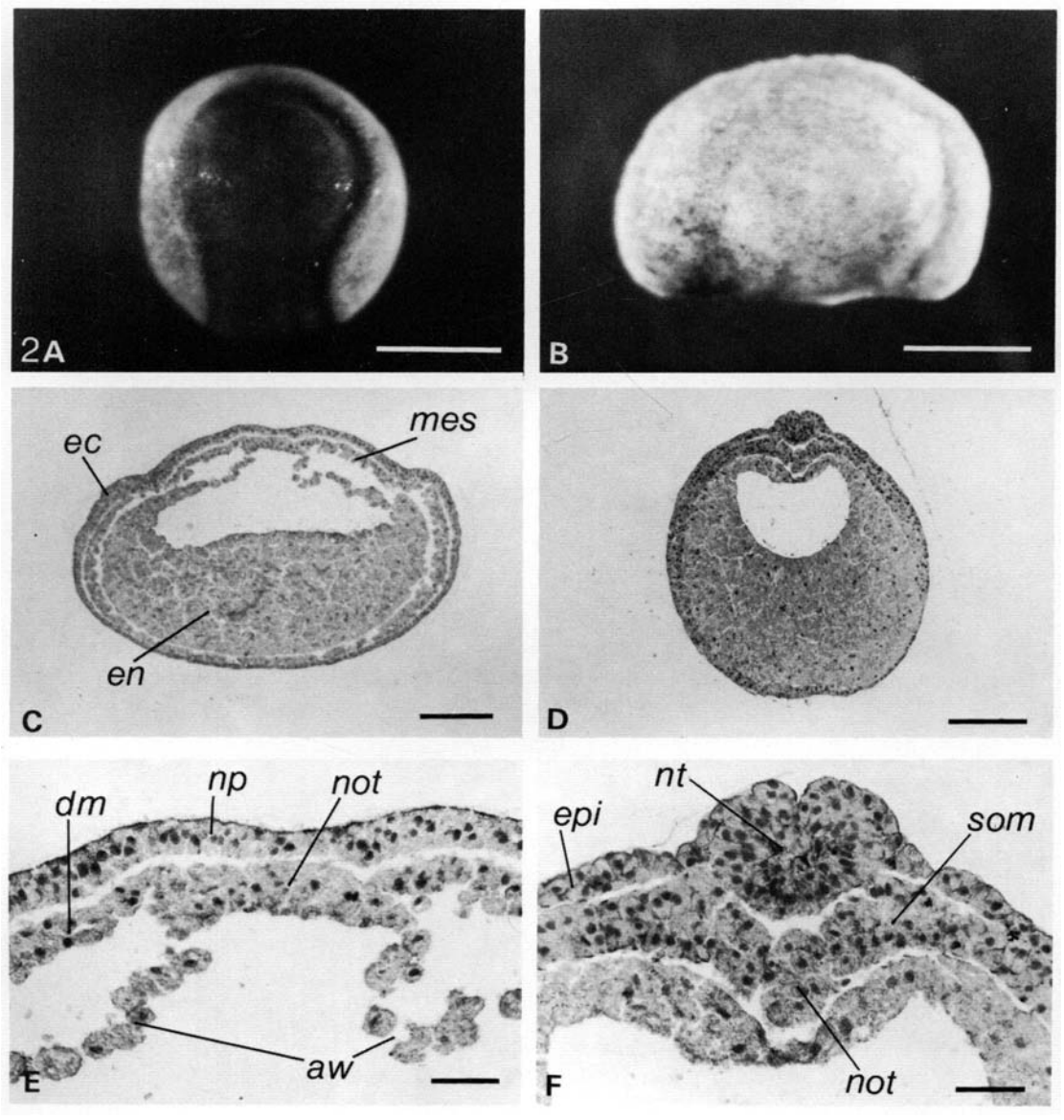


Fig. 2. Normal embryos at stage 14 (A, C, E) and stage 20 (B, D, F). (A, B) whole embryo, Scale bars 1 mm; (C, D) transverse section through trunk, ec ectoderm, mes mesoderm, en endoderm, Scale bars 0·5 mm; (E, F) axial structures, np neural plate, not notochord, dm dorsal mesoderm, aw archenteron wall, epi epidermis, som somite, nt neural tube, Scale bars 0·1 mm. Note that embryonic regions can clearly be identified by their relation to the whole but that individual cells are all undifferentiated.

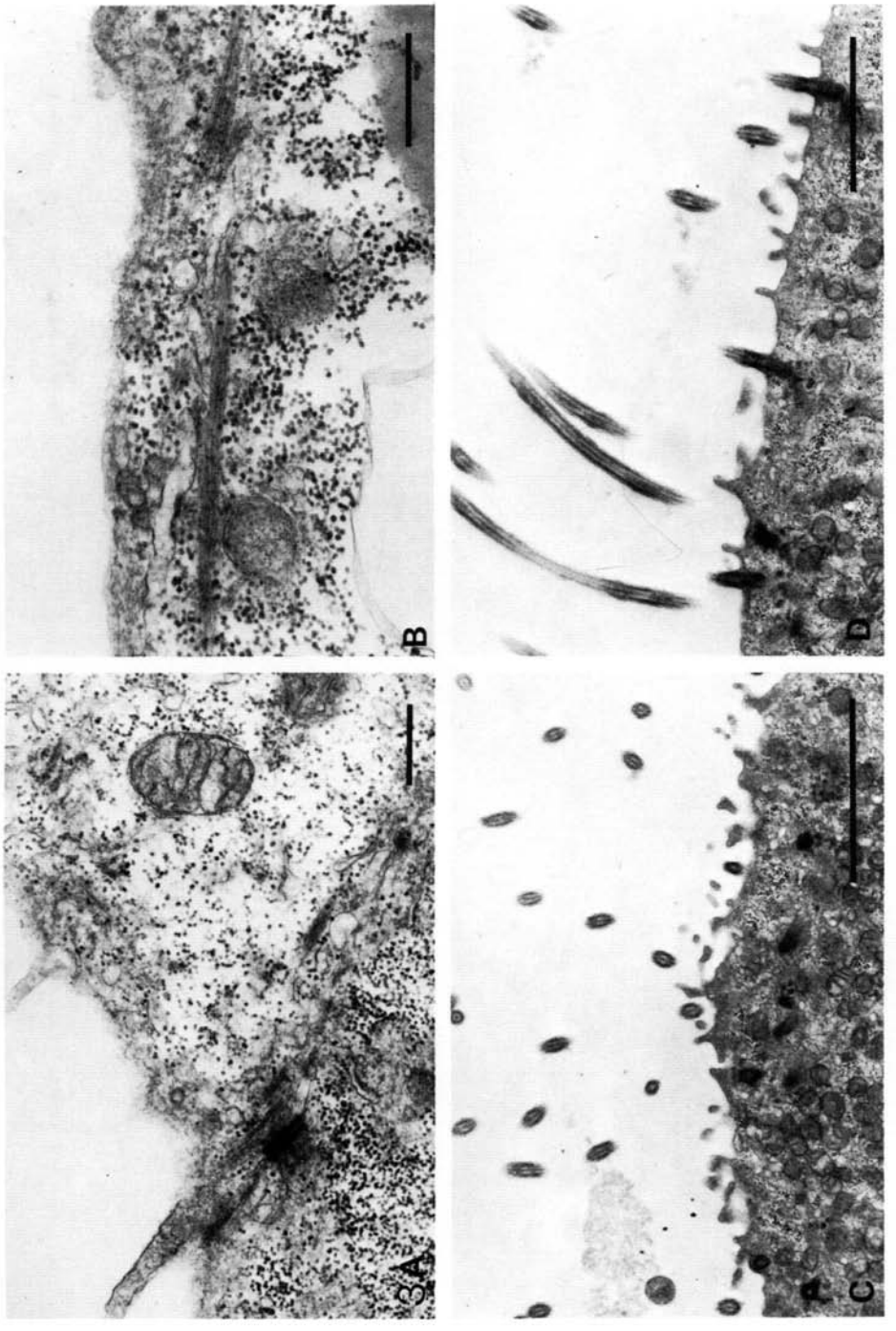


Fig. 3. Electron micrographs of epidermis at stage 20. (A) desmosome with associated tonofilaments, Scale bar 0·5 μm. (B) tonofilament bundles, Scale bar 0·5 μm. (C) cilia, Scale bar 5 μm. (D) cilia, Scale bar 2·5 μm.

