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AN INVESTIGATION INTO CELL SURFACE
VARIATION DURING THE EARLY DEVELOPMENT
OF XENOPUS LAEVIS

by

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This thesis is dedicated to my parents and to Ian -
without whose help and encouragement I would not have got this
far.

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DECLARATION

Except where this is otherwise acknowledged, the work described in this thesis is my own, and has not been submitted in any previous application for a degree. All published sources of information are noted by reference.

Jane R. Lewis

SUMMARY

Membrane proteins in *Xenopus laevis* embryos were analysed on 1- and 2-dimensional gels. Proteins showing temporal variation in their expression in the membrane during *Xenopus* development were detected. The overall range of membrane proteins present between fertilisation and late neurula stage embryos was found to stay essentially constant, with few proteins showing temporal variation.

Microinjection of ^{35}S -methionine into *Xenopus laevis* embryos was used to investigate membrane protein synthesis. Dramatic changes in the composition of proteins synthesised during *Xenopus laevis* early development were noted, with most significant changes observed at the gastrula stage following the mid-blastula transition.

Cell membrane variation in *Xenopus laevis* embryos was studied using cell surface labelling with ^{125}I Iodine. Two proteins specific to labelling of disaggregated embryos were detected and these are thought to be specific to intercellular membranes. Cell surface labelling with ^{125}I Iodine was also used to characterise overall changes in the cell membrane during *Xenopus laevis* early development. Histo-autoradiography of ^{125}I Iodine surface labelled embryos was used to confirm the origin of cleavage furrow membrane in the dividing embryo, and the source of ciliated cells from the double layered epidermis in *Xenopus laevis* neurulae.

2B12, a monoclonal antibody raised against adult *Xenopus laevis* brain tissue, was characterised on embryo and tissue sections using indirect immunofluorescence. Biochemical analysis of 2B12 activity has indicated that the antigen may be a ganglioside, specific to myelin and other nervous tissue membrane.

ABBREVIATIONS

APS	Ammonium persulphate
bis, bisacrylamide	N,N'-methylene bisacrylamide
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CJM	Cell junctional molecule
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dw	Distilled water
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
g	Gram, gravity
GAM	Goat anti mouse
GAR	Goat anti rabbit
GSL	Glycosphingolipid
HAT	Hypoxanthine, Aminopterin, Thymidine
hr	Hour
HCG	Human chorionic gonadotropin
Hepes	N-2-hydroxyethylpiperazine -N'-2-ethanesulphonic acid
HRP	Horseradish peroxidase

Ig	Immunoglobulin
IMP	Intramembranous particle
I.P	Intraperitoneal
i. u.	International units
m	Metre
MBT	Midblastula transition
min	Minute
Mr	Relative molecular mass
mRNA	Messenger RNA
NBCS	New born calf serum
Nephge	Non-equilibrium pH gradient gel electrophoresis
NP40	Nonidet P40
OD	Optical density
page	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMSF	Phenyl methyl sulphonyl fluoride
PPO	2,5-diphenyloxazole
RAM	Rabbit anti mouse
RNA	Ribonucleic acid
sec	Second
SAM	Substratum adhesion molecule
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
Tris	Tris(hydroxymethyl)aminomethane

TX100

Triton-X100

TX114

Triton-X114

uv

Ultra violet light

v

Volume

w

Weight

CHAPTER 1
INTRODUCTION

The aim of the work described in this thesis is to investigate and characterise observed changes in cell surface membranes during the early development of *Xenopus laevis*.

This introduction will review of work in related fields. An outline of development in *Xenopus laevis* embryos is given followed by a discussion of the specialised nature of the *Xenopus* embryo cell membrane. The last two sections will consider the role of the cell membrane in pattern formation and in morphogenesis.

SECTION 1.1 AN OUTLINE OF XENOPUS LAEVIS EARLY DEVELOPMENT

The eggs and embryos of Xenopus laevis have been widely studied by experimentalists over the past hundred years. Xenopus laevis was first noted in Europe in 1802 when Daudin described the organism and named it Bufo laevis, meaning smooth skinned toad like creature. From this the organism has become inappropriately known as the South African Clawed toad. Xenopus is in fact more closely related to the Pipa genus of frogs and belongs to the family Pipidae. A full classification of Xenopus laevis is given in Nieuwkoop and Faber (1967).

Xenopus laevis has become a commonly chosen animal for studies of development. This interest derives from a number of technical factors. The fertilisation of Xenopus eggs occurs externally leaving the developing embryo accessible to investigation and experimentation at all stages. Furthermore, the eggs and embryos can be obtained by hormonal stimulation throughout the year. The eggs are large (around 1.2mm in diameter), and are therefore suitable for micro-operative procedures. Fragments of early Xenopus embryos will continue to develop independently if incubated in simple isotonic or hypotonic salt solutions. This is due to all cells in the embryo having a supply of yolk platelets to serve as nutrient. Finally, Xenopus laevis shows rapid early development enabling short term culturing experiments to be carried out.

In this section an outline of Xenopus laevis early

development will be described with the role of the plasma membrane in development emphasised. Throughout this thesis, stages of Xenopus laevis embryo development will refer to those outlined in Nieuwkoop and Faber (1967). For a more detailed review of development in Xenopus laevis see Balinsky (1981) or Nieuwkoop and Faber (1967).

1.1a Oogenesis

The morphological aspects of oocyte development in Xenopus laevis have been described by Balinsky and Devis (1963), and by Dumont (1972) and Coggins (1973). The oocyte is surrounded during its entire growth and maturation in the ovary by follicle cells. Desmosomes connect the oocyte and follicle cell membranes, and hold the cells together in close contact (Wartenberg, 1962). As the oocyte develops, microvilli form on both the oocyte and follicle cell membranes with the microvilli from both cells interdigitating (Kemp, 1956). Through the base of the microvilli the oocyte is thought to absorb fluids from the follicle cell by pinocytosis (Press, 1959).

The oocyte remains transparent until it reaches a diameter of around 300 μ m when it starts to accumulate yolk from small capillaries surrounding the follicle cells. Yolk in the oocyte is formed into yolk platelets inside modified mitochondria (Balinsky and Devis, 1963). These yolk platelets ultimately become unevenly distributed in the cytoplasm of the egg being most dense towards the vegetal pole. The amphibian oocyte also stores lipid in the form of lipochondria (Holtfreter, 1946) and glycogen in the form of

small cytoplasmic granules.

Following the onset of yolk uptake (vitellogenesis), the oocyte starts to synthesise pigment in the form of granules. These granules are mainly restricted to the cortex underlying the oocyte surface. Initially the pigment granules cover the entire surface of the oocyte but are gradually restricted to one half as the oocyte increases in size. The pigmented side of the oocyte is termed the animal pole since this eventually gives rise to the more 'animal' structures of the frog (e.g. the nervous system) and the unpigmented side is termed the vegetal pole since this side gives rise to the 'lower' functions of the frog (e.g. the gut). The uneven distribution of pigment in the oocyte indicates a polarisation within the cytoplasm of the cell along the animal/vegetal axis (see Wittek, 1952; Kemp, 1953).

One of the most significant aspects of oocyte development is that of the cortex. The cortex is the layer of viscous cytoplasm underlying the plasma membrane, which is known to comprise a high concentration of cytoskeletal components. The cortex has been suggested to comprise only the cell membrane and the particles immediately attached to it (Mercoer and Wolpert, 1962), or alternatively, to comprise a layer of cytoplasm around 2-3 μ m in thickness (Hiramoto, 1957; Mitchison, 1956). An important aspect of cortical differentiation is the formation of cortical granules (Kemp, 1956). These are spherical bodies of around 2 μ m diameter formed in the Golgi apparatus (Bacc and Zamboni, 1967). The granules are surrounded by a simple membrane and contain acid mucopolysaccharides. From the Golgi they move to

the periphery and come to underly the surface of the oocyte. Cortical granules are important at fertilisation when the granules burst and the contents contribute to the accumulation of fluid around the fertilised egg.

During the later vitellogenic stages of growth the oocyte increases in size to a diameter of around 1.2mm. This entire growth phase takes between 3 months to a year. Full grown oocytes may then remain several months in the ovary before undergoing maturation. This occurs as a result of a gonadotropin produced in the pituitary gland of the frog which induces the follicle cells to produce progesterone, which in turn induces the full grown oocytes to mature (Masui and Clarke, 1979). On oocyte maturation significant changes in the cell occur including an increase in the overall level of protein synthesis (Wasserman et al 1982). The oocyte nucleus or germinal vesicle moves to the pigmented animal pole/^{and breaks down} displacing pigment granules and forming a visible white disc. At this stage the oocyte is radially symmetrical, having no prospective dorsal or ventral side.

This maturation process can be mimicked experimentally by the action of alternative gonadotropins such as Human Chorionic Gonadotropin (HCG). Injection of such hormones into female frogs causes oocyte maturation and the frog will lay eggs. When the eggs leave the ovary they are surrounded by the vitelline envelope. Further on in its passage through the oviduct the mature oocyte is coated with several layers of jelly.

1.1b Fertilisation

In a natural mating, the male frog grips the female around the waist (amplexus) and when he feels her laying eggs he releases sperm. The relative positions of the male and female frogs ensure the sperm reach the eggs as soon as they are expelled by the female. The Xenopus egg is penetrated by a single sperm in the animal hemisphere (Elinson, 1980). On fertilisation the vitelline envelope undergoes several changes. An electron dense layer appears on the outside of the vitelline membrane (Grey et al. 1974), and the membrane becomes impermeable to sperm (Grey et al. 1976). Biochemical studies of the pre- and post-fertilisation vitelline membranes have shown the presence of additional proteins in the post fertilisation membrane derived from the innermost layer of jelly and from cortical granules (Wolf et al. 1976).

Changes in the cortex of the egg also occur on fertilisation. There is release of the cortical granules and the expulsion of the contents into the perivitelline space. This causes the amount of fluid present in the space to increase and this in turn causes the vitelline membrane to rise. The egg then rotates under gravity until the pigmented animal pole is uppermost. This rotation occurs around fifteen minutes post fertilisation. One result of the expulsion of cortical granules is that membrane from the granules fuses with the plasma membrane around the cell. This increases the amount of membrane present and changes its composition (Afselius, 1988). Such an alteration in membrane composition may be significant for the future development of the embryo. Another change occurring in the cortex on fertilisation is

the depolarisation of the plasma membrane. Prior to fertilisation the inside of the plasma membrane is electrically negative with respect to the outside medium. On fertilisation this polarity is shifted to a small positive potential by a rapid influx of sodium ions. This positive potential is the 'fertilisation potential' and lasts only minutes before the polarity of the membrane reverts back to its previous negative value. (Jaffe, 1978; Cross and Elinson, 1980). The depolarisation of the membrane is found to constitute an effective, but not total, block to polyspermy.

Significant changes in the cytoplasm occur on fertilisation. An example is the movement of yolk platelets from the vegetal pole further into the interior of the egg, their places in the vegetal pole being taken up by the smaller sized yolk platelets. The reorganisation of the egg cytoplasm on fertilisation is manifested externally by the appearance of the grey crescent in the animal pole directly opposite the point of sperm entry. The grey crescent is an area of reduced pigmentation caused by the redistribution of pigment granules. The cortical cytoplasm underlying the egg surface which contains the majority of the dark pigment granules, is thought to rotate on fertilisation to create this area of lighter pigmentation in the animal hemisphere directly opposite the sperm entry point (Ansel and Vitemberger, 1948; Gerhart et al, 1981). The grey crescent coincides with the future position of the dorsal lip of the blastopore. At this stage the egg is no longer radially symmetrical and shows bilateral symmetry. The dorsal-ventral axis of the embryo is therefore effectively determined by the

position of sperm entry.

1.1c Cleavage and the Formation of the Blastula

The first egg cleavage in Xenopus laevis eggs occurs around 90 minutes post fertilisation and is vertical, bisecting the grey crescent and separating the egg into right and left halves. Subsequent cleavages occur initially at periods of around 15 minutes. The second cleavage is at right angles to the first and separates dorsal from ventral halves. The third cleavage is equatorial separating animal from vegetal halves. The large cells resulting from these initial egg cleavages are termed blastomeres. The initial fast rate of cell division in the embryo is maintained for around 11 cycles, and is accompanied by high rates of DNA synthesis and synthesis of proteins necessary for cell duplication. The embryonic cells do not grow between the divisions and there is little or no RNA synthesis during the initial cleavage steps (Newport and Kirschner, 1982a).

Cleavage in the early Xenopus embryo is asymmetric and the yolky vegetal cells are considerably larger than the corresponding animal pole cells since the presence of yolk slows down the cleavage process. As cleavage continues a fluid filled cavity termed the blastocoel is formed within the embryo and, because of the asymmetric cell size, this is located in the animal hemisphere and is flattened on the vegetal edge. At this stage of development the embryo is termed a blastula.

From the beginning of cleavage the blastomeres are joined together by intercellular junctions. Tight junctions

are the first to form (Straelin and Hull, 1978) and at the blastula stage these seal off the interior blastocoel of the embryo from the outside medium. Gap junctions, which provide a means of communication between cells of the embryo, develop around the 16 cell stage and are widespread in the embryo on formation of the blastula. Development of the embryo up to the mid-blastula stage proceeds entirely on maternal stores of RNA and protein present in the oocyte. 7-8 hours after fertilisation, cell division becomes slower and asynchronous due to the exhaustion of the maternal stores needed for the formation of sygotic nuclei. At this stage sygotic gene expression begins at a high level (Newport and Kirschner, 1982a; 1982b). The onset of this period is termed the mid-blastula transition (MBT).

1.1d Gastrulation

The next stage of development in the embryo is one of extensive morphogenetic movements termed gastrulation. In the final stages of the blastula the animal pole cells start to move down over the lower surface of the embryo. The vegetal cells (or future endoderm) are then pushed into the interior of the embryo. The blastocoel is extended down the insides of the embryo by the upward and inward shift of the endoderm and the downward spread of the animal pole cells. This process of overgrowth of the embryo by the moving animal pole cells (ectodermal cells) is termed epiboly. During gastrulation these movements are extended and enhanced until the endoderm is completely surrounded by ectoderm (Keller, 1985).