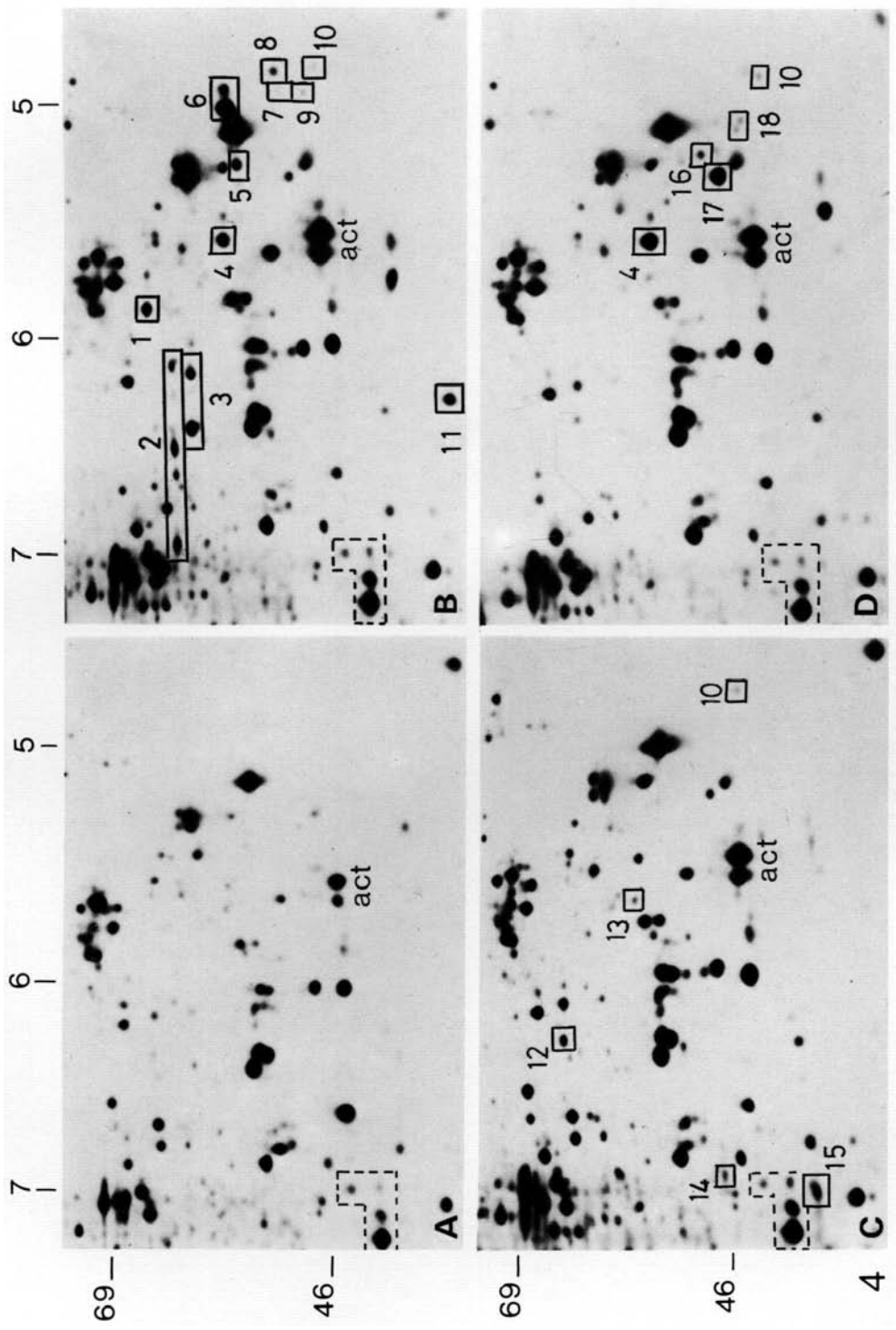


Fig. 4. Two-dimensional gels of newly synthesized proteins made by explants. (A) gastrula ectoderm, (B) neurula epidermis, (C) neurula neural plate, (D) neurula notochord. CPM x weeks exposure equalized for all four gels. Actin (β and γ forms) is labelled act. The dashed line encloses proteins made by all explants which are precipitated by anti-keratin. Labelling was for 3 h in 50 μCi [^{35}S]methionine. pH values are shown at the top, and relative molecular mass markers ($\times 10^3$) on the left side.

Protein synthesis

All of the neurula subdivisions detailed in Fig. 1 were labelled with [^{35}S]-methionine either for 3 h or until controls had reached stage 20 (about 18 h) and the newly synthesized proteins were analysed using both uniform 10 % and 5–15 % gradient SDS polyacrylamide gels. No qualitative differences were apparent and it was concluded that regional markers are insufficiently abundant relative to 'housekeeping' functions to show up following this type of analysis.

Accordingly the neural plate, epidermis, notochord and whole archenteron roofs (=notochord+dorsal mesoderm+underlying flaps of archenteron wall) were analysed by two-dimensional gel electrophoresis. The patterns of spots were compared with each other and with the patterns obtained from stage-10 gastrula ectoderm. These experiments were repeated two or three times with different batches of embryos since in this type of work differences are sometimes not reproducible. At least 18 spots or groups of isoelectric variants were found to increase dramatically in relative intensity between gastrula and neurula stages (Fig. 4). Of these only one (No. 10) was found in all tissues examined. Nine were found only in the epidermis (Nos. 1,2,3,5,6,7,8,9,11). Four appeared specific to neural plate after a 3 h label but appeared also in epidermis following an overnight label (12,13,14,15). No. 4 was found in epidermis and notochord, Nos. 16 and 17 were enhanced in epidermis but further enhanced in notochord, and No. 18 was specific to notochord (or whole archenteron roof).

In view of the fact that the epidermis is known to contain a network of tonofilament bundles from the beginning of neurulation (see previous section and accompanying paper) it was thought likely that some of the epidermis specific proteins might be cytokeratins. This was tested by immunoprecipitation using an anti-human keratin antibody. A two-dimensional gel of the precipitate is shown in Fig. 5 and it will be seen that six of the eleven epidermal features are indeed cytokeratins. There is also present in the precipitate a group of spots of M_r 40×10^3 and isoelectric point 7·0–7·5 which are synthesized by all the tissues (enclosed by dashed line on figures). The epidermis-specific cytokeratins fall into five M_r groups of 62(pI 5·8), 59(pI 5·9–6·9), 54(pI 5·1), 51(pI 5·0) and 46(pI 5·1) $\times10^3$.

The doublet No. 18 was specific to notochord but no other major spots wre found reproducibly to appear in either the neural plate or the notochord cultures alone, although there are a number of quantitative and combinatorial differences which make the patterns recognisable to an experienced observer.

Glycoprotein synthesis

Since the main object of this work is to find markers of tissue regions which can be used in experiments on induction it is preferable to be able to use a one-dimensional rather than a two-dimensional analytical separation. This allows many more samples to be analysed and makes comparison easier. One way of reducing the complexity of one-dimensional gels of total protein is to use a

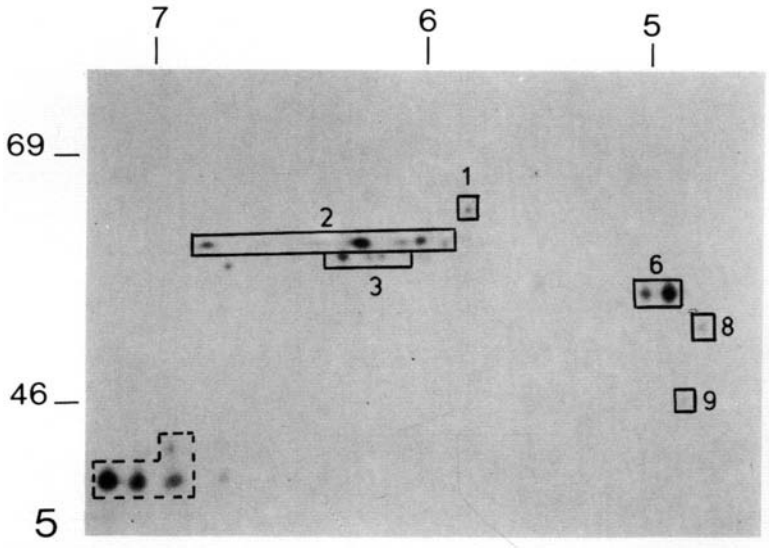


Fig. 5. Two-dimensional gel of proteins newly synthesized by epidermis and precipitated by anti-human keratin antibody. Numbering of spots is the same as Fig. 5. pH values are shown at the top and relative molecular mass markers ($\times 10^3$) on the left side.

radiolabel more selective than an amino acid. In this section results will be described which are obtained by labelling the explants with tritiated sugars. These preferentially label glycoproteins and provide patterns simple enough for analysis by one-dimensional gradient gels. Glycoproteins were selected for study because they are often located at the cell surface or in the extracellular matrix and are thought to play an important role in selective cell adhesion or specific cell recognition – functions which we expect to be displayed by cells of the early embryo.

It was not practicable to prepare pure plasma membranes from numerous small tissue samples and so the samples were processed by a simple three-way andfractionation procedure. After cell disruption and nucleasing, the large yolk granules were removed by very low speed centrifugation. This pellet contained little radioactivity and on analysis was found to contain no unique components. The reason for removing yolk is that it produces a large unsightly bulge of unlabelled material on the gels and distorts the radioactive bands in the same track. The supernatants were separated into a 'soluble' and 'particulate' fraction by ultracentrifugation and material was recovered from the soluble fraction by ethanol precipitation. Examination of these fractions by electron microscopy showed that the 'soluble' fraction contained large lipid droplets along with precipitate and the 'particulate' fraction consisted of all the other inclusions visible in embryonic cells, namely mitochondria, smooth membranes, ribosomes and small yolk granules (Fig. 6).

In all the gels shown here the labelling was carried out using 25 μCi of [^{3}H]-galactose plus 25 μCi of [^{3}H]mannose although similar results are obtained using mannose alone. It became clear early in the work that regional differences were concentrated in the high M_r zone. Because of this, and because glycoprotein relative molecular masses are more reliably measured with gradient than with uniform gels, the standard analysis was carried out with linear SDS polyacrylamide gradient gels.

In Fig. 7 is shown a time course of glycoprotein synthesis in whole embryos. The labelling periods for the three tracks were: stage 7→10 (blastula), stage 10→12 (gastrula), stage 14→20 (neurula) respectively and the products of one embryo were loaded onto each track. It can clearly be seen that there is no appreciable glycoprotein synthesis until neurulation. There is some radioactivity at the buffer front and as we shall see in the following section this is probably mainly phospholipid labelled in the glycerol moiety. Six bands or doublets are labelled S1–S6 (S for sugar label) and there is also a certain amount of polydisperse high M_r material ($>500\times10^3$) at the top of the gel. The comparison of soluble and particulate fractions shows that only S4 and the high M_r material are found in the former while all are found in the latter. S2 and S3 are doublets although the relative proportions of the doublet components may vary between preparations.

In the same figure are shown experiments in which particulate preparations have been incubated with trypsin and collagenase prior to gel separation. All the bands except S4 and the high M_r material appear sensitive to trypsin and can therefore be identified as glycoproteins. The S4 band broadens and migrates a little faster but seems otherwise resistant to trypsin. All bands are

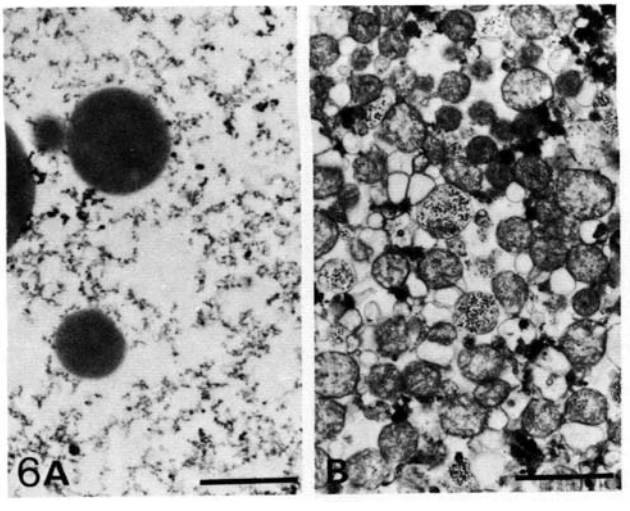


Fig. 6. Electron micrographs of cell fractions. (A) 'soluble', Scale bar 2 μm. (B) 'particulate', Scale bar 2 μm.

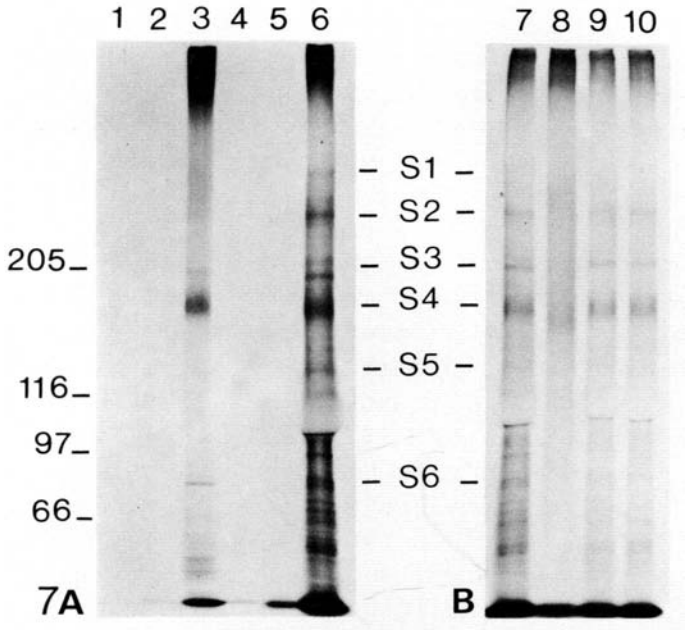


Fig. 7. Synthesis of glycoproteins by whole embryos. Relative molecular mass markers shown on left ($\times 10^3$), band designations in centre. A. Tracks 1–3, soluble fractions from embryos labelled respectively stage 7→10, 10→12, 14→20. Tracks 4–6, particulate fractions from the same specimens. (Products of one embryo are loaded on each track.) B. Particulate fractions treated with enzymes. Track 7 trypsin control, track 8+trypsin, track 9 collagenase control, track 10+collagenase.

resistant to collagenase and so it is unlikely that any of them are collagens despite the fact that they are in the right M_r range for the various procollagens ($100–200\times10^3$).

The regional distribution of glycoprotein synthesis is shown in Fig. 8. In this experiment each track represents an equal amount of protein in the initial supernatants. S1, S3 and S5 are made by all regions of the neurula. S2 is concentrated in the notochord and dorsal mesoderm. S2.2 and S3.2 are made only by the notochord. S4 is made only by the epidermis and S6 is concentrated in the notochord. This type of experiment has been carried out four times with different batches of embryos and has given consistent results. The mean relative molecular masses and regional distribution of the glycoproteins are presented in Table 3.