The mesoderm in the embryo is the region demarcated by the grey crescent and forming the inner equatorial region of the blastocoel roof. The mesodermal cells also move into the interior of the embryo during gastrulation. The deep mesodermal cells at the site of the grey crescent slide round the lower edge of the blastocoel to lie over the endoderm on the floor of the blastocoel. These cells are then propelled further into the embryo which causes a visible slit to appear in the endoderm in the area beneath the grey crescent (the dorsal-ventral quadrant of the embryo). This slit is the dorsal lip of the blastopore. The slit is lined with endoderm cells and increases in size by the addition of more endoderm cells that roll over the edge of the slit. The slit then elongates and becomes a complete circle with cells invaginating around its circumference.

The material invaginating through the blastopore is double sided and the cavity within the two layers is termed the archenteron. This is the primitive gut of the embryo and, as invagination continues, it grows in size and the blastocoel is pushed to one side. The visible endodermal tissue within the blastopore is the yolk plug, and acts effectively as a plug for the archenteron which fills with fluid probably derived from the shrinking blastocoel. By the end of gastrulation the archenteron becomes the principal cavity at the expense of the blastocoel, and the embryo has rotated such that its dorsal side is uppermost.

The embryo at this stage has definitive dorsal-ventral and cranio-caudal axes in addition to the bilateral symmetry imposed at fertilisation. The three germ

layers have also reached their definitive positions: ectoderm outside, endoderm inside and mesoderm in between.

1.1e Neurulation

During neurulation the dorsal ectoderm becomes differentiated as the central-nervous system (see Nieuwkoop and Florschütz, 1950; Schroeder, 1970). Neurulation occurs at about 25 hours post fertilisation as a smooth continuation of gastrulation. It is a result of an inductive signalling event between invaginated dorsal ectoderm and underlying dorsal mesoderm (Spemann, 1938).

Following closure of the blastopore, the presumptive area of the nervous system becomes differentiated in the form of the neural plate. The cells of the neural plate become elongated and arranged into a columnar epithelium. The edges of the neural plate thicken and rise above the dorsal surface of the embryo as neural folds. A shallow groove then forms between the folds and, as the folds become higher, they meet and fuse to form the neural tube. The cavity of the neural tube is broadest in the anterior region of the embryo corresponding to the presumptive brain. After closure the neural tube becomes covered by ectoderm from beyond the folds differentiated as epidermis. Tissue from the neural folds which comes to lie as an irregular flattened mass dorsal to the neural folds forms the neural crest. Neural crest cells are thought to contribute later to the formation of ganglia outside of the central nervous system, to Schwann cells, to pigment cells and to the visceral skeleton (see Hamilton, 1976).

During neurulation the embryo becomes elongated and flattened from side to side, and smaller in a dorsal/ventral direction. This contraction is more pronounced in the posterior of the embryo accentuating the difference between the presumptive brain and spinal cord.

Neurulation is also accompanied by development of mesodermal structures. The notochord starts to differentiate from dorsal mesoderm at stage 13. It begins as a narrow strip of cells which become surrounded by the notochord sheath. This structure acts as the first axial skeleton of the embryo. The notochord enlarges by vacuolisation of its cells, and during tailbud stages of development this process accounts for the elongation of the embryo. Later, when the tadpole starts to metamorphose, the notochord becomes obsolete and eventually disappears. Mesodermal cells lying adjacent and lateral to the notochord differentiate into paired somites (Hamilton, 1969). The initial function of the somites is for locomotion, since they form the axial musculature of the embryo. The dorsal part of the inner wall of the somite is the eventual source of somatic muscle in the frog and is termed the myotome.

1.1f Further Development

By the end of neurulation the rudiments of all the major structures of the body are in their definitive positions. The mesoderm of the trunk region develops into myotomes, pronephros and mesonephros, and into ventral blood islands. The heart and limbs are already present in rudimentary form within the mesodermal tissue. The ectoderm

becomes the epithelial components of the pharynx, stomach, liver, intestine and rectum. The tail is formed from the tailbud.

The central nervous system is formed from the neuro-epithelium of the neural tube. Since the later work described in this thesis involves the characterization of a monoclonal antibody specific for tissue of the nervous system, a brief outline of further nervous system development in Xenopus laevis embryos is given below. A more detailed description of later stages of Xenopus development can be found in Nieuwkoop and Faber (1987); Hamilton (1976) and in general embryology textbooks such as Balinsky (1981). For detail of the development of the nervous system in Xenopus embryos, see Jacobson (1970).

The neural tube develops eventually into the brain and spinal cord of the embryo. Little is known about the fate and determination of cells within the central nervous system. The early brain becomes divided by constriction into three gross regions, each associated with one of the pairs of sense organs. The forebrain is linked to the olfactory organs, the eyes become functionally linked to the midbrain (which is also associated with the pituitary and pineal bodies), and the hindbrain is associated with the ears and the lateral line system.

Two types of nerve cell are formed which connect the central nervous system to the periphery. Efferent nerves develop from cells within the spinal cord and send projections to the somites and viscera. Each somite receives one efferent nerve at the middle of its length. Afferent

nerves are derived from neural crest cells which are located outside the neural tube but which send projections into the tube and to both visceral and somatic peripheral sites. Collections of sensory nerve cell bodies are grouped at intervals to form dorsal root ganglia and these are arranged between the somites. All nerve axons lying outside the central nervous system are myelinated by Schwann cells derived from the neural crest. Cells within the central nervous system are also myelinated but by cells of neural tube origin.

The eyes develop from optic vesicles formed from the lateral walls of the forebrain (see Reyer, 1977). Formation of the retina occurs by the folding of pre-existing sheets of cells. The optic vesicles grow out from the forebrain to touch overlying ectoderm tissue which is thought to be induced to form the lens. The walls of the optic vesicles then collapse to form the optic cup which is attached to the brain by the narrow optic stalk. The outer cells of the optic cup comprise a thin epithelium which becomes heavily pigmented. In the inner layer of the optic cup the sensory cells are tall and closely packed. Cell bodies eventually come to lie on the inside of the optic cup and nerve fibres lie on the outside. The edges of the optic cup fold over between the lens and the ectoderm to form the iris which does not contain photoreceptive cells (see Strasznisky and Gase, 1971). Contact between the optic cup and the overlying ectoderm then loosens and mesodermal cells enter the optic cup to form the transparent cornea. The eyes are surrounded by the mesodermal choroid coat for blood

supply, and the sclera for support. It is thought that the eye muscles may be derived from the first three pairs of somites in the embryo.

SECTION 1.2 THE CELL MEMBRANE IN XENOPUS EMBRYOS

In this section the nature and biochemistry of the Xenopus embryo cell membrane is described with emphasis placed on the highly specialised nature of the embryo membrane. A detailed review of the biochemistry of eukaryotic membranes is not included, and more information on this subject can be obtained from the following textbooks and reviews: Finean et al (1978); Lodish et al (1979); Bretscher (1973); Harrison and Lunt (1980) and Sim (1982).

1.2a The Composition and Biosynthesis of Eukaryotic Cell Membranes

Eukaryotic cell membranes are sheetlike structures composed primarily of protein and lipid. They appear under the electron microscope as two layers of electron dense material approximately 50Å thick, separated by a less dense interval of about 60Å. Membrane lipids are amphipathic molecules and are organised in this bilayer structure with the hydrophilic 'heads' of the molecules pointed outwards, and the hydrophobic 'tails' pointing inwards. This lipid bilayer is highly stable and will form spontaneously in water. The bilayer alone is impervious to ions and polar molecules and it is the membrane proteins that are responsible for most of the functions of the membrane. These proteins constitute between 18% (myelin) and 75% (internal mitochondrial membranes) of total membrane dry weight. Membrane proteins may be integral within or spanning the

membrane, or associated only with one side or other of the bilayer.

The functions of eukaryotic cell membranes are numerous. They act as selective permeability barriers allowing the passage of specific molecules across the membrane through the use of molecular pumps and gates. They can be involved in mediating the flow of information between cells through the use of specific messengers and receptors, and through membrane junctions. Membranes can provide an anchoring system for cytoplasmic enzymes and components of the cell cytoskeleton. Membranes can also mediate cell-cell adhesion or the adhesion of the cell to the substratum. Some of these functions of the cell membrane will be studied in more detail with reference to the role of the plasma membrane in the development of *Xenopus laevis* embryos in Section 1.4 of this introduction.

In 1972, Singer and Nicholson put forward a model for the organisation of biological membranes. Their Fluid Mosaic Model suggests that membranes are 2-dimensional solutions of oriented globular proteins and lipids, and that the lipid bilayer has a dual role acting as both a solvent for integral membrane proteins and as a non specific permeability barrier. A small proportion of the membrane lipids will interact specifically with particular membrane proteins and may be necessary for their function. The model also suggests that membrane proteins and lipids are free to diffuse laterally in the lipid bilayer unless restricted by specific interactions, but that membrane proteins are not free to flip-flop across the bilayer.

This Fluid Mosaic Model has been widely upheld by experimental evidence and is the generally accepted model for most biological membranes. However, the model does not adequately describe the *Xenopus* embryo cell membrane indicating its highly specialised nature. The reasons for this suggestion will be discussed in more detail below.

The requirement for new membrane in embryo development is a major metabolic demand since one cell division requires an approximate doubling in the amount of membrane present. There is also evidence to suggest a continuous turnover of membrane material in the cell which enables a fast response to any changes in membrane function required from a varying environment. The synthesis of membrane lipids occurs from cytosolic precursors by membrane bound enzymes present on the cytoplasmic surface of the endoplasmic reticulum (ER). How lipids are moved from the ER to the cell surface is not clear (Bell *et al.*, 1981). Lipids may move with proteins in vesicles from the ER through the Golgi complex to the cell surface (Dower *et al.*, 1982; Pfenninger and Johnson, 1983; Mills *et al.*, 1984). Alternatively lipids may be transferred through the cytoplasm on lipid transfer proteins (Wirtz, 1974; De Grella and Simoni, 1982; Yaffe and Kenedy, 1983; Sleight and Pagano, 1983).

Integral membrane proteins are synthesized on polysomes on the rough ER, with simultaneous synthesis and insertion of proteins into the membrane (Blobel and Dobberstein, 1975; Walter *et al.*, 1984). Most plasma membrane proteins are glycosylated (Bretscher and Raff, 1975), and

N-linked core glycosylation takes place during translocation through the ER membrane (Bothman and Lodish, 1977; Sabatini et al., 1982). From this compartment the proteins are transported to the Golgi for further processing and extension of carbohydrate side chains (Hacover and Lennars, 1983; Bothman, 1981; Staneloni and LeLoir, 1982; Tartakoff, 1983). Other membrane proteins are synthesized on free polyosomes and are subsequently attached to, or inserted into the membrane (Lodish et al., 1981).

1.2b The Specialised Nature of the Xenopus Embryo Plasma Membrane

Experimental evidence suggests that the Xenopus embryo plasma membrane is specialized to its particular role in development. The plasma membrane of fertilised Xenopus eggs[‡] differs in comparison with other biological membranes. In particular the membrane has an unusually high specific resistance ($74 \text{ k}\Omega/\text{cm}^2$; de Laat et al., 1973), it lacks permselectivity for cations (de Laat et al., 1975), and is highly insensitive to the action of cytochalasins (Bluemink, 1971a; 1971b; 1978). Freeze fracture electron microscopy of the plasma membrane of the 1-cell fertilised Xenopus egg has shown that protein particles within the membrane (intramembranous particles or IMP's) were larger than generally observed in biological membranes, and were mainly restricted to the exoplasmic face of the membrane (Bluemink et al., 1978). In addition, the distribution of IMP's differed between the animal and vegetal halves of the egg suggesting a polarity in the membrane of the fertilised uncleaved egg

[‡] The cell surface represents a small and unknown amount of total cell membrane.

(Bluemink and Tertoolen, 1978).

The existence of animal/vegetal polarity in the *Xenopus laevis* embryo plasma membrane has been confirmed by scanning electron microscopy (Elinson, 1980) and by the measurements of lipid mobility in the plane of the membrane. In the unfertilised egg, lipids in the vegetal pole membrane have a five-fold higher mobility than lipids in the animal pole membrane. Upon fertilisation this difference is enhanced over 100 fold, and the lipids in the animal pole membrane become almost totally immobile (Dictus et al., 1984). This is most likely due to the fusion of large amounts of membrane during cortical granule expulsion.

New membrane formation in the dividing *Xenopus* embryo is restricted to the site of furrow formation (Bluemink and de Laat, 1973; 1977). Lipid mobility measurements on the furrow membrane indicate a diffusion coefficient for the lipid molecules which is intermediate between that of the animal pole membrane lipids and those of the vegetal pole (Tetteroo et al., 1984). The membrane of the dividing *Xenopus* egg therefore appears to comprise three distinct membrane domains: the animal pole membrane, the vegetal pole membrane and the membrane of the cleavage furrow. The presence of these membrane domains indicates that the plasma membrane of the *Xenopus* egg and embryo is not adequately described by the Fluid Mosaic Model outlined above.

The membrane of the cleavage furrow differs from the pre-existing animal and vegetal pole membrane in its biochemical properties. In particular, cleavage furrow

membrane shows distinct ultrastructural organisation (Bluemink and de Laat, 1973), has unusual permeability properties (de Laat and Bluemink, 1974; de Laat et al., 1974), and there are differences in binding properties for lanthanum (Bluemink and de Laat, 1973) ruthenium red (Singal and Sanders, 1974) and lectins (Geuskens and Tencer, 1979; Nosek, 1978; Roberson and Armstrong, 1979; 1980). The presence of lipid domains in the plasma membrane of developing embryos is not specific to *Xenopus laevis*, and similar membrane domains have been observed in other animal embryos, for example mouse eggs (Wolf et al., 1981a; Klausner and Wolf, 1980; Peters and Richter, 1981), sea urchin eggs (Campisi and Scandella, 1980; Wolf et al., 1981b) and molluscan eggs (Speksnijder et al., 1985).

The significance of the membrane domains in *Xenopus* embryos is not fully understood. It has been suggested that regional differences in the lateral mobility of plasma membrane components in the unfertilised egg could provide a mechanism for controlling the localisation of sperm recognition sites (Grey et al., 1974). It is also possible that the enhanced lipid immobilisation occurring in the animal pole membrane upon fertilisation is required for the polyspermy block (Dictus et al., 1984). The lipid environment is known to affect the functioning and properties of membrane proteins such as enzymes receptors and transport proteins (Singer and Nicholson, 1972; Warren et al., 1975; Edidin and Petit, 1977; Sanderman, 1978; Rothfield and Romeo, 1971; Magee and Schlessinger, 1982; Boonstra et al., 1982; van Zoelen et al., 1983; de Laat et al., 1983). Differences in

membrane lipid properties could determine major regional differences in plasma membrane functioning. This could be achieved either by the specification of the location of a particular protein, or by affecting the properties of the protein locally (Dietus *et al.* 1984). Some experimental evidence exists to support this suggestion. Amphibian blastulae have a polarised water transport system required for the formation of the blastocoel, and the activity of this system has been ascribed to animal/vegetal differences in plasma membrane functioning (Tuft, 1957; 1961a; 1961b; 1962; Hamilton and Tuft, 1972).