None of the glycoproteins was labelled with [^{35}S]sulphate although the high M_r polydisperse material was labelled and so it seems probable that this material is proteoglycan.

'Epimucin'

Because of its prominence and tissue specificity the band labelled 'S4' was selected for more intensive characterisation. We have already seen that it is soluble in dilute buffer and has a subunit M_r around 170×10^3.

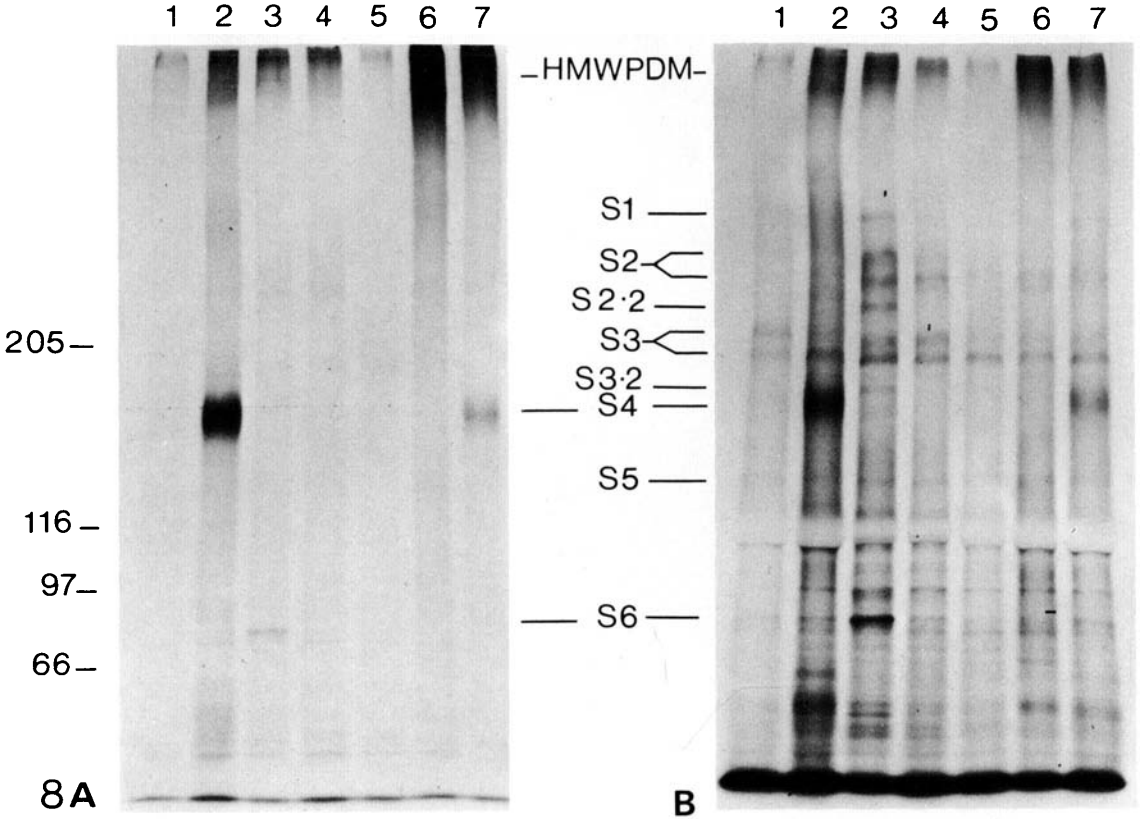


Fig. 8. Synthesis of glycoproteins by different regions of neurulae. A, soluble fractions; B, particulate fractions. For each: track 1 neural plate; track 2 epidermis; track 3 notochord; track 4 dorsal mesoderm; track 5 ventrolateral mesoderm; track 6 archenteron wall; track 7 whole embryo. The amounts loaded correspond to equal amounts of protein (150 μg) in the supernatant prior to the ultracentrifuge spin. Relative molecular mass standards ($\times 10^3$) are shown to right and left and glycoprotein designation in the centre. HMWPDM = high relative molecular mass polydisperse material.

Table 3. *High molecular weight glycoconjugates synthesised by the axolotl neurula*

Species	Relative molecular mass ($\times 10^3$)	Regional specificity
HMWPDM	>500	Mainly endoderm
S1	430	All tissues
S2	315	Mainly mesoderm
S2·2	265	Notochord only
S3	210	All tissues
S3·2	185	Notochord only
S4 ("epimucin")	170	Epidermis only
S5	135	All tissues
S6	82	Mainly notochord

The native M_r was determined by gradient gel electrophoresis of a preparation made by extracting labelled epidermis with tris-borate buffer (Fig. 9A). In this technique electrophoresis is continued until bands have come to a standstill because of the molecular sieve effect of the polyacrylamide. The relative molecular mass was determined by a log–log plot using Pharmacia high M_r standards and came to 470×10^3 (mean of two experiments on different preparations which gave 460 and 480). Clearly this is not a simple multiple of the subunit

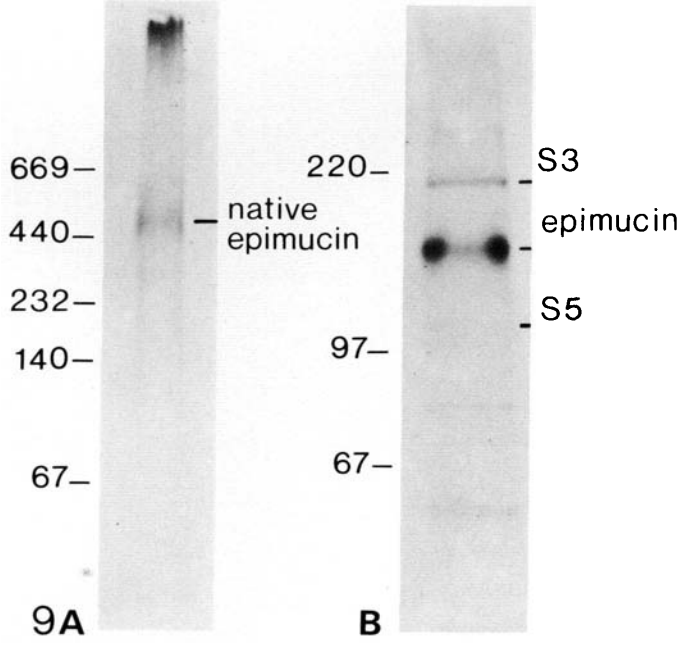


Fig. 9. Characterisation of epimucin. A. Electrophoresis on non-denaturing gradient gel with relative molecular mass markers. B. Epidermal glycoproteins labelled with [^{35}S]methionine and retained by lentil lectin Sepharose.

M_r of 170×10^3. It is possible that there are two 170×10^3 subunits in the molecule and the other 130×10^3 is lost as small fragments on denaturation. Alternatively it might be that the subunit M_r is overestimated by the SDS gel because of glycosylation or underestimated by the gradient gel because of asymmetrical shape.

As mentioned above, S4 is comparatively trypsin resistant. Since it was not visible on one-dimensional gels of [^{35}S]methionine-labelled epidermis or by Coomassie blue staining, it seemed possible that it might not contain any polypeptide at all. However it became visible as an epidermis-specific band of the expected M_r when [^{35}S]methionine-labelled preparations were absorbed onto lentil lectin Sepharose and eluted with α-methyl mannoside (Fig. 9B). This procedure selects out glycoproteins containing mannose or glucose and the removal of the high background of nonglycosylated protein means that components of relatively low abundance can become visible.