The polarity of the Xenopus embryo membrane is consistent with the known polarity present in the cytoplasm. Studies on the distribution of RNA, cortical granules, yolk, pigment and cytoskeletal elements has shown that at the cytoplasmic level, animal/vegetal polarity exists before and after fertilisation and is in fact laid down during oogenesis (Capco and Jeffery, 1981; Capco, 1982). The true significance of lipid domains and animal/vegetal polarity in Xenopus embryo membranes remains unclear. These properties of the embryo membrane may exist purely as a result of changes occurring in the embryo during development and may play little or no role in morphogenesis. However more work should be undertaken to investigate regional differences in the embryo plasma membranes, and to question their possible significance during development.

1.2c Variation in Cell Surface Membranes During *Xenopus laevis* Development

There is evidence to indicate that the *Xenopus* cell membrane undergoes change throughout the development of the embryo. This is particularly evident at fertilisation when the egg plasma membrane undergoes a substantial reorganisation (Charbonneau and Picheral, 1983). On fertilisation there is an increase in the overall polarity of the membrane and an associated decrease in lipid mobility in the animal pole (Dictus et al., 1984). This is accompanied by changes in the composition of the membrane due to cortical granule fusion. There is a dramatic drop in the membrane potential (the 'fertilisation potential', Cross and Elinson, 1980) and an increase in membrane endocytosis (Bernadini et al., 1987).

Substantial changes are thought to occur in all embryo cell membranes during development. Changes in the overall composition of the embryo cell surface have been shown for mammals (for a review see Johnson and Calarco, 1980; also Kimber and Bird, 1985; Keil-Dlouha et al., 1980), *Drosophila* (Woods et al., 1987) *Flaurodelaes waltlii* (Darriberes et al., 1982) and *Dicotyostelium* (Boswaro and Merkl, 1985; Galtosky et al., 1978). In myoblasts there is evidence for a total remodelling of the cell membrane during development (Kaufman and Foster, 1985; Wakshull et al., 1983).

No thorough analysis of variation in the *Xenopus laevis* plasma membrane during development has been carried out and this will form the basis of the work attempted in this thesis.

1.2d Analysis of Cell Surface Polarity in Epithelia

The Xenopus embryo plasma membrane is unusual in many respects. Not least of these is the animal/vegetal polarity present within the membrane before and after fertilisation (see 1.2b above). Comparison with other embryo membranes suggests that a polarity in the Xenopus plasma membrane may also exist at later stages of development. Analysis of mouse morulae embryos indicates polarization of the plasma membrane at compaction of the embryo, with microvilli being localised mainly on the outer surface of the embryo, and microtubules and mitochondria localised beneath areas of intercellular membrane (Ducibella et al., 1977). Antibody, enzyme and lectin studies have also revealed differences in the membrane components present in these two domains in both mice and rat embryos (Isquierdo et al., 1980; Isquierdo and Ebensperger, 1982; Handyside, 1980; Lois and Isquierdo, 1984). Cell surface labelling studies of Pleurodeles waltlii embryos have indicated differences in the proteins present in the outer and newly synthesised inner membranes at very early stages of development (Darribere et al., 1982). A similar investigation of Xenopus laevis embryos has been carried out as part of the work in this thesis, and comparable results are obtained (See Chapter 4).

Polarity in the Xenopus embryo membrane exists between the animal and vegetal pole membranes around fertilisation, and between intercellular and external plasma membranes at later stages of development. Cell surface polarity seen in the embryo membranes at later stages of

development can be partly ascribed to the presence of epithelial cells which develop during the formation of the blastula. Epithelial cells are polarised and a substantial amount of work has been carried out to investigate how this polarity is established and maintained. A brief discussion of this topic is included below to illustrate the nature of this specialised membrane system, and to enable comparison of the cell surface polarity in epithelial cells with that present in the Xenopus embryo around fertilisation. For a review of cell surface polarity in epithelial cells, see Simons and Fuller (1985).

Epithelial cells are organised into sheets that separate compartments within the organism (Berridge and Oschman, 1972). The cell sheets maintain concentration gradients between the compartments that they separate. Cells in the epithelium are linked through membrane junctions and, as such, form a selective permeability barrier. Examples of these cells include transporting epithelia (e.g. those in the renal tubule), absorptive epithelia (e.g. those of the intestine) and secretory epithelia (e.g. hepatocytes).

Epithelial cells maintain concentration gradients by localising distinct sets of cell surface components to separate cell surface domains. The plasma membrane of epithelial cells is comprised of an apical domain (which is often covered with microvilli and faces the external side of the organism or organ), and the basolateral domain (which is often attached to an extracellular matrix arranged as a basal lamina).

Evidence suggests that the lipid composition of

apical and basolateral membranes in epithelial cells differs (Forstner *et al.*, 1968; Douglas *et al.*, 1972; Kawai *et al.*, 1974; Brasitus and Schoachter, 1980). This suggests that the lipids in the separate membrane domains are not able to diffuse freely. Epithelial cell membrane proteins are restricted to one surface domain or the other. A protein assigned to one domain in one epithelial cell type is usually found in the same domain in all other epithelial cells (Simons and Fuller, 1985). No proteins are known which are distributed without polarity to the two surface domains.

The polarity of the epithelial cell membrane arises during biosynthesis from the sorting of membrane components to the separate membrane domains. The sorting of membrane proteins is believed to occur in the trans compartment of the Golgi in recognition of a signal on the protein thought to be part of the amino acid sequence. No specific primary sequence responsible for directing proteins to a particular membrane domain has been identified (Matlin, 1986), but other signals involved in intracellular transport have been characterised (Sohats and Butow, 1983; Hurt *et al.*, 1984; Calderon *et al.*, 1984; Lanford and Butel, 1984; Dingwall, 1985; Blobel, 1980; Walter *et al.*, 1984). Transport of the proteins (and possibly also membrane lipids) to the correct membrane domain occurs through selective membrane transport via budding and fusion of membrane vesicles (Palade, 1975; 1982).

Polarity in epithelial cell membranes is maintained by the presence of tight junctions which act as a fence to limit the diffusion of membrane constituents (Diamond, 1977). The tight junctions are organised alongside intermediate

junctions into a junctional complex which encircles the apex of each cell (Farquhar and Palade, 1963). This complex inhibits diffusion of membrane proteins and lipids (Diamond, 1977).

1.2e Summary

The Xenopus embryo cell membrane is specialised to its particular role in development. This is demonstrated by the unusual nature of the membrane in terms of its biochemical and permeability properties, and in the polarisation of the membrane into separate domains. Substantial changes occur within the Xenopus laevis cell surface membrane throughout development. These changes appear to be related to cell differentiation and to varying functions of the membrane in morphogenesis. Examples of changes in the cell membrane relating to characterised functions of the membrane in development will be discussed in section 1.4 below.

The polarity in epithelial cells was discussed because these cells are present in developing Xenopus embryos at a fairly early stage, and because membrane protein distribution between inter-cellular and external cell surface membranes is relevant to the work carried out in this thesis in Chapter 4. The polarity in epithelial cells is not similar to that seen in Xenopus eggs (indeed the mechanism involved in maintaining the separate membrane domains in fertilised and unfertilised Xenopus eggs is unknown). No evidence exists for the presence of a permeability barrier to explain membrane domains in Xenopus embryos, such as the tight junction in

epithelial cells (Tetteroo et al, 1984).

SECTION 1.3 THE XENOPUS EMBRYO CELL MEMBRANE IN
DEVELOPMENTAL CONTROL

One of the central problems of development concerns the nature of the control mechanisms whereby cells of a common lineage are caused to differentiate into distinct cell types. In this section aspects of developmental control will be discussed with the emphasis placed on potential areas of interaction by the cell membrane.

1.3a Developmental Control

There are two stages to consider in the emergence of organisation from an initial early embryo. The first stage, pattern formation (also called regional specification or spatial organisation), is the process whereby cells acquire instructions as to how they should undergo change. This is outlined in more detail below. The second stage is a combination of cell differentiation and morphogenesis. Differentiation refers to the irreversible acquisition of new properties by cells, and morphogenesis to the way in which cells use these properties to build new structures. Cell differentiation is a consequence of pattern formation and involves the synthesis of new protein species. The immediate controls on cell differentiation are therefore controls on gene transcription and translation. A discussion of this area of research is beyond the scope of this thesis. The mechanisms of morphogenesis such as cell communication, cell adhesion and movement are fundamental to development, and the

role of the membrane in morphogenesis will be considered in Section 1.4 below.

1.3b Pattern Formation

Pattern formation in embryos is thought to occur by a process of epigenesis whereby the fertilised egg has a simpler spatial organisation than the adult and the embryo gradually becomes more complex with the emergence of new cellular properties. In some organisms (e.g. the ascidian), the information governing cell fate appears to be present in the egg and this information is passed on to individual blastomeres. In such cases development is said to be mosaic. In other embryos the blastomeres appear to be equivalent and the eggs are termed regulative since isolated blastomeres must regulate their behaviour to produce a normal embryo. The amphibian embryo is regulative in the sense that individual blastomeres can form a complete embryo, but the polarity of the dorsal/ventral axis in the embryo is established by cytoplasmic factors.

Cytoplasmic localisation and embryonic induction are the only two general processes known which can account for the mechanisms of pattern formation. There is little clear understanding of either of these, and in neither case is the biochemical basis of the phenomena clear. Cytoplasmic localisation and induction will be considered in more detail below, with the emphasis placed on the role of these processes in the development of Xenopus laevis embryos, and the associated function of the cell membrane.

1.3c Cytoplasmic Localisation

Cytoplasmic localisation is a mechanism of pattern formation involving the assymetric distribution of regulatory molecules in the cytoplasm of stem cells. In the course of cell division, the two daughter cells inherit different materials and consequently enter different states of determination. There is now extensive evidence to suggest that cytoplasmic determinants are important in pattern formation in a number of organisms (for reviews see Davidson, 1976; Johnson and Pratt, 1983). Cytoplasmic determinants are also thought to be involved in the generation of spatial organisation in Xenopus embryos, particularly in the establishment of animal/vegetal polarity in Xenopus embryos during oogenesis and in the dorsal/ventral polarity established after fertilisation.

During the early stages of Xenopus oogenesis, there is a polarised organisation of components within the cytoplasm of the cell. At one end of the oocyte (the future vegetal pole) is a cytoplasmic bridge that remains from incomplete cytokinesis (Coggins, 1973). Nearer the other end of the cell (the future animal pole) is a centrosome surrounded by mitochondria, germ plasma precursors and Golgi vesicles. Nearer still the future animal pole of the cell lies the germinal vesicle or nucleus. It is thought that this polarised organisation may be exploited by the oocyte to localise cytoplasmic materials which could determine the primary axis of the egg and hence the future organisation of the embryo.

There is little clear evidence concerning the

nature of molecules in the oocyte which are involved in the establishment of the primary embryonic axis. Determinants may be present in the germinal vesicle which contains both nuclear proteins and nuclear membranes in vast quantities (Gerhart et al. 1981). When the nucleus breaks down at meiotic maturation nuclear proteins are distributed throughout the animal pole and to the periphery of the vegetal pole (Hausen et al. 1985). Yolk platelets present in the vegetal hemisphere have also been implicated as a possible source of determinant molecules. Yolk platelets nearest the vegetal pole of the oocyte differ from other platelets in their bouyant density which could indicate a difference in protein composition (Radice et al. 1981).

Embryonic determinants may be located in the plasma membrane and cortex of the oocyte. Support for this suggestion comes from the known polarity of the *Xenopus* egg membrane before and after fertilisation (discussed previously). The plasma membrane of the animal and vegetal halves of the *Xenopus* egg differs in lipid fluidity, intramembraneous protein particles and microvilli location (Dictus et al. 1984). Pigment granules are located in the animal hemisphere cortex in greater numbers than in the vegetal hemisphere and cortical granules of the two regions differ in their number and size (Campanella and Andreucetti, 1977). Evidence for the presence of determinant molecules within the cortex of the oocyte has come from work on the Vg-1 RNA (Melton, 1987), which is discussed in Section 1.3d below.

The future dorsal and ventral regions of the

Xenopus embryo are established at the outset of development. Ligation experiments have shown that segregation of egg cytoplasm influences the formation of the normal dorsal/ventral axis of the embryo (see Spemann, 1938). Egg cytoplasm is therefore thought to contain information which controls the future pattern formation of the embryo.

The unfertilised Xenopus egg is radially symmetric about its animal/vegetal axis. On fertilisation, the sperm enters randomly in the animal hemisphere (Elinson, 1975) with the sperm entry point (SEP) indicating the side of the egg destined for ventral structures (Ubbels, 1977; Kirschner et al., 1980; Gerhart, 1980). If the egg is prevented from developing a dorsal/ventral axis, it develops radially arranged ventral structures as a default (Nieuwkoop, 1973; Spemann, 1938). The cytoplasmic processes following sperm entry are not well understood but are thought to involve a co-ordinated reorganisation of the egg's contents midway through the first cell cycle in response to positional cues from the sperm aster formed at the SEP (Elinson, 1980). On fertilisation, the cortex shifts relative to the endoplasm leaving the grey crescent region on the prospective equatorial dorsal side. This is accompanied by a simultaneous rearrangement of the endoplasm (Pasteels, 1964; Klag and Ubbels, 1975). The reorganisation process is thought to localise regulatory agents on the prospective dorsal side of the embryo which will determine the future development of the embryonic axis (see Gerhart et al., 1981).

Information concerning the nature of dorsal/ventral determinants comes from experiments using ultra-violet (uv)

light and other perturbing stimuli such as cold shock or pressure. Amphibian embryos irradiated with uv light on the vegetal hemisphere in the first cell cycle, show a dose-related reduction in dorsal structures present in the tadpole. At high doses of uv no dorsal structures are seen in the embryo (Grant and Wacaster, 1972; Malacinski et al, 1975; 1977). This sensitivity to uv is limited to the period from the point of fertilisation to around two thirds of the time to first cleavage, which corresponds to the period of grey crescent formation (Manes and Elinson, 1980; Scharf and Gerhart, 1980). Ultra violet irradiation is thought to destroy agents needed for axis formation, or to inhibit their production or localisation. Further experiments involving artificial rotation of eggs under gravity have supported the view that uv interferes with the production or localisation of dorsal/ventral determinants (Ansel and Vitemberger, 1948; Born, 1885; Kirschner et al, 1980; Scharf and Gerhart, 1980). Treatment of the egg with uv in some way inhibits the normal cortical/cytoplasmic rotation of the egg which is vital for the development of dorsal structures. Other inhibitory treatments besides uv include brief cold shock and high hydrostatic pressure (Scharf and Gerhart, 1983). These experiments have lead to the suggestion that the normal rotation of the embryo occurring after fertilisation cues local activation of widespread, but latent, cytoplasmic agents needed for the dorsalisation of the embryo (Gerhart and Keller, 1988).

In 1982, Curtis suggested that the cell cortex may be involved in the determination of the dorsal/ventral axis.

Curtis carried out grafting experiments on Xenopus embryos where he removed a piece of grey crescent cortex from an egg shortly before first cleavage and grafted it into the equatorial surface of the prospective ventral side of a recipient egg at, or close to, first cleavage. All eggs receiving grafted cortex developed double embryonic axes. Curtis concluded that the grey crescent cortex was a locus of the dorsalising determinant in the egg. Recent results have since questioned this interpretation (Gerhart et al., 1981). It appears that the double axis formation occurring in Curtis' grafted embryos did not arise from determinants present in the grafted grey crescent cortex, but rather from the effects of gravity on artificial rotation of the eggs during the grafting experiments" (see Ancel and Vitemberger, 1948). The grey crescent is now thought to be a result of the early dorsalising process, but is not itself a transplantable dorsal determinant (Gerhart et al., 1981).

A thorough understanding of cytoplasmic localisation in Xenopus eggs and embryos can occur only with the detection and characterisation of the region specific determinant molecules. Work has recently been carried out on RNA species detected in the unfertilized Xenopus egg which are restricted to either the animal or vegetal hemispheres (Rebagliati et al. 1985, Melton 1987). One such RNA is Vg-1, which is localised in the cortical region at the vegetal end of mature Xenopus oocytes (Melton, 1987). Vg-1 RNA is synthesised during oogenesis, is inherited by eggs and persists in the cleaving embryo up until the gastrula stage when the RNA is degraded. The Vg-1 RNA is known to encode a

member of the transforming growth factor- β (TGF- β) family of proteins (Weeks and Melton, 1987). These are molecules which can enhance the activity of fibroblast growth factor (FGF) to induce muscle in Xenopus animal pole tissue (Kimelman and Kirschner, 1987). It is now suggested that localised maternal mRNA's in the egg may act as cytoplasmic determinants by encoding proteins that can specify cell types during early development.

1.3d Embryonic Induction

Induction is the process whereby competent embryonic tissue becomes differentially determined in response to chemical signals from other regions of the embryo. Embryonic induction is known to be an important mechanism in the pattern formation of a large number of vertebrates (see Wessels, 1977; Nieuwkoop *et al.*, 1985). It is thought that three different major inductive interactions occur during early Xenopus development and these are outlined briefly below.

The first inductive interaction in amphibian development occurs around the 64 cell stage, and results in the formation of mesodermal tissue around the equator of the embryo (Jones and Woodland, 1987). Mesoderm is induced from animal pole tissue by the action of a signal from vegetal pole tissue. In isolation, tissue from the animal pole will form only epidermis (ectodermal), whereas isolated vegetal pole tissue does not develop. When animal and vegetal pole tissue are combined, large amounts of mesoderm are formed, and the use of cell markers has confirmed that this

mesodermal tissue is derived from the animal pole component (Nieuwkoop 1969; 1973; Dale et al., 1985).

The second inductive interaction believed to occur in Xenopus embryos is dorsalisation, which is closely related to mesodermal induction described above. Following fertilisation, the vegetal hemisphere of the Xenopus egg is thought to be divided into dorsal and ventral components. These characters are then transmitted to the mesoderm as part of the mesodermal induction process (Slack and Forman, 1980; Boterenbrood and Nieuwkoop, 1973; Smith et al., 1985; Dale and Slack, 1987). The ventral mesoderm produced as a result of mesodermal induction becomes further subdivided into different regions or territories as a result of an inductive interaction by the dorsal mesoderm, or the 'organiser' (Slack and Forman, 1980; Smith and Slack, 1983). This dorsalisation interaction occurs during gastrulation of the embryo.

The third established inductive interaction in early Xenopus development is neural induction which involves the formation of the neural tube from ectoderm tissue brought into contact with dorsal mesoderm (the organiser). Cell movements during gastrulation bring dorsal mesoderm into close proximity with ectodermal tissue in the region of the archenteron roof. Neural induction is not a simple process since each region of the archenteron roof induces a specific region of the nervous system, i.e there is anterior/posterior patterning (Mangold, 1933; Horst, 1948). It is thought, therefore, that a number of inducing signals are involved. In the absence of the neural inducing signal, ectodermal cells have been shown to form epidermis (Gimlich and Cooke, 1983,

Jacobson, 1984; Slack et al, 1984).

Little is known about the molecular nature of induction, and much work has been centred on the identification of the inducing molecules particularly for mesodermal induction. A protein isolated from 9-13 day chick embryos has the characteristics expected of the mesoderm inducing factor. (Tiedemann and Tiedemann, 1959; Born et al. 1972; Geithe et al. 1981). This 'vegetalising factor' has a molecular weight of 28-30,000 Daltons and, when applied in the form of an insoluble pellet to amphibian blastula ectoderm, it causes the formation of a range of mesodermal cell types (Asashima and Grunz, 1983). A molecule with similar activity is thought to be present in early Xenopus embryos (Faulhaber, 1972; Faulhaber and Lyra, 1974).

More recently, the Xenopus XTC tissue culture cell line has been shown to secrete mesoderm inducing activity into the culture medium (Smith, 1987). If isolated Xenopus animal pole tissue is cultured in XTC medium it will differentiate into mesodermal tissue. Preliminary investigations have suggested that this inducing factor is heat stable, trypsin sensitive, non-dialysable and has an apparent reduced molecular mass of 16,000 Daltons. Work is being carried out to characterise the factor further, and to determine whether it is present in normal Xenopus embryos.

An investigation has been carried out into the role of growth factors in induction. This follows the discovery that both bovine basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF) at very low concentrations and with high specificity, mimic the action of

the mesodermal inducing signal (Kimelman and Kirschner, 1987). Vg-1 has previously been mentioned in reference to cytoplasmic determinants (Section 1.3c). The discovery that Vg-1 RNA encodes a protein from the transforming growth factor- β (TGF- β) family (which enhance the mesoderm inducing activity of FGF), has led to the suggestion that molecules related to FGF and TGF- β may be the natural inducers of mesoderm in vertebrate development.

In contrast to mesodermal induction which appears a specific process, neural induction can be evoked by a wide range of substances many of which are clearly unrelated to possible inducing agents *in vivo* (for a review see Witkowski, 1985). This is particularly true of experiments carried out on urodele amphibians where the ectoderm tends to autoneuralise with high frequency. Work on neural induction has also supported the role of the membrane in the inductive process. Neural induction is thought to involve the recognition of the external signal which is first perceived and converted by neural target cells at the plasma membrane (Tiedemann and Born, 1978; Takata *et al.*, 1981; Gualandris and Duprat, 1987). Neural induction depends critically on the structural organisation and composition of the target tissue (Duprat *et al.*, 1982; Gualandris *et al.*, 1983; 1985), and lectin binding to the target cell surface leads to a rearrangement of membrane glycoconjugates which strongly inhibits the inductive process. This implies that neural induction requires a particular molecular organisation of the plasma membrane of the target cells (Gualandris and Duprat, 1987).

SECTION 1.4 THE ROLE OF THE XENOPUS LARVIS MEMBRANE IN MORPHOGENESIS

Embryonic development is brought about by the combination of two types of embryonic event, primary determinative events of pattern formation where cells respond to specific signals to undergo new developmental direction, and secondary processes where the more 'mechanical' aspects of development occur. In this section the role of the embryo cell membrane in these secondary processes will be discussed.

1.4a Cell-Cell Adhesion

The main guiding forces in embryo morphogenesis are thought to involve the adhesion of cells mediated by a small number of specific cell surface molecules (Edelman and Thiery, 1985; Edelman 1984a; 1976). There are three major types of adhesion molecule: cell adhesion molecules (CAM's) which play a role in initial boundary formation, embryonic induction, cell migration and in tissue stabilisation and regeneration; substratum adhesion molecules (SAM's) which are important in cell migration, stabilisation of epithelia, and in the development of hard tissues; and cell junctional molecules (CJM's) which are important in the formation of specialised cell connections, in cell communication and the sealing of surfaces by epithelial sheets (see Edelman and Thiery, 1985; Edelman, 1986). The role of SAM's and CJM's in Xenopus development will be discussed in Sections 1.4b and 1.4c below. In this section the role of CAM's in Xenopus

laevis development will be considered. More information can be obtained from the following reviews: Grinnell (1978); Frazier and Glaser (1979); Lilien et al (1979); Letourneau et al (1980); Garrod and Nicol (1981); Damsky et al (1984); Edelman (1984a); (1984b); (1984c); (1985a); (1985b); (1986). Until recently little work had been carried out on cell adhesion in Xenopus laevis embryos, and for this reason much of the discussion of CAM's in this section will involve results obtained from work on chick and mouse embryos.

There are two major types of CAM, primary CAM's which are expressed on early embryonic cells, and secondary CAM's which appear later in development and which show a very restrictive distribution in the embryo (Cunningham, 1986). The number of primary CAM's in an organism is believed to be small and these are likely to be the most crucial as control factors in development. Two of these primary CAM's have been purified and chemically characterised, these are NCAM (neural cell adhesion molecule, Thiery et al, 1977; Hoffman et al, 1982) and LCAM (liver cell adhesion molecule; Bertolotti et al, 1980; Gallin et al, 1983). Both NCAM and LCAM appear early in development on derivatives of all three germ layers (Thiery et al, 1982; Edelman et al, 1983; Thiery et al, 1984; Crossin et al, 1985a). LCAM is a member of the class of calcium dependent cell adhesion molecules termed cadherins, and as such is believed to be homologous to E-cadherin and uvomorulin (Yoshida and Tacheichi, 1982; Peyrieras et al, 1983). The secondary cell adhesion molecule NgCAM (neuron-glia cell adhesion molecule) has also been purified and characterised. This appears only on postmitotic cells in

derivatives of the neurectoderm (Grumet and Edelman, 1984; Grumet et al., 1984; Cunningham, 1986). Other secondary CAM's are known to include N-cadherin (Hatta et al., 1985; Hatta and Takeichi, 1986) and P-cadherin (Nose and Takeichi, 1986).

NCAM, LCAM AND NgCAM are large integral membrane glycoproteins present on the cell surface (Hoffman et al., 1982; Gennarini et al., 1984a; 1984b). The three molecules show different binding specificities: NCAM shows calcium independent homophilic binding, LCAM shows calcium dependent binding which is thought to be homophilic, and NgCAM shows calcium independent heterophilic binding. CAM's undergo cell surface modulation during development with variation occurring in the amount, distribution or chemical properties of the molecule at the cell surface (Edelman, 1976). One example of the modification of CAM's is the embryonic to adult (E to A) conversion of NCAM seen in brain (Rothbard et al., 1982; Hoffman et al., 1982), muscle (Rieger et al., 1985) and skin (Chuong and Edelman, 1985a; 1985b). This is a gradual but large decrease during development in the polysialic acid content of the NCAM molecule. Changes in the amount of sialic acid are thought to act as a mechanism to modulate NCAM activity in vivo (Edelman, 1985a). This suggestion is supported by the observation that the E to A transition of NCAM is delayed in the mouse cerebellar mutant staggerer (Edelman, 1985a).

Cell adhesion in embryos is thought to be important from very early developmental stages. In mammalian embryos, compaction of cells occurs at the 8-cell stage to form a trophoblast. This compaction is caused by dramatic changes in

cell shape accompanied by increased intercellular adhesion (Ducibella, 1980). CAM's are also known to play a role in many different aspects of morphogenesis (see Edelman, 1985a). NCAM is thought to be involved in the assembly and interconnection of neural tissues in chick embryos, particularly in the mediation of retinal cell aggregation (Brackenbury *et al.*, 1977), in neurite fasciculation (Rutishauser *et al.*, 1978; Rutishauser and Edelman, 1980), and in the formation of neuromuscular connections (Grumet *et al.*, 1982; Rutishauser *et al.*, 1983). NCAM antibodies have been used to alter retinal histogenesis *in vitro* (Rutishauser *et al.*, 1978; Buskirk *et al.*, 1980), and neurite pathways in retinotectal projection *in vivo* (Thanos *et al.*, 1984; Silver and Rutishauser, 1984).

NCAM is thought to play a direct role in the mechanism of primary neural induction in the chick embryo (Edelman, 1983, 1984a, 1984b, 1985a; Crossin *et al.*, 1985a; Chuong and Edelman, 1985a; 1985b). The addition of anti-LCAM antibodies has been shown to inhibit induction of chick feather formation *in vitro* (Gallin *et al.*, 1983), and it has been suggested that the activity of CAM's may be vital during embryonic induction (Edelman, 1986). Defects in CAM expression and function are also associated with disease. NCAM levels are altered in fetal neural tube defects (Jorgensen and Norgaardpedersen, 1981) and in various muscle diseases (Walsh and Moore, 1985). Characterisation of central nervous system tumours suggests patterns of CAM expression that differ in tumours of different origin (Edelman, 1986) and CAM modulation is also perturbed in certain genetic

diseases (Rieger et al., 1985). NCAM and LCAM appear together in the earliest embryonic cells. Later in development they are differentially expressed suggesting that CAM specificity and activity is crucial in establishing boundaries between different cell populations (Edelman, 1985a).

The role of NgCAM in development has also been studied using monoclonal antibodies. Addition of anti-NgCAM antibodies disrupts neural fasciculation of ganglion cells in vitro (Friedlander et al., 1986; Stallcup et al., 1985), and inhibits characteristic migration of external granule cells in cerebellar tissue slices (Lindner et al., 1983; Grumet et al., 1984a; 1984b).