In order to gain some insight into the sugar composition, samples of S4 labelled with [^{14}C]glucose and with [^{3}H]galactose were hydrolysed in acid and the products separated by thin-layer chromatography with unlabelled sugars as standards (Fig. 10). It is clear that most of the radioactivity from the galactose-

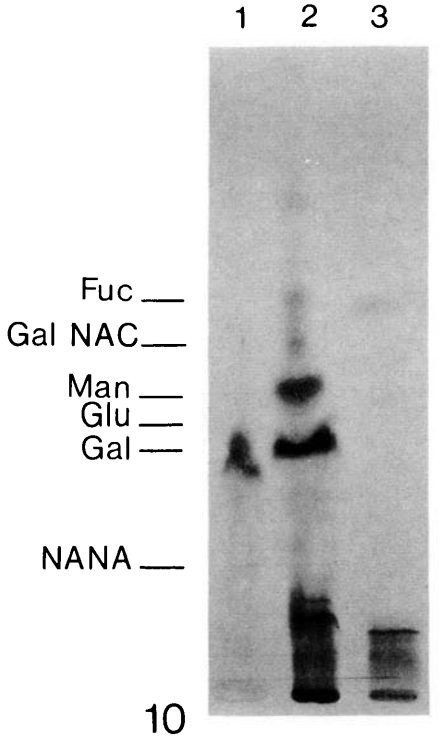


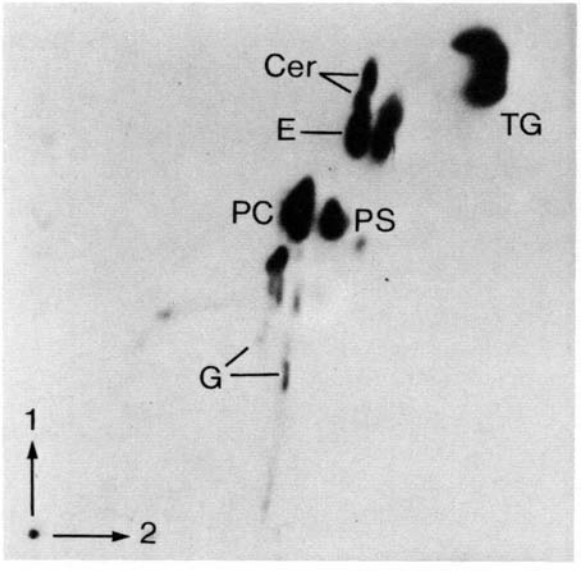
Fig. 10. Characterisation of epimucin. Thin-layer chromatography of acid hydrolysates of epimucin. Track 1: [^{3}H]galactose-labelled epimucin; Track 2: [^{14}C]glucose-labelled epimucin, complete hydrolysis; Track 3: [^{14}C]glucose-labelled epimucin, mild hydrolysis. Positions of standard sugars shown on left.

labelled samples can be recovered in the precursor sugar. In the case of [^{14}C]-glucose we would expect much more metabolic redistribution of the label, and in fact several different labelled species are recovered. fucose, mannose and galactose can tentatively be identified on the basis of the chromatography.

To summarise, S4 is a species with a native M_r of 470 $\times 10^3$ and an apparent subunit molecular weight of 170 $\times 10^3$. It is labelled by [^{35}S]methionine, [^{3}H]galactose, mannose, glucosamine (data not shown) and [^{14}C]glucose but not by [^{35}S]sulphate. It contains fucose, mannose, galactose and other sugars. The relative labelling by the different precursors, together with the trypsin resistance and lack of staining by Coomassie blue suggests a high ratio of carbohydrate to polypeptide. It is soluble in dilute buffers and binds to lentil lectin Separose. These properties do not correspond to those of any named molecule known to the author (see Discussion) and so the name '*epimucin*' is proposed in accordance with its tissue specificity and high sugar content.

Glycolipid analysis

Explants from all stages and regions examined incorporated [^{3}H]galactose into lipid. [^{35}S]sulphate was found not to be incorporated into lipids at all. In early experiments total lipid extracts were analysed by one-dimensional thin-layer chromatography. It soon became clear that most of the labelled bands were not glycolipid but triglyceride and phospholipid. This is shown clearly on Fig. 11 in



11

Fig. 11. Thin-layer chromatography of total lipid extract from heads of tailbud-stage embryos labelled in [^{3}H]galactose. TG triglyceride, Cer cerebroside, PE phosphatidyl ethanolamine, PC phosphatidyl choline, PS phosphatidyl serine, G gangliosides. Solvent 1: chloroform: methanol: ammonia 45: 55: 15. Solvent 2: n-butanol: acetic acid: water 50: 20: 30.

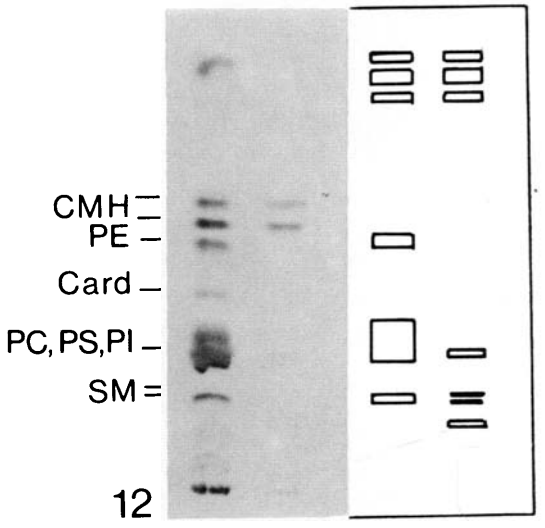


Fig. 12. Thin-layer chromatography of tailbud-stage lipids from yolk mass labelled with [^{3}H]galactose. Left track: total lipid extract (as Fig. 11), right track: after mild alkaline hydrolysis. On the left are shown positions of chromatographic standards and on the right a drawing of the sample tracks after exposure to iodine vapour. Solvent: chloroform: methanol: water 60: 30: 5.

which total lipid extracts from late (stage-30) embryos labelled with [^{3}H]galactose are fractionated by two-dimensional TLC. The identification of spots depends on the positions of standards run on parallel plates and visualized with iodine vapour or with the α-naphthol reagent. There are however some labelled spots which have the chromatographic behaviour of glycolipids: cerebroside and certain gangliosides can be identified with some confidence.

Once it was realized that there was considerable metabolic relocation of the galactose label the samples were routinely subjected to mild alkaline deacylation followed by solvent partition. This gave a much simpler pattern which could be analysed by one-dimensional TLC (Fig. 12). The removal of all radioactivity from triglyceride and phospholipid bands by this procedure shows that the label must be in a water-soluble moiety, presumably glycerol.

The standard one-dimensional TLC separation of alkali-stable embryo lipids (Fig. 12) showed two iodine staining bands near the solvent front which had the chromatographic behaviour of fatty acid methyl esters and cholesterol. These were unlabelled. There were three iodine-positive bands in the polar region. All three gave a positive reaction with molybdenum blue reagent (Sigma) indicating that they are phospholipids. The middle one was unlabelled and comigrated with sphingomyelin standard. The first and third were labelled and are probably alkyl diether and alkyl monoether glycerophosphatides labelled in the glycerol moiety. It should be noted that the neutral glycolipid standards give doublets on thin-layer chromatography. This is because they are mixtures of forms with

different fatty acid composition and the trailing band is hydroxylated while the leading band is not.

The regional distribution of glycolipid synthesis in early embryos is shown in Fig. 13. This shows chromatograms of alkali stable [^{3}H]galactose-labelled lipids extracted from explants dissected as shown in Fig. 1, and labelled until controls had reached stage 22. All tissues show some radioactive bands in the region of ceramide mono-, di-, tri- and tetrahexoses with the monohexose predominating greatly over the others. There is a certain quantitative variation between the tracks, the epidermis showing significantly more counts in the putative CTH and less in the putative C4H than the other tissues. It also seems to lack the tight band present in all the others. There is also variation in the region near the origin which is expected to contain gangliosides and complex neutral glycolipids. In general however the tissue variations are quantitative rather than qualitative.