Information on inter-cellular adhesion in Xenopus embryos has only recently come to light. In 1986, Nomura et al. confirmed the presence of both calcium dependent (CDS) and calcium independent (CIDS) cell adhesion systems in Xenopus laevis embryos. Using monoclonal antibodies against mouse E-cadherins (believed to be identical to LCAM) they were able to demonstrate antigenic similarity between Xenopus CD adhesion molecules and the mouse E-cadherin. Their studies on the reaggregation kinetics of cell adhesion in Xenopus confirmed that both CDS and CIDS show similar functional properties to mammalian systems.

Characterisation of NCAM expression in Xenopus embryos has been carried out using monoclonal antibodies (Balak et al., 1987; Kintner and Melton, 1987). Substantial differences in the pattern of expression of NCAM in Xenopus embryos to that previously observed in chick has been noted. Antibodies against NCAM in chick embryos stain the neural

plate, notochord and myotomes but not the surrounding ectoderm (Edelman et al, 1983). Antibodies against Xenopus NCAM show a more complex pattern of immunoreactivity. Initial antibody staining occurs in the neural ectoderm, somitic mesoderm and chordamesoderm. During formation of the neural tube NCAM immunoreactivity becomes restricted to the neurectoderm and its derivatives, during closure of the neural tube NCAM is then expressed in a radial pattern in coronal sections of the neural tube. NCAM is expressed in neural crest cells before migration and after formation of cranial and spinal ganglia. During the period of initial neurite outgrowth NCAM is concentrated in the developing central nerve fibre pathways. NCAM is seen on peripheral nerves from the time of their initial outgrowth, and is strongly expressed at neuromuscular junctions during the period of their formation (Balak et al, 1987). In summary, NCAM is expressed in Xenopus laevis embryos following neural induction and is thought to function during morphogenesis of the neural plate and tube, some neural crest derivatives, development of nerve fibre tracks and formation of neuromuscular connections (Balak et al, 1987).

The observation that NCAM first appears in Xenopus embryos in the neurectoderm at stage 14 (shortly after formation of the neural plate, and several hours after primary neural induction) has led to the conclusion that NCAM is unlikely to be involved in the mechanisms of primary embryonic induction. NCAM immunoreactivity is now believed to be a reliable marker of the differentiation of neural cells in Xenopus laevis following neural induction (Jacobson and

Rutishauser, 1988). NCAM in *Xenopus* embryos is specific for neural tissue and is not present on embryonically derived muscle cells (Kay *et al.* 1988).

Studies on the localisation of NCAM mRNA in *Xenopus* embryos using *in situ* hybridisation have shown that the RNA can be detected at very early stages of neural development, and that the expression of the NCAM gene is dependent on neural induction (Kintner and Melton, 1987). These findings are in agreement with those of Jacobson and Rutishauser (1988) discussed above, and it is suggested that activation of NCAM gene expression may be a direct and nearly immediate genetic consequence of neural induction.

Cell-cell interaction is an important process in morphogenesis with the modulation of a relatively small number of cell surface molecules thought to have a significant effect in the overall development of the embryo (Edelman, 1984a). This is supported by the discovery that in certain cases cell adhesion can regulate gene expression (Newell *et al.* 1971; Parish and Schmidlin, 1979; Pfohl and Giudice, 1987). In *Xenopus laevis* embryos the synthesis of many proteins which are directed by new transcripts at the neurula stage is dependent on cellular interactions (Shickawa *et al.* 1985). For example, activation of muscle specific alpha-actin genes is dependent on cell contacts during blastula stages (Sargent *et al.* 1986). This is not the case for genes studied from ectodermal and endodermal proteins.

There is no single pattern of NCAM expression in tissues from different animal embryos. This is consistent with the role of NCAM in mediating adhesion during

morphogenesis, where differences in expression of NCAM between species reflect variation in tissue patterns (Balak et al. 1987). The spatio-temporal pattern of CAM's in embryonic cell membranes implies a precise control of the expression of different CAM's during development. If this were not the case cell segregation and association would occur incorrectly during body formation, leading to abnormal morphogenesis.

1.4b Cell Surface Interactions with Extra-Cellular Materials

Many types of animal cell secrete organised materials into the region outside the plasma membrane. In epithelial and endothelial cells the extra-cellular material is deposited in a polar fashion around the cell to produce a thin network of components termed the basement membrane (Simons and Fuller, 1985). In other cells extracellular material is deposited in the form of a general extracellular matrix (ECM) on all sides of the cell (Ekblom et al. 1986). Cellular interaction with extracellular materials can be important during embryonic development and plays a role later in the regulation of growth and in the maintenance of normal tissue function. The ECM acts mainly to provide attachment sites for cells through the interaction of matrix components with substrate adhesion molecules present in the membrane (SAM's, see Edelman, 1985a). Although it is likely that the ECM in amphibians has a composition similar to that described in other embryos (Hay, 1981; Yamada, 1983) as yet only fibronectin has been characterised in detail in Xenopus laevis embryos (Boucaut and Darribere, 1983b).

Extracellular matrices contain a number of glycoproteins, proteoglycans and collagens (Ekblom *et al.*, 1988). Collagens form the major structural component of the ECM and are present as a network of interconnecting fibrils (see Kafalides *et al.*, 1979). Collagens comprise a unique group of glycoproteins which are characterised by high glycine content and which contain the modified amino acids hydroxylysine and hydroxyproline. There are several different types of collagen and cells at different developmental stages in the same tissue can produce different molecular forms. Collagen is thought to be present in the ECM in close affinity to glycosaminoglycan molecules (GAG's). These are large highly charged chains of repeating disaccharides with carboxyl and/or sulphate groups, and which usually contain attached proteins (Toole, 1978). GAG's are thought to play a structural role in the ECM and are important for filtration (Ekblom *et al.*, 1988).

The adhesion of cells to the ECM is thought to be mediated by glycoproteins. Fibronectin (FN) is an adhesive matrix glycoprotein found in the ECM, on the cell surface and in plasma (Vaheri and Mosher, 1978). FN is a large molecule with subunits around 220-250,000 Mr, and functions primarily in promoting cellular adhesion to solid substrates, particularly to collagen. The FN molecule comprises several protease resistant functional domains that can independently bind a number of substrates including fibrin, collagen, and heparin (Chen, 1981; Hughes *et al.*, 1980; Rees *et al.*, 1980). The glycoprotein laminin (LN) is a major constituent of all basement membranes but is absent from other extracellular

sites (Farquar, 1981). LN is composed of three A chains (200,000 Mr each) and one B chain (400,000 Mr) which are covalently linked by disulphide bonds to form a molecule of approximately 1,000,000 Mr. LN domains will bind the cell surface (Liotta, 1986), heparin (Edgar *et al.*, 1984) and type IV collagen (Vlodavsky and Gospodarowisc, 1981), but little is known concerning the structure of these binding domains. Other ECM glycoproteins include entactin, a 150,000 Mr sulphated glycoprotein initially found in the matrix of endodermal cell lines (Carlin *et al.*, 1981; Hogan *et al.*, 1982). Entactin may be the same molecule as nidogen, Mr 150,000 (Dziadek *et al.*, 1985). Chondronectin is an ECM glycoprotein with a high degree of specificity for mediating the adhesion of chondrocytes to type II collagen (Hewitt *et al.*, 1980; 1982).

The interaction of cells with the ECM is thought to occur through receptors present in the plasma membrane. An example is the binding of matrix FN to the cell surface which occurs through a 140,000 Mr membrane receptor complex (Akiyama and Yamada, 1985; Akiyama *et al.*, 1986; Pytela *et al.*, 1985). Recently much interest has been shown in the discovery of short peptide sequences which are thought to be involved in protein recognition in the membrane receptor complexes. Using small synthetic peptides deduced from the primary structure of the FN cell-attachment domain, the tri-peptide Arg-Gly-Asp (RGD) was shown to be the minimal FN amino acid sequence with cell attachment activity. The sequence is normally present as part of the tetrapeptide Arg-Gly-Asp-Ser (RGDS) (Pierschbacher *et al.*, 1983; Pierschbacher and

Ruoslahti, 1984a; 1984b). A second ECM glycoprotein with cell attachment properties is vitronectin (VN). The VN receptor in the cell membrane has a different molecular weight to that of the FN receptor and is clearly distinct from it, but it also recognises the RGD sequence as part of the tetra-peptide sequence Arg-Gly-Asp-Val (Hayman *et al.* 1983; 1985; Suzuki *et al.* 1985; Pytela *et al.* 1985). Fibronectin also interacts with the cytoskeleton through a plasma membrane receptor complex CSAT (Horwitz *et al.* 1988). CSAT is a receptor for fibronectin and laminin in the ECM, and also for the cytoskeletal protein talin which is located at sites of cell matrix adhesion where actin filaments meet the plasma membrane. The binding of FN to CSAT is inhibited by the RGDs sequence, but the tetrapeptide does not inhibit the binding of talin. This indicates that the binding sites for talin and fibronectin are distinct, and has led to the suggestion that the RGD sequence may be specific for ECM components. Fibronectin is also involved in the attachment of cells to collagen (Klebe, 1974; Pearlstein, 1976; Vaheri and Mosher, 1978). However, membrane proteins that bind collagen directly have been described (Oclind *et al.* 1980; Chiang and Kang, 1982; Saito *et al.* 1986).

The nature of the ECM present in *Xenopus laevis* embryos at different stages of development is not known. Experiments on the frog *Rana* have indicated an increase in the overall level of synthesis of ECM components during gastrulation which corresponds to a period of extensive morphogenetic movement (Johnson, 1977a; 1977b; 1977c). The appearance of ECM components in mammalian embryos during

development have been characterised, and the ECM is found to be present at very early stages. In the 16 cell compacted mouse morula laminin is present lining the cell borders (Leivo *et al.*, 1980; Wartiovaara *et al.*, 1980). Following the blastocyst stage, type IV collagen appears (Adamson and Ayers, 1979; Leivo *et al.*, 1980) and fibronectin is deposited in the ECM between the germ layers (Wartiovaara *et al.*, 1972). At later stages of development these ECM components are found in extra-embryonic membranes and in other tissue structures.

FN is present in many embryos from an early stage, including mouse (Zetter and Martin, 1978), chick (Critchley *et al.*, 1979), sea urchin (Spiegel *et al.*, 1980) fowl (Newgreen and Thiery, 1980) and in amphibians (Boucaut and Darribere, 1983a; Lee *et al.*, 1984). In *Xenopus laevis* embryos FN is synthesised at a low rate from maternally derived mRNA during oogenesis, its translation then increases rapidly during the late blastula and gastrula stages (Lee *et al.*, 1984; Darribere *et al.*, 1984). FN is first detected on *Xenopus* embryo cells at the early blastula stage where it is associated with the surface of ectodermal cells facing the blastocoel. At the end of gastrulation FN is present between the basal surface of ectodermal cells and migrating mesodermal cells (Boucaut *et al.*, 1985; Lee *et al.*, 1984). FN is organised into a three-dimensional fibrillar matrix which exclusively coats the inner surface of the blastocoel roof from early blastula to late gastrula stages (Boucaut *et al.*, 1985).

Cell interactions with the ECM are important in several different aspects of morphogenesis. Fibronectin interacts with the cell surface via the cell binding domain

to mediate the attachment and migration of cells during development (Critchley *et al.*, 1979; Newgreen and Thiery, 1980; Mayer *et al.*, 1981; Heasman *et al.*, 1981; Duband and Thiery 1982a; 1982b; Thiery *et al.*, 1982; Sanders 1982; Wylie and Heasman, 1982). One example of the role of FN in the movement of cells is the migration of mesodermal cells during gastrulation in *Xenopus* embryos. The migrating cells are thought to interact directly with the ECM, with the orientation of cell migration determined by fibronectin in the ECM (Nakatsuji *et al.*, 1982; Nakatsuji and Johnson, 1984). In the presence of anti-FN antibodies there is no migration of mesodermal cells along the blastopore roof (Boucaut *et al.*, 1985). FN is not produced by the migrating mesodermal cells, but by the ectodermal cells over which the cells pass (Boucaut *et al.*, 1985; Lee *et al.*, 1984). This is consistent with observations for migration of neural crest cells (Newgreen and Thiery, 1980) and primordial germ cells (Wylie and Heasman, 1982). The control of cell migration in development is thought possibly to be linked to the behaviour of FN receptors in the cell membrane (Darribere *et al.*, 1984; Lee *et al.*, 1984).

Laminin is an abundant protein component of the basement membrane in vertebrate embryos (see McCarthy and Burger, 1987). In mouse embryos it is one of the earliest ECM components to appear preceding the establishment of an organised basement membrane, and is thought to be involved in the co-ordination of basement membrane formation (Leivo *et al.*, 1980; Wu *et al.*, 1983; Dziadek and Timpl, 1985). The expression of laminin on the surface of early mouse

blastomeres indicates that it may play a role in the establishment of cell polarity, in cell adhesion during compaction and in the mediation of cell interactions during preimplantation development (Dziadek and Timpl, 1985). Many cell types in the embryo bind laminin and this can alter cell behaviour (Palotie *et al.* 1983; Engvall and Ruoslahti 1983; Goodman and Newgreen 1985). Laminin alone is an effective stimulator of cell differentiation (Edgar *et al.* 1984), an effect enhanced when laminin is complexed to other basement membrane components (Hadley *et al.* 1985).

There is substantial research on the role of collagens in morphogenesis (see Hay, 1981; and Treisted, 1984 for reviews), and they are known to play an essential role in the development of most tissues and organs in vertebrate embryos (Wessels, 1977). Collagen in the ECM of embryos can influence cell proliferation (Adams, 1983; Gay *et al.* 1974; Liotta *et al.* 1978; Kleinman *et al.* 1981), cell differentiation (Hauschka and Konigsberg, 1986; Kosher and Church, 1975; Reddi and Anderson, 1976; Bunge and Bunge, 1978), cell migration (Bard and Hay, 1975; Couchman *et al.* 1982; Rovasio *et al.* 1983) and specific gene expression (Lee *et al.* 1984; 1985).

The GAG molecules of the ECM are thought have a specific role in development. GAG's can bind water and swell in size to 10,000 times larger than normal. This swelling can exert considerable pressure on neighbouring tissues and is involved in the formation of cell-free spaces in certain organs (Bard and Abbot, 1979). An example is the appearance of the GAG hyaluronic acid in the chick embryo which

correlates with an increase in the cell-free space near the head and appears to permit the subsequent migration of neural crest cells (Porter, 1973). An example of the role of collagen and GAG's in development is in the development of mouse embryo salivary glands. The dependence of normal salivary gland morphogenesis on an intact cell coat at the epithelial surface is attributable to hyaluronidase sensitive acid mucopolysaccharide/collagen complexes (Bernfield and Wessels, 1970; Bernfield et al., 1972).