To prove that the labelled bands really were glycolipids, unlabelled alkali-stable lipid preparations were labelled *in vitro* with galactose oxidase and sodium[^{3}H]borohydride. This technique specifically labels molecules containing a terminal galactose or galactosamine. Unfortunately there is some non-specific enzyme-independent labelling, but the results show that at least four species are labelled (Fig. 14). The first three have the approximate mobilities of CMH, CTH and C4H respectively. The patterns are, of course, expected to differ from those of Fig. 13 because this type of experiment detects *presence* of glycolipid rather

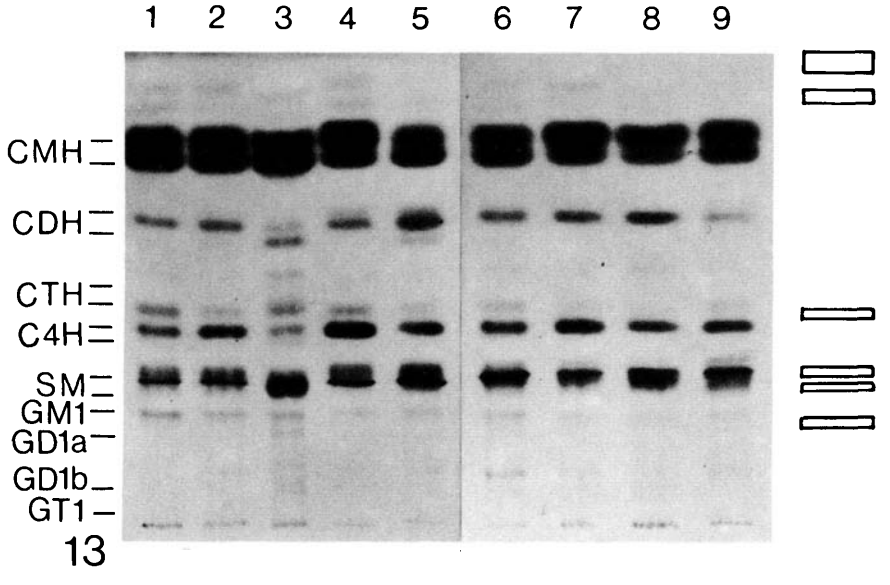


Fig. 13. Thin-layer chromatography of [^{3}H]galactose-labelled alkali stable lipids from different regions of axolotl neurulae. Track 1 anterior neural plate, 2 posterior neural plate, 3 epidermis, 4 notochord, 5 dorsal mesoderm, 6 ventrolateral mesoderm, 7 archenteron wall, 8 yolk mass, 9 whole embryo. Solvent: chloroform: methanol: water 55: 45: 10. Chromatographic standards are shown on left. The iodine positive pattern, which is the same for all samples, is shown on the right.

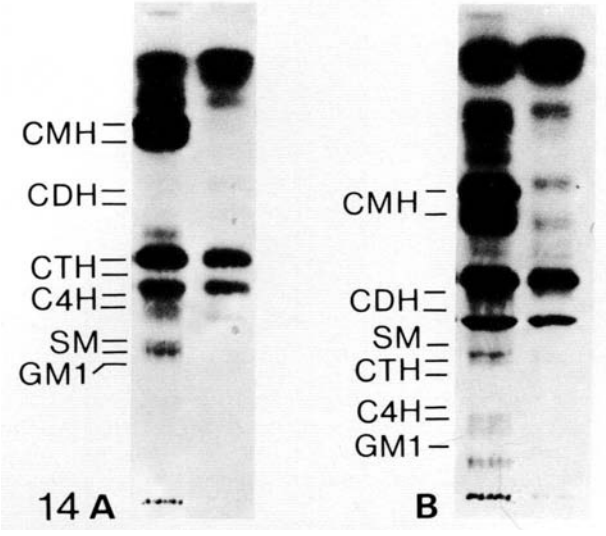


Fig. 14. Thin-layer chromatography of lipids isolated from whole tailbud-stage embryos and labelled *in vitro* by the galactose oxidase method. Left track experimental, right track control without enzyme. A. Solvent: chloroform: methanol: water 55: 45: 10. B. Solvent: chloroform: methanol: ammonia 55: 45: 10.

than new synthesis. Elution, acid hydrolysis and thin-layer chromatography of the CMH band showed that the end group was indeed galactose, and so this molecule can confidently be identified as galactocerebroside. Insufficient counts were available for this analysis to be conducted on the other bands.

Because of the absence of specific glycolipid markers at the neurula stage, no detailed investigation was made of the time course of synthesis of the glycolipids described. However early experiments which involved labelling of whole embryos by injection with labelled sugars showed that galactocerebroside is synthesised throughout early development from the zygote onwards. This is in striking contrast to the glycoproteins, which we have seen only start to be synthesized from the onset of neurulation.

Gangliosides

Although the amount of label obtained in the ganglioside region of the chromatograms was too small to be sure about regional differences in synthesis, it was felt desirable to confirm that the embryos actually did contain gangliosides. This was done by preparing gangliosides from unlabelled tailbud stage embryos and then labelling them *in vitro* by the mild periodate oxidation followed by sodium[^{3}H]borohydride. This is specific for the side chains of neuraminic acid and its derivatives. There are five clearly labelled bands, three of which comigrate with the standards GM1, GD1b and GT1 (Fig. 15). The fastest band is probably GM3 for which no standard was available at the time. A chemical measurement of ganglioside by the method of Jourdian *et al.* (1971) showed that

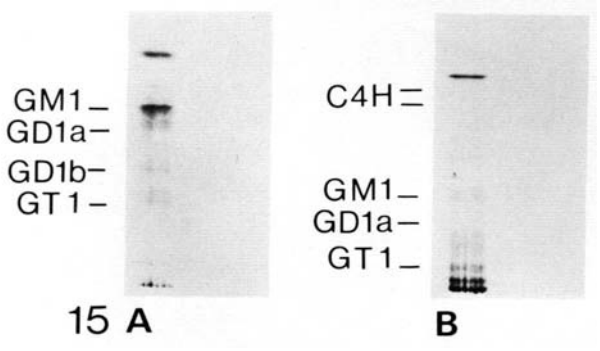


Fig. 15. Thin-layer chromatography of gangliosides isolated from tailbud-stage embryos and labelled *in vitro* by the periodate method. Left track experimental, right track control without periodate. A. Solvent: chloroform: methanol: water 55: 45: 10 two runs. B. Solvent: chloroform: methanol: ammonia 55: 45: 10 two runs.

the preparation used was 33 % ganglioside by weight, which means 0·13 μg ganglioside per embryo assuming complete recovery.

DISCUSSION

It is a familiar idea that different histological cell types show major qualitative differences in their biosynthesis, and indeed the differences have to be major because they become visible as differences in shape, size and staining properties down the microscope. They are necessary concomitants of functional specialisation of the cell: for contraction, for impulse conduction, for secretion, for mechanical strength, and so on.

It would be a mistake to expect the same degree of biochemical difference between the histologically undifferentiated cells of the early embryo since most of the functions characteristic of specialized cells in the adult are as yet undeveloped. It is notable for example that the regional biochemical markers known in the early mouse embryo are mainly found in extraembryonic tissue which are already differentiated and have functions concerned with the support and nutrition of the embryo (Hogan, Barlow & Tilly, 1983). There are however certain transient functions within the embryo itself which we might expect to be associated with specific macromolecules: the guidance of cell movements, the junctions and extracellular materials responsible for cell adhesion, the permeability of cell sheets, and the selective transport of inorganic ions and of nutrients across the cell membrane. These might be expected to appear as transient biosynthetic differences in substances of at least intermediate relative abundance and to be useful as markers for experiments on induction.

More interesting than these physiological functions are the mechanisms underlying regional pattern formation (Slack, 1983a). In order to account for

the progressive spatial organisation of the embryo it is necessary to assume that the sequential inductive interactions lead to regionally specific activation of combinations of biochemical switches. The combination of switches active in a particular cell at a particular stage is its epigenetic coding (or 'positional value' (Wolpert 1969)). The totality of codings is the 'second anatomy' of the organism (Slack 1982b, Smith & Slack 1983) which causes the later spatial pattern of cytodifferentiation. Of course we do not know whether the cell states that we call codings are associated with specific macromolecules or not. They might instead depend on quantitative differences in a large number of substances, either in the realm of macromolecules or of intermediary metabolism. However at present we have the technology to look for specific macromolecules and so it would be foolish not to do so, even if the search should eventually prove unsuccessful.

It is because of the above considerations that the present work has concentrated specifically on the problem of *regional* specificity. This is in contrast to much biochemical embryology in which the *temporal* sequence of events in whole embryos is emphasized (Davidson 1976). The techniques which have been used in the present work are not sufficiently sensitive to detect substances of low abundance, ie, less than about 10 000 molecules per 10^{-10} cm^3 tissue where the latter is roughly the size of a mammalian cell. In the case of [^{35}S]methionine this is because so many substances of high abundance are labelled that extensive purification is necessary to lower the background. In the case of ^{3}H-sugars it is because the isotope itself is of too low specific activity; using fluorographic methods the detection limit is about 10^{11} atoms of ^{3}H. It follows that the markers which have been discovered in the present work must be of intermediate rather than low abundance and so it seems likely that they represent physiological functions rather than epigenetic codings.

Cytokeratins

Most of the major epidermis specific spots on the two-dimensional gels are regarded as cytokeratins on the basis of immunoprecipitation with specific antibody. It is probable that these proteins make up the tonofilament network which is specific to epidermis and also makes its first appearance at the end of gastrulation (Eakin & Lehmann 1957; Burnside 1971). In a previous two-dimensional gel study of limb regeneration in the axolotl (Slack 1982a) a number of epidermis specific spots were described which were synthesized by the epidermis of the regeneration blastema. Many but not all of these are the same as the cytokeratins of neurula epidermis described here.

It is of some interest that in other embryos which have been examined, cytokeratins are the first intermediate filament protein type to appear. For example in the mouse they are made in the extraembryonic trophectoderm at the blastocyst stage (Jackson *et al.*, 1980).

Glycoproteins

A specific glycoprotein, here named epimucin, is synthesized only by the epidermis. It is possible that this represents the external mucous secretion of the organism. However the characteristic secretory granules do not appear in the epidermis until the late tailbud stage which is about three days later than the end of the incubation period used here. Epidermis labelled at this stage shows a band resembling epimucin but with a slightly higher mobility, and also a number of new glycoproteins not seen during neurulation. Another possibility might be that epimucin is a new basement membrane component.

A number of high relative molecular mass glycoproteins have been characterized and named over the last few years and serious consideration has been given to the possibility that epimucin mught be the same as one of them. Of candidates with SDS-relative molecular masses in the range 150–200 $\times 10^3$, N-CAM (Hofman *et al.* 1982) and entactin (Carlin, Jaffe, Bender & Chung 1981) seem unlikely since they require detergent for extraction and the latter is characteristically sulphated. Type IV procollagen (Bornstein & Sage 1980) should be sensitive to collagenase, and laminin (Timpl *et al.* 1979) has a much higher native M_r. The closest match is probably with thrombospondin (Lawler, Slayter & Coligan 1978; Jaffe *et al.* 1983) which has native and subunit molecular weights M_r of 450 and 150 ($\times 10^3$) respectively and is becoming recognized as a widespread component of the extracellular matrix. However thrombospondin has quite a low sugar content and is broken down into fragments of less than 100×10^3 by trypsin. It is therefore thought that epimucin is not homologous to this or any other previously described molecule and that a new name is appropriate.

It was thought possible that the universal band S3 might be fibronectin (Hynes & Yamada, 1982) but it proved not possible to precipitate it with anti-*Xenopus* fibronectin antibody (kindly donated by Dr C. Wylie, St Georges). The bands S2.2 and S3.2 are specific to the notochord and may perhaps be components of the characteristic notochord sheath, although this is not yet visible even down the electron microscope at the stages considered. These glycoproteins will be more fully characterized in due course.

Glycoproteins are not synthesized until the neurula stage whereas glycolipids are synthesized throughout early development and the high relative molecular mass polydisperse material is synthesized during gastrulation. Since this is labelled by ^{3}H-sugars and ^{35}S-sulphate but not by [^{35}S]methionine it is presumably proteoglycan, perhaps that demonstrated histochemically by Kosher & Searls (1973) and by Johnson (1977, 1978). The present study shows that its synthesis is concentrated in the endoderm.

Glycolipids

The results presented have shown that glycolipids are both present and

synthesized in the early embryo. These substances are probably the best candidates for positional coding factors since their molecular structure allows for extensive combinatorial specificity, they are located on the outer surface of the plasma membrane and although their functions are at present completely unknown they show qualitative changes during cell differentiation in the adult and also on neoplastic transformation (Hakomori 1981).

The results show two species whose synthesis is greatly enhanced in the epidermis and one which is diminished. There is not much regional variation among the other tissue explants studied but this may be because the complex glycolipids, which are potentially the most interesting, are insufficiently abundant to be clearly visible after tritium labelling. It is unfortunate that glycolipids are composed only of C, H, N and O and so it is not possible to label with an isotope of higher specific activity than tritium. There are now available some potentially more sensitive detection methods involving ^{125}I-labelled lectins or antibodies (Magnani, Brockhaus, Smith & Ginsburg 1982) but these will of course detect presence rather than new synthesis.

The epidermis

It is noteworthy that all the methods used in this study have revealed unique markers in the epidermis whereas only the high M_r glycoproteins differ significantly between the other regions. Perhaps the epidermis should be considered as a differentiated tissue as early as the beginning of neurulation, its function being to act as a barrier separating external from internal environments. However it is not *terminally* differentiated at this stage since it is still destined to form specialized structures such as lens or other placodes in response to signals from the underlying tissue, so the features displayed are presumably reversible until somewhat later in development.

I am grateful to James Vernon for electron microscopy, Harvey Jacobs for photography and Jim Smith for stimulating discussions.

REFERENCES

BALLANTINE, J. E. M., WOODLAND, H. R. & STURGESS, E. A. (1979). Changes in protein synthesis during the development of *Xenopus laevis*. *J. Embryol. exp. Morph.* **51**, 137–153.

BONNER, W. M. & LASKEY, R. A. (1974). A film detection method for ^{3}H-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.

BONNER, W. M. & STEDMAN, J. D. (1978). Efficient fluorography of ^{3}H and ^{14}C on thin layers. *Anal. Biochem.* **89**, 247–256.

BORDZILOVSKAYA, N. P. & DETLAFF, T. A. (1979). Table of stages of normal development of axolotl embryos and the prognostication of timing of successive developmental stages at various temperatures. *Axolotl Newsletter* No. 7.

BORNSTEIN, P. & SAGE, H. (1980). Structurally distinct collagen types. *Ann. Rev. Biochem.* **49**, 957–1003.

BRAVO, R. & KNOWLAND, J. (1979). Classes of protein synthesized in oocytes, eggs, embryos and differentiated tissues of *Xenopus laevis*. *Differentiation* **13**, 101–108.

BROCK, H. W. & REEVES, R. (1978). An investigation of *de novo* protein synthesis in the South African clawed toad, *Xenopus laevis*. *Devl Biol.* **66**, 128–141.

BURNSIDE, B. (1971). Microtubules and microfilaments in newt neurulation. *Devl Biol.* **26**, 416–441.

CARLIN, B., JAFFE, R., BENDER, B. & CHUNG, A. E. (1981). Entactin, a novel basal lamina associated sulphated glycoprotein. *J. biol. Chem.* **256**, 5209–5214.

DAVIDSON, E. H. (1976). *Gene Activity in Early Development*, 2nd edn, New York: Academic Press.