1.4c Intercellular Membrane Junctions

Membrane junctions are easily recognised under the electron microscope as regions where membranes of two adjacent cells are held more closely in contact than the normal 20-30nm interspace. There are three major classes of membrane junction: adhesive junctions which hold cells together and maintain them in a fixed position in the tissue, sealing junctions which prevent the diffusion of molecules between cells, and communicating junctions which provide transmembrane channels enabling direct flow of ions and small molecules between cells (See Wolfe, 1981). The structure and composition of these junctions will be outlined briefly before considering the role of membrane junctions in development.

The primary type of adhesive junction are the desmosomes. Spot desmosomes function like individual rivets to reinforce a localised point of cell-cell contact (Grinnell, 1978). The polypeptides composing spot desmosomes have not been fully characterised, but they are thought to

consist of a membrane domain comprising three antigenically distinct glycoprotein families (Cohen *et al.*, 1983), and a cytoplasmic plaque (Geiger *et al.*, 1983) that contains non-glycosylated desmoplakins and other peripheral proteins (Franke *et al.*, 1981; Mueller and Franke, 1983; Gorbakky *et al.*, 1985). The plaque is anchored to cytokeratin type tonofilaments (Franke *et al.*, 1978; Lazarides, 1980) and these filaments may be connected to desmosomes in adjacent cells or to half desmosomes in the extracellular surface (Gipson *et al.*, 1983). Spot desmosomes are present between all cells bound together into tissues, and they appear early in development when cells take up permanent positions in tissues (Wolfe, 1981).

Another type of adhesive junction is the intermediate junction or belt desmosome. This junction forms a continuous band of reinforcement around cells such as that seen in epithelial cells. The intermediate junction is composed of three parts: an integral membrane site containing a 135,000 Dalton protein and uvomorulin (Boller *et al.*, 1985), a membrane bound cytoplasmic plaque composed of vinculin and possibly additional proteins (Geiger *et al.*, 1981), and a contractile cytoplasmic actin filament bundle that forms a belt around the cell and connects the junctional complex to the actin network in the cytoskeleton.

Sealing junctions or tight junctions join cells together preventing cavity and extracellular fluids from mixing (Farquhar and Palade, 1963). Tight junctions were originally thought to form impervious seals between cells but electrophysiological studies have revealed that the junctions

act as gates, selectively regulating the passage of ions between the cells (Diamond, 1977). Freeze fracture electron microscopy has revealed that tight junctions are composed of a vast network of strands (Goodenough and Revel, 1970; Pinto da Silva and Kachar, 1982). The tightness of the junction appears to be related to the number of strands in the network (Claude, 1978; Easter *et al.*, 1983; Marcial *et al.*, 1984). Strands of the tight junctions are thought to represent inverted lipid micelles in which the exoplasmic areas of the lipid bilayers from adjacent cells have fused (Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982). Tight junctions are deformed in the absence of calcium ions and a monoclonal antibody has been identified that blocks the reformation of tight junctions in the presence of calcium and which reacts with a polypeptide of about 120,000 Daltons (Martinez-Palomo *et al.*, 1980).

Communicating junctions or gap junctions form channels in the membrane to allow the passage of small molecules between adjacent cells (Hertzberg and Gilula, 1978; Loewenstein, 1981; Hertzberg and Skibbens, 1984). Gap junctions enable the transfer from cell to cell of molecules up to around 1000 Mr (Simpson *et al.*, 1977). Electronmicroscope and Xray analyses have revealed the structure of the gap junction to comprise clusters of transmembrane channels called connexons. Connexons in neighbouring membranes are paired end to end with those of the opposite cell membrane to form the complete channel (Revel *et al.*, 1988; Unwin and Zampighi, 1980; Unwin and Ennis, 1984). Models of gap junctions depict connexons as

consisting of four to six protein subunits forming the cylindrical wall of the transmembrane channel (Casper *et al.*, 1970; Markowski, 1985). Gap junction proteins vary in junctions isolated from different tissues and different organisms (Revel *et al.*, 1985). Gap junctions in the rat liver have been most thoroughly characterized and there is evidence for a 27,000 Mr protein as a major component of the junction (Revel *et al.*, 1985). Antibodies have indicated immunogenically similar proteins in gap junctions from other sources and it appears this 27,000 Mr protein is a major constituent of gap junctions in most tissues (Dermietzel *et al.*, 1984; Hertzberg and Skibbens, 1984).

Gap junctions are found in many cell types. They provide avenues for extremely rapid communication and electrical coupling between cells. Gap junctions can be regulated directly or indirectly by phosphorylation; a cAMP dependent protein kinase raises junctional permeability, and c-src protein tyrosine kinase reduces it (Wiener and Loewenstein, 1983). Cells within the embryos of most species are able to communicate directly with each other through gap junctions (Warner *et al.*, 1984). Gap junction communication becomes less widespread throughout the embryo as development occurs (see discussion in Wolfe, 1981).

The majority of work carried out on membrane junctions in development has concentrated on the gap junction and little has been done to characterize the appearance and significance of tight junctions and the adhesive junctions in Xenopus development. The presence of epithelial cells in the

blastula stage *Xenopus laevis* embryo indicates that these junctions are likely to be present at very early stages of development (Straelin and Hull, 1978). Ducibella et al have described the appearance of the different intercellular junctions in mammalian development and these findings are outlined briefly below (Ducibella et al, 1975).

Tight junctions in mammalian embryos first appear during the morula stage of development which corresponds to the formation of the trophoblast (Ducibella et al, 1975). The appearance of these junctions signifies the formation of an epithelial cell layer which separates the outer maternal environment from that of the embryo (Ducibella et al, 1975). The tight junction provides a physical barrier by which a specialised environment can be maintained within the morula. The establishment of the permeability barrier between the oviductal fluid and the inner cell mass is consistent with a period of major change occurring in the physiology and biochemistry of the embryo (Biggers and Stern, 1973; Stern et al, 1971; Barbehenn et al, 1974; Woodland and Graham, 1969; Piko, 1970; Monesi and Salfi, 1967). The microenvironment created inside the embryo by the formation of tight junctions is thought to be required for cell determination and is necessary for the accumulation of fluid during the expansion of the blastocoel (Enders, 1971; Ducibella et al, 1975).

Spot and belt desmosomes are first observed in the mammalian embryo at the blastocyst stage as part of a trophoblast junctional complex which is also characterised by the presence of gap and tight junctions (Schlafke and Enders, 1967; van Blerkom et al, 1973). The desmosomes and gap

junctions are thought to fix the inner central mass to one pole of the embryo and to partially isolate it from the blastocoel. The anchoring nature of desmosomes is additionally important at this stage because, on formation of the trophoblast, the cells become more attenuated and the area of contact between them decreases (Ducibella, 1975). Gap junctions also act at this stage to couple the blast cells together in a co-ordinately functioning population (Ducibella, 1975).

Cells in the early Xenopus embryo are able to communicate with each other through a direct cell to cell communication pathway mediated by the gap junction (Ito and Lowenstein, 1966; Palmer and Slack, 1970; Warner, 1985). Similar communication pathways have been described in all embryos so far studied (Warner et al., 1984). Patterns of junctional communication within the Xenopus embryo vary significantly from cell to cell, and as development proceeds (Guthrie, 1984). Direct cell to cell communication in Xenopus embryos both appears and disappears between groups of cells with different developmental fates as the embryo develops (Dixon and Cronley-Dillon, 1972; 1974; Blackshaw and Warner, 1976; Keeter et al., 1977). One example are the cells of the neural plate and lateral ectoderm which lose electrical connections on closure of the neural tube. New communication pathways are then formed between ectoderm cells originally separated by the neural plate (Warner, 1973). As development proceeds, gap junction communication becomes compartmentalised within the embryo (Lo and Gilula, 1979) with the borders of gap junction compartments coinciding with

developmental borders as described by fate maps (de Laat et al. 1980) and cell lineage experiments (Warner and Lawrence, 1982).

The variation observed in gap junction expression in the Xenopus embryo during development supports the concept that these junctions transmit the inducing signals that control regional specification of the embryo (see Section 1.3d). Warner et al. (1984) used a channel blocking antibody against the 27,000 Mr protein to inject into one cell of the eight cell Xenopus embryo. The antibody blocked gap junction communication in the progeny cells and caused clear developmental defects that were consistent with the notion that the block of gap junctional communication interferes with neural induction (Warner et al., 1984). However, a direct role for gap junctions as channels for the neural inducing signal has been ruled out in view of the fact that the early stages of neural induction can occur in the absence of any cell contact (Toivonen et al., 1975). Experiments have also indicated that inhibition of gap junction activity did not prevent the induction of muscle gene activation in the Xenopus embryo (Warner and Gurdon, 1987). The precise role of gap junctions in embryonic induction remains unclear. It is thought that gap junctions may be involved in mediating the inducing signal in homeogenetic induction (i.e the spread of neural inducing signal from nervous system to induce further nervous system, Mangold, 1933). However the presence of homeogenetic induction in Xenopus embryos and the associated role of gap junctions remains unproven.

1.4d Cell Surface Interactions with the Cytoskeleton

The major fibrous elements of the cytoskeleton in eukaryotic cells are microtubules, microfilaments and intermediate filaments (Goldman and Knipe, 1973). Microtubules are long straight unbranched rods, 20-25nm in diameter which appear hollow in cross section. They comprise two non-identical subunits of the protein tubulin and numerous other proteins termed microtubule associated proteins (MAPs). Microtubules are important as supporting structures in the cell and in defining cell shape. Their role in cell movement is particularly evident in the motion of cilia and flagella, and they are involved in cell division through formation of the spindle apparatus responsible for directing chromosome movement (see Dustin, 1984).

Microfilaments are shorter and finer than microtubules and comprise individual 9nm filaments. They are built by the end to end association of actin molecules, and appear in the cell in connection with additional proteins such as myosin, tropomyosin and alpha-actinin. Microfilaments are found throughout eukaryotic cells in tightly packed arrays that support projections of the plasma membrane such as microvilli. Microfilaments are also involved in changes in cell shape and in cytokinesis (see Wolfe, 1981).

Intermediate filaments are midway in size between the larger microtubules and smaller microfilaments (7-11nm diameter). There are five distinct types of intermediate filament found in eukaryotic cells that differ in their protein monomers. Keratin (or tono-) filaments are found in epithelial cells and cells of epithelial origin, desmin

filaments are found predominantly in muscle cells, vimentin filaments are found in mesenchyme cells and cells of mesenchyme origin, neurofilaments are found in neuronal cells, and glial filaments found in glial cells. Intermediate filaments are thought to position the nucleus within the cell, and are important in strengthening sheets of cells such as the skin and gut lining which are subject to shearing or stretching forces (see Lazarides, 1980).

In Xenopus the cytoskeleton is formed during the early stages of oogenesis and is thought to be involved in the establishment of the animal/vegetal polarity inherited by the egg (Wylie et al., 1985). Work has been carried out to investigate the distribution of cytoskeletal components in the cells of developing embryos. At early stages actin and other microfilament proteins are found to be concentrated in a peripheral layer in the cortex of the cell. This has been shown for rat and mouse embryos (Lehtonen and Badley, 1980), sea urchin embryos (Wang and Taylor, 1978) and in Xenopus oocytes and eggs (Wylie et al., 1985; Gall et al., 1983). In cleavage stage mouse embryos tubulin appears evenly distributed throughout the cell with some concentration at the perinuclear region and at areas of wide intercellular contact (Lehtonen and Badley, 1980). Microtubules in the 4 cell mouse embryo are arranged orthogonally to the cell surface but by the 8 cell stage microtubules appear parallel to the membrane at the cell contact area (Ducibella and Anderson, 1975; Ducibella et al., 1977). In contrast to the localised distribution of microfilament and microtubule components within the cell, intermediate filament protein is

found to be evenly distributed within the cytoplasm of cleavage stage mouse embryos without apparent concentration (Lehtonen and Badley, 1980).

Changes in the form and distribution of cytoskeletal filaments are thought to be important in cell differentiation. In particular, cytoskeletal components play a role in cell movement, in cell anchorage to the substratum, in the maintenance of cell shape and in the movement of membrane proteins (Lehtonen and Badley, 1980). The role of microtubules in development has been investigated using colchicine (Olmsted and Borisy, 1973), which inhibits microtubule assembly. Microtubules have been shown to be associated with changes in cell shape during development, particularly in the elongation of cells and in the maintenance of the elongated form. An example is the growth of nerve cell processes. When colchicine is applied to a nerve cell, the nerve axon loses its rectilinear shape and may eventually become withdrawn into the cell body. When the elongating cells of the chick primitive streak are treated with colchicine the elongating cells shorten, and the primitive groove straightens out (Wessels *et al.*, 1971). A similar effect is seen with elongating cells of the neural plate (Karfunkel, 1970).

Microfilaments are contractile elements and are consequently found in positions where their contraction could cause a constriction of the cell. In cells undergoing cleavage, microfilaments appear in the form of a contractile ring just below the cleavage furrow. As the ring narrows the cleavage furrow cuts deeper and eventually separates the

daughter cells. When cleaving eggs are treated with cytochalasin B which destroys microfilaments (Spooner, 1978), cleavage stops immediately and the forming cleavage furrows are reversed (Schroeder, 1972). Nuclear divisions however still occur resulting in multinucleated cells. Microfilaments are also involved in the changes of cell shape required for the folding of cells in epithelial cell layers. An example are the folding mechanisms involved in the closure of the neural tube in *Xenopus* embryos (Balinsky, 1981; Baker and Schroeder, 1987).

Less is known about the role of intermediate filaments during development but they are believed to be structural, tension bearing elements (Hubbard and Lazarides, 1979; Lazarides, 1980). Tonofilaments are present as part of the plaque in intermediate junctions and in spot desmosomes (Franke *et al.*, 1981), and as such they are important in the formation of epithelial sheets during development (see Section 1.2d above).

The mechanisms by which cytoskeletal components interact with the plasma membrane has proved a recent area of active research. Microfilament-plasma membrane associations occur at sites of cell-substratum or cell-cell adhesion, or at sites of cell contractility where cytoskeletal activities can mediate events such as cytokinesis, endocytosis and cell movement (Rogalski, 1987). It is suggested that the actin filaments of non-muscle cells bear the same relationship to the plasma membrane as they do in the Z disc of striated muscle. Thus, they are in a position to exert force at the cell surface where it can be used to change the shape of the

cell or to pull on other cells connected to the substratum.

Particular emphasis has been placed on the determination of membrane receptors for cellular actin and several possible candidates have been suggested. Vinculin is a peripheral membrane protein known to co-distribute in the membrane with actin microfilaments (Geiger, 1983), and with other cytoskeletal proteins such as alpha-actinin (Geiger *et al.* 1981) and talin (Burridge and Connell, 1983). This co-distribution occurs at both junctional and non-junctional sites within the membrane (Tokuyasu *et al.* 1981; Craig and Pardue, 1983). Sgp-130 is an integral membrane sialo-glycoprotein closely correlating with microfilament distributions in cultured cell types at both intercellular and cell-substrate contact sites (Rogalski and Singer, 1985). Experiments have indicated that sgp-130 assumes at least three tissue specific isoforms that are developmentally regulated (Rogalski, 1987). However, the molecular basis for both vinculin and sgp-130 interactions with actin microfilaments and the plasma membrane remain largely unresolved and the significance of these interactions in development is unclear. Connectin is a cell surface protein (Mr 70,000) isolated from mouse fibrosarcoma cells that binds both the ECM protein laminin and cytoskeletal actin with high specificity (Brown *et al.* 1983). Connectin is an example of a trans-membrane protein that mediates the interaction of a cell with its extracellular matrix (Sugrue and Hay, 1981). The existence of membrane receptors for ECM and cytoskeletal components may provide a mechanism for the modulation of cell behaviour by the ECM. Another cell surface protein with links

to both the ECM and the cytoskeleton has been isolated from murine mammary epithelial cells. This receptor can strongly bind both collagen and actin (Rapraeger and Bernfield, 1982). A more direct link from the cytoskeleton to the ECM may arise through fibronectin interactions (Yamada, 1983, Koteliensky et al, 1982).

CSAT, the cell substratum attachment receptor complex (Horwitz et al, 1985; Knudsen et al, 1985), was mentioned above in Section 1.4b. CSAT is a receptor for fibronectin and for the cytoskeletal component talin which is located specifically at sites of cell substratum adhesion where actin filaments meet the plasma membrane. CSAT is thought also to have secondary associations with one of the *sgp-130* isoforms (Rogalski, 1987). In erythrocytes the membrane proteins ankyrin and band 4.1 play a role in the anchorage of the submembraneous skeleton to the plasma membrane (see Marchesi, 1985). These proteins are particularly involved with the binding of spectrin and actin to the membrane, and analogues of these proteins are now known to exist in many different cell types (Davis and Bennett, 1984; Moon et al, 1985; Aster et al, 1984).

1.4c The Role of Membrane Glycolipids in Development

The previous discussion of the role of the cell surface membrane in morphogenesis has concentrated largely on membrane protein interactions. Other components of the membrane are also thought to be important in development. Glycolipids are ubiquitous membrane components with an essential role in stabilising the membrane bilayer and

conferring structural rigidity on the cell (Sharom and Grant, 1977; Kinsky, 1972; Yamakawa and Nagai, 1978). Glycolipids may also be involved in the regulation of cell growth and in mediating cellular interaction (Hakomori, 1981). Evidence for this suggestion has come from observations of dramatic changes in glycolipid composition following the oncogenic transformation of cells (Hakomori and Murakami, 1988; Brady and Fishman, 1975; Richardson *et al.*, 1976). Glycolipids also play a role in contact inhibition (Hakomori, 1970; 1981, Yogeeswaran and Hakomori, 1975), and changes in the cell cycle have been observed after the exposure of glycolipids at the cell surface (Gahmberg and Hakomori, 1974; 1975).

Correct functioning of membrane proteins can be controlled by glycolipids through interactions with the lipid annulus (Singer and Nicholson, 1972; Yamakawa and Nagai, 1978), or through the requirement of certain glycolipids as cofactors for membrane bound enzymes (Svennerholm, 1980; Partington and Daly, 1979). An example are the gangliosides in human brain microsomes which greatly activate the Mg dependent adenosine triphosphatase but not the Na/K dependent enzyme (Caputto *et al.*, 1977). Observed changes in glycolipid composition and synthesis observed during development have lead to the suggestion that lipids are involved in cellular recognition. An example are the undifferentiated 'crypt' cells of mammalian intestinal epithelia (Marsh, 1961; Bouhours and Glickman, 1969). Glycosphinglipids (GSL's) are also important as cell surface antigens in the immune response (Marcus and Schwarting, 1976; Rapport and Graf, 1981), and as cell surface receptors for several biofactors.

Examples include bacterial toxins (Van Heyningen, 1974; Morita *et al.*, 1980; Ledley *et al.*, 1977; Kaito and Naiki, 1976), glycoprotein hormones (Mullin *et al.*, 1976; Lee *et al.*, 1977), viruses (Haywood, 1974; Holmgren *et al.*, 1980), and interferon (Besancon and Ankel, 1974; Ankel *et al.*, 1980). Membrane glycolipids also interact directly with fibronectin (Kleinman *et al.*, 1979).

Despite the evidence for the role of glycolipids in cellular interaction, very little work has been done to analyse the significance of glycolipids in animal development. Characterisation of the regional specificity of glycolipids has been carried out in *Xenopus laevis* embryos (Slack *et al.*, 1985) and active synthesis was found to occur from fertilisation onwards. This is thought to indicate the requirement for large amounts of membrane in the cleaving embryo. Analysis of alkali stable lipids from *Xenopus* neurulae indicated significant regional variation only in the epidermis (Slack *et al.*, 1985).

1.4f Summary: The Role of the *Xenopus* Embryo Membrane in Morphogenesis

Several different aspects of membrane interaction in development have been outlined above. Each of these areas is a research field in its own right and no attempt was made to cover a full review of all relevant data on the role of the cell surface membrane in development. The aim was to illustrate the diversity of membrane components involved in morphogenesis and to emphasise the degree of co-operation and co-ordination that exist between them. The cell surface

membrane is involved in many different aspects of morphogenesis including cell adhesion, cell movement, cell cleavage, cell communication, cellular recognition and in tissue stabilisation. These are clearly essential and fundamental processes of development, and more than one set of membrane components may be involved in each of these processes. An example is cell adhesion which occurs through CAM's, CJM's and through interactions with the ECM.

Other aspects of membrane interactions during development were not mentioned and are outside the scope of this thesis. These include the membrane receptor response to hormones, the role of membrane bound enzymes during development, and the surface charge of the membrane which is thought to be involved in cell migration.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

All chemicals not otherwise mentioned below were of 'Analar' grade from BDH Chemicals (Poole, Dorset).

Amersham International (Amersham, Bucks); ³⁵S-methionine (15mCi/ml), (¹²⁵I) NaI (100mCi/ml), ¹⁴C-protein molecular weight markers, Amplify.

Beckman-RITC Ltd. (High Wycombe, Bucks); Ready solv EP scintillant.

(Watford, Herts); Temed,
4-chloro-1-naphthol, Biorad protein assay concentrate.

BRI(UK) Ltd. (Cambridge, Cambs); Ultrapure urea.

(Rochester, New York, USA) N',N'-methylene
bis-acrylamide.

Fisons Chemicals Ltd. (Loughborough, Leics); 30% (v/v)
hydrogen peroxide, glass tubing.

Flow Laboratories (UK) Ltd. (Irvine, Ayrshire, Scotland);
RPMI-1640 medium.

Fluka AG (Buchs, Switzerland) Triton-X114.

Gibco Ltd. (Paisley, Renfrewshire, Scotland); Freund's adjuvant (complete and incomplete), foetal calf serum, new born calf serum.

(Greenford, Middlesex); sodium benzyl penicillin (crystopen), streptomycin sulphate (BP).

LKB Instruments Ltd. (South Croydon, Surrey); Ampholines pH 3.5-10 and 5-7.

May and Baker (Dagenham, Essex); Glacial acetic acid, TCA, chloroform, acetone.

Miles-Yeda Ltd. (Rehovot, Israel); FITC and enzyme conjugated antibodies.

Pierce and Warriner (UK) Ltd. (Chester, Cheshire); Iodogen.

Sigma (Poole, Dorset); Tris, DTT, PMSF, MS-222, gelatin, BSA, hypoxanthine, thymidine, aminopterin, lactoperoxidase, glucose oxidase.

South African Snake Farm (Windhoek, Cape province, RSA); Male and female Xenopus laevis.

Whatman Laboratory Products Ltd. (Maidstone, Kent); Whatman number 1, Whatman number 50 and 3MM filter paper.

2.2 Methods

M.1 Frogs

Xenopus laevis frogs were kept in tanks of water at 19-21°C and fed twice weekly on a diet of blow-fly larvae and minced beef. The frogs were looked after and fed by Mrs C. Kwasnik.

M.2 Obtaining Xenopus Eggs and Embryos

Female frogs were induced to lay by injection of 100-150 i.u. of FSH 1-8 days prior to laying, followed by a second injection of 500-600 i.u. of HCG 8-16 hours prior to laying. Both injections were into the dorsal lymph sac of the frog. Iced water was occasionally used after the injection of HCG to slow down the initial production of eggs. Injection of HCG alone without the initial priming injection of FSH was also found to be effective at inducing laying and this reduced the number of frogs that laid eggs in 'strings' of jelly.

Embryos were obtained by one of two methods: through the natural mating of frogs or by artificial fertilisation of eggs. In the first case the male frog was injected with 100 i.u. of FSH at the same time as the female frog received her injection of HCG. The frogs were then placed in a tank of water and left for between 8 to 24 hours until the mating was complete. An in vitro fertilisation was carried out when a large number of embryos at synchronous stages of development were required. An adult male Xenopus laevis frog was killed by injection of 300µl of Euthatal into

the dorsal lymph sac. Both testes were then removed and stored at 4°C for up to 2 weeks in Barths saline (see below). In vitro fertilisation of the eggs was carried out as follows: eggs were collected in a petri dish and the medium removed, a piece of testis was stroked over the eggs and a small amount of distilled water added to promote sperm motility. The eggs were then left for a minute before flooding the petri dish with distilled water. If the fertilisation was successful rotation of the embryos was observed after 15 min. Barths saline (Barth X) was comprised of the following:

88mM NaCl

1mM KCl

24mM NaHCO₃

0.82mM MgSO₄

0.33mM Ca(NO₃)₂

0.41mM CaCl₂

15mM Tris.HCl pH 7.6

10mg/litre Benzyl penicillin

10mg/litre Streptomycin sulphate

Xenopus eggs and embryos were dejellied by swirling in a solution of 1% or 2% cystein pH 8.0, for 5-10 min. The eggs and embryos were then washed thoroughly and stored in Barths saline, or in 1/10 diluted Barths saline until use.

M.3 Purification of Crude Membrane from Xenopus Embryos

Aproximately 300 Xenopus eggs or embryos were

homogenised in 2ml of homogenisation buffer (10% w/v sucrose, 150mM NaCl, 10mM Mg-acetate, 20mM Tris.HCl pH 7.6, 1mM PMSF). The homogenate was spun in an eppendorf centrifuge for a very brief period to remove yolk. An excessive spin at this stage was found to cause loss of membranes. The supernatant was removed avoiding the superficial lipid layer. This was divided into four aliquots and these were layered onto 1ml of 20% sucrose buffer (20% w/v sucrose, 50mM NaCl, 10mM Mg-acetate, 20mM Tris.HCl pH 7.6) in a centrifuge tube. The gradients were spun at 17,000g for 30 min at 4°C in a Beckmann High Speed 18 centrifuge. The crude membrane was found to pellet at the bottom of the tube and cytoplasmic proteins were present at the interface between the two sucrose layers.

M.4 Triton-X114 Purification of Membrane Proteins

This method is adapted from that described by Bordier (1981). Crude membrane pellets were resuspended in 200-300µl of 500mM KCl, 100mM Tris.HCl pH 7.2. 50-80µl of recondensed and saturated TX114 (see below) was added to each tube. The samples were vortexed and sonicated on ice using a sonic probe for 15 sec each. They were then left at 4°C for between 30 min and an hour. The tubes were spun at full speed in an eppendorf microfuge for 10 min after which the detergent and aqueous phases were mixed and removed into clean tubes. The pigment containing pellets were discarded. The samples were spun again for 10 min, the aqueous and detergent phases were mixed and layered onto 600µl of sucrose containing buffer (8% sucrose, 150mM KCl, 10mM Tris.HCl pH

7.5, 0.0075% TX114). The tubes were warmed to 30°C for 3 min and spun at high speed for 10 sec. The uppermost 400µl was then removed and mixed with 20µl of TX114 in a clean eppendorf. This was layered over the original sucrose cushion which was warmed to 30°C and spun for 10 sec at high speed. The uppermost aqueous layer was removed and stored as TX114 insoluble proteins (or TX114 supernatant).

1ml of wash 1 (500mM KCl, 10mM Tris.HCl pH 7.2) was added to the TX114 fraction. The tube was whirlmixed, warmed to 30°C for 3 min until cloudy, cooled on ice for 3 min until clear, and warmed again to 30°C for 3 min until cloudy. The mixture was spun for 10 sec and the aqueous phase removed and discarded. This washing procedure was repeated using 10mM Tris.HCl pH 7.2. The final TX114 fraction was stored at -20°C or acetone precipitated.

To recondense TX114, 20g of uncondensed TX114 was added to 16mg of butylated hydroxytoluene and 980ml of buffer (400mM KCl, 10mM Tris.HCl pH 7.4). The detergent was dissolved at 4°C then left overnight at 37°C. The aqueous phase was removed and replaced with fresh buffer and the method repeated. After the third recondensation the TX114 layer was removed and stored at 4°C.

M.5 Purification of Plasma Membranes

This is a method described by Bretzel *et al.*, 1986. Unless otherwise mentioned all procedures were carried out on ice, and all sucrose solutions were filtered through millipore filters.

Xenopus embryos were sorted, dejellied and washed

in homogenisation buffer (0.25M sucrose, 1mM CaCl₂, 50mM Tris.HCl, pH 7.4). The embryos were then homogenised in 9 volumes of homogenisation buffer in a glass homogeniser using three strokes of the plunger. The homogenate was centrifuged for 10 min at 3,100g in a bench top centrifuge. The supernatant was removed and the sediment homogenised in 9 volumes of buffer (25% sucrose, 70mM CaCl₂, 35mM Tris.HCl pH 7.4) in a glass homogeniser using three strokes of the plunger. This was centrifuged briefly at 8-9g, and the large yolk sediment discarded.