EAKIN, R. M. & LEHMANN, F. E. (1957). An electron microscopic study of developing amphibian ectoderm. *Wilhelm Roux Arch. EntwMech. Org.* **150**, 177–198.

FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. biol. Chem.* **226**, 497–509.

GARRELS, J. I. (1979). Two dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. *J. biol. Chem.* **254**, 7961–7977.

GERHART, J. C. (1981). Mechanisms regulating pattern formation in the amphibian egg and early embryo. In "*Biological Regulation and Development*" Volume 2 (ed. R. Goldberger), pp. 133–316. New York: Plenum Press.

GURDON, J. B. (1974). *The Control of Gene Expression in Animal Development.* Oxford University Press.

HAKOMORI, S. I. (1981). Glycosphingolipids in cellular interaction, differentiation and oncogenesis. *Ann. Rev. Biochem.* **50**, 733–764.

HAMES, B. D. & RICKWOOD, D. (1981). *Gel Electrophoresis of Proteins: a Practical Approach.* London: IRL Press Ltd.

HOFMAN, S., SORKIN, B. C., WHITE, P. C., BRACKENBURY, R., MAILHAMMER, R., RUTIHAUSER, U., CUNNINGHAM, B. A. & EDELMAN, G. M. (1982). Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J. biol. Chem.* **257**, 7720–7729.

HOGAN, B. L. M., BARLOW, D. P. & TILLY, R. (1983). F9 Teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surveys* **2**, 115–140.

HOLTZER, H. (1978). Cell lineages, stem cells and the "quantal" cell cycle concept. In "*Stem Cells and Tissue Homeostasis*" (ed. B. I. Lord, C. S. Potten & R. J. Cole), pp. 1–27. Cambridge: Cambridge University Press.

HYNES, R. O. & YAMADA, K. M. (1982). Fibronectins: multifunctional globular glycoproteins. *J. Cell Biol.* **95**, 369–377.

IUPAC-IUB COMMISSION ON BIOCHEMICAL NOMENCLATURE (1978). The Nomenclature of Lipids. *Biochem. J.* **171**, 21–35.

JACKSON, B. W., GRUND, C., SCHMID, E., BURKI, K., FRANKE, W. W., & ILLMENSEE, K. (1980). Formation of cytoskeletal elements during mouse embryogenesis. I. Intermediate filaments of the cytokeratin type and desmosomes in preimplantation embryos. *Differentiation* **17**, 161–179.

JAFFE, E. A., RUGGIERO, J. T., LEUNG, L. L. K., DOYLE, M. J., MCKEOWN-LONGO, P. J. & MOSHER, D. F. (1983). Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix. *Proc. natn. Acad. Sci., U.S.A.* **80**, 998–1002.

JACOBSON, M. (1982). Origins of the nervous system in amphibians. In "*Neuronal Development*" (ed. N. C. Spitzer), pp. 45–99. New York: Plenum Press.

JOHNSON, K. E. (1977). Extracellular matrix synthesis in blastula and gastrula stages of normal and hybrid frog embryos. III. Characterization of galactose and glucosamine labelled materials. *J. Cell Sci.* **25**, 335–354.

JOHNSON, K. E. (1978). Extracellular matrix synthesis in blastula and gastrula stages of normal and hybrid frog embryos. IV. Biochemical and autoradiographic observations on fucose, glucose and mannose labelled materials. *J. Cell Sci.* **32**, 109–136.

JOURDIAN, G. W., DEAN, L. & ROSEMAN, S. (1971). The Sialic Acids XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. *J. biol. Chem.* **246**, 430–435.

KOSHER, R. A. & SEARLS, R. L. (1973). Sulphated mucopolysaccharide synthesis during the development of *Rana pipiens*. *Devl Biol.* **32**, 50–68.

LAWLER, J. W., SLAYTER, H. S. & COLIGAN, J. E. (1978). Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. *J. biol. Chem.* **253**, 8609–8616.

MAGNANI, J. L., BROCKHAUS, M., SMITH, D. F. & GINSBURG, V. (1982). Detection of glycolipid ligands by direct binding of carbohydrate binding proteins to thin layer chromatograms. *Meth. Enzymol.* **83D**, 235–241.

MOHUN, T. J., TILLY, R., MOHUN, R. & SLACK, J. M. W. (1980). Cell commitment and gene expression in the axolotl embryo. *Cell* **22**, 9–15.

MORMOI, T., ANDO, S. & MAGAI, Y. (1976). High resolution preparative column chromatographic system for gangliosides using DEAE Sephadex and a new porous silica – iatrobeads. *Biochem. Biophys. Acta* **441**, 488–497.

NAKAMURA, O. & TOIVONEN, S. (eds) (1978). *Organizer – A Milestone of a Half Century from Spemann*. Amsterdam: Elsevier/N. Holland.

NARASIMHAN, R., HAY, J. B. GREAVES, M. F. & MURRAY, R. K. (1976). Studies on the glycolipids of sheep thymus and of normal and Concanavalin A stimulated sheep peripheral lymphocytes. *Biochem. Biophys. Acta* **431**, 578–591.

NIEUWKOOP, P. D. (1973). The "organization centre" of the amphibian embryo, its origin, spatial organization and morphogenetic action. *Adv. Morphogen.* **10**, 1–39.

O'FARRELL, P. H. (1975). High resolution two dimensional electrophoresis of proteins. *J. biol. Chem.* **250**, 4007–4021.

SCHROEDER, T. E. (1970). Neurulation in *Xenopus laevis*. An analysis and model based upon light and electron microscopy. *J. Embryol. exp. Morph.* **23**, 427–462.

SLACK, J. M. W. (1982*a*). Protein synthesis during limb regeneration in the axolotl. *J. Embryol. exp. Morph.* **70**, 241–260.

SLACK, J. M. W. (1982*b*). Regeneration and the second anatomy of animals. In "*Developmental Order*" *40th Symposium of Society for Developmental Biology* (ed. S. Subtelny & P. B. Green), pp. 423–436. London: Academic Press.

SLACK, J. M. W. (1983*a*). *From Egg to Embryo. Determinative Events in Early Development.* Cambridge: Cambridge University Press.

SLACK, J. M. W. (1983*b*). How I get eggs out of axolotls. *Axolotl Newsletter* **13**, 28–30.

SMITH, J. C. & SLACK, J. M. W. (1983). Dorsalisation and neural induction: properties of the organiser in *Xenopus laevis*. *J. Embryol. exp. Morph.* **78**, 299–317.

STURGESS, E. A., BALLANTINE, J. E. M., WOODLAND, H. R., MOHUN, P. R., LANE, C. D. & DIMITRIADIS, G. J. (1980). Actin synthesis during the early development of *Xenopus laevis*. *J. Embryol. exp. Morph.* **58**, 303–320.

SUZUKI, Y. & SUZUKI, K. (1972). Specific radioactive labelling of terminal N-acetyl galactosamine of glycosphingolipids by the galactose oxidase – sodium borohydride method. *J. Lipid Res.* **13**, 687–690.

TIEDEMANN, H. (1976). Pattern formation in early developmental stages of amphibian embryos. *J. Embryol. exp. Morph.* **35**, 437–444.

TIMPL, R., ROHDE, H., ROBEY, P. G., RENNARD, S. I., FOIDART, J.-M. & MARTIN, G. R. (1979). Laminin – a glycoprotein from basement membranes. *J. biol. Chem.* **254**, 9933–9937.

UBBELS, G. A. & HENGST, R. T. M. (1978). A cytochemical study of the distribution of glycogen and mucosubstances in the early embryo of *Ambystoma mexicanum*. *Differentiation* **10**, 109–121.

VEH, R. W., CORFIELD, A. P., SANDER, M. & SCHAUER, R. (1977). Neuraminic acid specific modification and tritium labelling of gangliosides. *Biochem. Biophys. Acta* **486**, 145–160.

WOLPERT, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. theor. Biol.* **25**, 1–47.

(Accepted 12 January 1984)