18ml of supernatant containing the plasma membranes was layered onto a gradient comprising 7.5ml 55% (w/v), 3.5ml 50% (w/v), 7.0ml 47% (w/v) sucrose and centrifuged for 15 to 20 hr at 100,000g (27,000 rpm) in a Beckman SW-28 rotor. All solutions were made up in 70mM KCl, 35mM Tris.HCl pH 7.4. The upper half of the band in the 47% sucrose layer and the sucrose layer above this were removed with a pasteur pipette and diluted with 19 volumes of 1mM NaHCO₃, 500mM CaCl₂, pH 7.4. The samples were then homogenised with ten strokes of the plunger in a glass homogeniser. This was centrifuged for 15 min at 27,000g. The pellets from four to eight gradients were pooled and homogenised in a glass homogeniser in 12.5ml of 25% sucrose, 70mM KCl, 35mM Tris.HCl pH 7.4. The homogenate was then layered onto a second discontinuous sucrose gradient comprising 8ml 55% (w/v), 4.5ml 50% (w/v), 4.5ml 45% (w/v), 4.5ml 42% (w/v) sucrose, and centrifuged for 10-12 hr at 100,000g (27,000 rpm) in a Beckman SW-40 rotor. Plasma membranes band in the 42% sucrose layer and in the upper half of the 47% sucrose layer.

M.6 Purification of Myelin

The method used to purify myelin was an adaptation of that described by Norton and Poduslo (1973). Xenopus laevis brains and spinal cords were collected and stored until use at -70°C . 1g weight of tissue (about 15 brains/spinal cords) was homogenised in 20ml of 0.32M sucrose and this was layered over 16ml of 0.85M sucrose in a 38ml Beckman centrifuge tube. The tube was centrifuged at 24,000rpm (75,000g) for 30 min in a Beckman SW-40 rotor (*). Crude myelin was present at the interface between the two sucrose layers.

The crude myelin was removed with a pasteur pipette and was homogenised in water to a final concentration of 38ml. This suspension was centrifuged at 24,000 rpm (75,000g) in the SW-40 rotor for 15 min. The pellet was homogenised in 38ml of water and was centrifuged at 9,500rpm (12,000g) in the SW-40 rotor for 10 min. This last centrifugation step was repeated. The pellet from the second osmotic shock was removed and resuspended in 20ml of 0.32M sucrose by homogenisation. This was layered over 16ml of 0.85M sucrose and the initial centrifugation step (*) repeated. Pure myelin was obtained at the interface between the two sucrose layers.

M.7 Acetone Precipitation of Proteins

A four to five fold excess of cold acetone (-20°C) was added to the protein solution and the samples were left at -20°C for an hour or more. The tubes were spun in an eppendorf centrifuge at 4°C for 5 min. The acetone was poured off and the pellets allowed to drain before evaporating to

dryness in a vacuum desiccator and resuspending in fresh medium.

M.8 Protein Determination

Protein determination was carried out using the Biorad protein assay procedure. For the standard assay, dilutions of BSA from 0.2 to 1.4mg/ml of protein were prepared in protein buffer. 50 μ l of these standards and appropriately diluted samples were placed in disposable 3ml plastic cuvettes along with a 'blank' consisting of the protein buffer alone. 2.5ml of diluted and filtered dye reagent was added, and the samples were mixed by inversion. After 5 mins the absorbance at 595nm was read from a Cecil spectrophotometer. The protein concentration of the samples was determined after construction of a standard curve .

Smaller quantities (1-20 μ g) of protein could be assayed using the microassay procedure. Standards of BSA containing 1-25 μ g of protein were prepared. 0.8ml of samples and standards were placed in disposable plastic microcuvettes (1ml), and 200 μ l of filtered concentrated dye reagent was added. The protein concentrations were then determined as for the standard assay procedure above.

M.9 Enzymatic Iodination of Cell Surface Membranes

This method for labelling the cell surface with ¹²⁵Iodine was adapted from that described by Darribere et al (1982). The main variation in the technique is the amount of label used for each batch of embryos. The iodination procedure was normally carried out in the fume-hood of the

radioisotope room.

Xenopus laevis embryos were dejellied, sorted and staged. The vitelline membranes were removed using watchmakers forceps, and the embryos placed in a single well of a 96 well disposable microtitre plate. 10 embryos were used for each sample and these were incubated in 125 μ l of either Barth X containing 5mM glucose if the embryos were to remain intact, or in calcium and magnesium free Barth X medium with 5mM glucose if the embryos were to be disaggregated.

Embryos were left for a sufficient period at room temperature to allow disaggregation to occur (1-6 hr). Control intact embryos were used to indicate the stage of development reached before labelling. 5 μ g of glucose oxidase (Sigma, 228 units/mg), 50 μ g lactoperoxidase (Sigma, 87 units/ μ g) and 100uCi of Na¹²⁵I (Amersham, 100mCi/ml) were added to the microtitre well. The embryos were left to label at room temperature for 30 min and then washed gently in a large excess (15ml) of Barth X medium made 150mM in unlabelled KI. The embryos were homogenised in SDS-page gel sample buffer or IEF lysis buffer (see Methods M.12 and M.13) in a 0.1ml glass homogeniser, or were fixed in Bouins fixative for wax embedding (see Method M.32).

M.10 ³⁵S-Methionine Labelling of Membrane Proteins

Embryos were collected, staged and dejellied. Each embryo in a batch of 200 was microinjected with 40nl of ³⁵S-methionine (15mCi/ml). Embryos were then incubated in 1/10 Barth X solution, at 18°C for 2 hr before staging.

Embryos were rinsed in crude membrane homogenisation buffer and a crude membrane preparation was carried out (see Method M.3).

M.11 Scintillation Counting of Radioactive Proteins

1 μ l volumes of radioactive protein samples were spotted onto small discs of Whatman No. 1 paper. The paper was washed first in ice cold 5% TCA and then in 5% TCA at room temperature for 15 min each. The papers were rinsed briefly in ethanol and in acetone before air drying. The papers were counted using Ready-solv EP aqueous scintillation fluid. This method was carried out in duplicate.

M.12 SDS Polyacrylamide Gel Electrophoresis (SDS-page)

A modified version of that proposed by Laemmli (1970) was used for the running of SDS-polyacrylamide gels. Protein samples were dissolved in sample buffer comprising 60mM Tris.HCl pH 8.8, 2% SDS, 5% β -mercaptoethanol, 10% glycerol and 0.001% Bromophenol blue. The samples were then boiled for 5 min and spun in an eppendorf centrifuge for 2 min before loading onto an SDS-page gel.

A 12X high bis gel was poured if the gel was later to be blotted onto nitrocellulose. This comprised 12% acrylamide, 0.32% bis-acrylamide, 370mM Tris.HCl pH 8.8, 0.1% SDS, and the gel was polymerised by the addition of 0.1% APS and 0.02% Temed. If the gel was to be stained or dried down for autoradiography a 15X low bis gel was poured. This comprised 15% acrylamide, 0.075% bis-acrylamide, 370mM Tris.HCl pH 8.8, 0.1% SDS, and was polymerised with 0.1% APS,

0.2% Temed. In both cases the stacking gel used was 3% acrylamide, 0.15% bis-acrylamide, 125mM Tris.HCl pH 6.8, 0.1% SDS and was polymerised by the addition of 0.1% APS and 0.05% Temed.

The running buffer used was 28.8g/litre glycine, 8g/litre Tris base and 1g/litre SDS. The gels were run overnight at 8mA each. When the dye-front had reached within a centimetre of the bottom, the gels were taken off and fixed in a solution of 10% glacial acetic acid, 45% methanol.

M.13 Isoelectric Focussing Gel Electrophoresis (IEF)

The method used for the running of isoelectric focussing gels is an adaptation of that first described by O'Farrell (1975). Gel tubing of internal diameter 2.5mm (Fisons) was cut into 13cm lengths using a diamond pencil. The ends of the tubing were flamed to remove the sharp edges and one end was sealed with Nescofilm. The gel solution was prepared as follows:

- 9.2M ultra pure urea
- 2% Nonidet P40
- 4% acrylamide
- 0.23% bis-acrylamide
- 0.67% ampholines pH 3.5-10
- 1.34% ampholines pH 5-7

5ml of solution was mixed for each of 8 tubes to be poured. 0.012% APS and 0.14% Temed were added and the mixture was poured into the tubes using a piece of thin plastic

tubing attached to the end of a plastic syringe. A Hamilton syringe was then used to remove the gel solution above a prepared mark of 11cm and the gels were overlain with 8M urea before leaving them to set for 1 hr at 37°C. The Nescofilm was removed from the tubes and the 8M urea replaced with 20 μ l of lysis buffer (9.5M ultra pure urea, 2% NP-40, 0.67% ampholines pH 3.5-10, 1.34% ampholines pH 5-7, 5% β -mercaptoethanol). The gels were pre-run at constant voltage for 15 min at 200V, 30 min at 300V and 30 min at 400V. The cathode buffer used was 0.02M NaOH, and the anode buffer was 0.01M orthophosphoric acid.

Protein samples were prepared in lysis buffer and were spun for 2 min before loading 50 μ l volumes onto the gels. 50 μ l of lysis buffer was loaded onto a control gel to enable determination of the pH range. The NaOH cathode solution buffer was replaced with fresh solution and the gels were electrophoresed for 19hr at 400V. This ensured that an equilibrium pH gradient had been established.

Gels were removed from their tubing by wrapping in foil and breaking the glass gently in a vice. The gels were stored frozen at -20°C in 5 ml each of equilibration buffer (70mM Tris.HCl pH 6.8, 2% SDS, 5% β -mercaptoethanol). The pH gradient gel was cut into 1cm pieces. These were shaken in 2ml each of degassed distilled water for 15 min and the pH determined using a pH electrode.

For the second dimension, 15% SDS-page gels were prepared as normal but without the use of a slot former in the stacking gel. The stacking gel was poured flat and the tube gels were sealed on top of the stacker using a solution

of agarose/Bromophenol blue (1% agarose, 72mM Tris.HCl pH 6.8, 0.2% SDS, 0.002% Bromophenol blue). The SDS page gels were then run as for normal 1-dimensional gels.

M.14 Nephge Gel Electrophoresis

The use of Nephge (Non-equilibrium pH gradient gel electrophoresis) as a variant on IEF gels was developed by O'Farrell et al (1977). The gels were set up and run essentially as for IEF gels (Method M.13) but with certain exceptions. 2% ampholines pH range 3.5-10 were used and the tube gels were polymerised using 0.28% Temed and 0.024% APS. The tube gels were overlain with water rather than 8M urea used for IEF gels. Nephge gels were run at reverse polarity to IEF gels and the samples were loaded onto the anode of the gel. The tube gels were electrophoresed at 400V for 4-5 hrs only without pre-running.

M.15 Western Blotting of Acrylamide Gels

Protein samples were run on a 12% high bis polyacrylamide gel at 8mA overnight. Western blotting onto nitrocellulose (Burnette et al. 1981) was carried out using the Biorad Trans-blot apparatus.

The gel and nitrocellulose were pre-equilibrated in blotting buffer comprising 150mM glycine, 20mM Tris base and 20% methanol. Electrophoresis was carried out at a current of 0.25 mA (60-70V) for 5-7 hr. After blotting the nitrocellulose could be stained with Ponceau S (5mg/ml in 5% TCA). Alternatively, the nitrocellulose was cut into strips and screened for antibody binding.

M.16 Protein Molecular Weight Markers

A solution of the following proteins was prepared in SDS-page sample buffer (Method M.12) at a concentration of 2mg/ml each.

<u>Protein</u>	<u>Mr</u>
cytochrome C	12,400
myoglobin	17,800
gamma-globulin	25,000 50,000
ovalbumin	45,000
bovine serum albumin	68,000
transferrin	77,000
B-galactosidase	130,000

The solution was boiled for 5 min and stored in aliquots at -20°C. 2µl was sufficient for use on silver-stained gels, 25µl was used on Coomassie Blue stained gels. When radioactive protein samples were run, the following ¹⁴C methylated protein mixture (Amersham) was used:

<u>Protein</u>	<u>Mr</u>
lysosyme	14,300
carbonic anhydrase	30,000
ovalbumin	45,000
bovine serum albumin	68,000
myosin	200,000

M.17 Silver-Staining of Acrylamide Gels

SDS-page gels were fixed and washed thoroughly in 3

changes of 50% methanol. It was important to be sure that all traces of acetic acid had been removed since the acid inhibits the staining procedure. The following solutions were then prepared: (A) 0.8g of AgNO_3 in 4ml of distilled water and (B) 21ml of 0.36% NaOH and 1ml of 35% NH_3 solution. Solution (A) was added dropwise to solution (B) whilst stirring and the final volume was made up to 100ml. This solution was used within 5 min.

The gel was incubated in the staining solution for 15 min. It was washed thoroughly for 5 min in 2 changes of distilled water and the developing solution added. This comprised 2.5ml of 1% citric acid, 0.25ml of 37% formaldehyde in a total volume of 500ml of distilled water. The stained bands developed within 5 min. The gel was then placed into 50% methanol to inhibit further development of the bands and was photographed.

M.18 Photography of Gels

Stained gels were photographed on a light box using Kodak ASA-32 Panatomic X film. This was developed for 5 min using Acutol developer diluted 1+9 and fixed for 5 min using Kodak Unifix diluted 1+3.

M.19 Autoradiography of Radioactive Gels

Radioactive gels were dried down and autoradiographed at -70°C , using Fuji X-ray film. Intensifying screens were used for gels containing ^{125}I iodine labelled proteins.

M.20 Fluorography of Protein Gels

Polyacrylamide gels containing ^{35}S radioactively labelled proteins were silver-stained (Method M.17) and photographed (Method M.18) before fluorography. Each gel was shaken in 3 changes of DMSO for 30 min each followed by an incubation of 2 hr 30 min in 20% (w/v) PPO in DMSO. The gels were then left under running water for 30 min before incubating overnight in 3% glycerol, 30% methanol to prevent shattering of the gel when drying. The gels were dried at 80°C for 90 min and autoradiographed at -70°C using Fuji X-ray film.

M.21 Alkaline Phosphatase Enzyme Assay

This enzyme was used as a marker for plasma membranes. It proved straightforward to assay using the method of Ribot *et al* (1983). 1ml of 5mM MgCl_2 , 5mM p-nitrophenol phosphate (PNP), 50mM Tris.HCl pH 10.5 was placed in a disposable spectrophotometer cuvette and 50 μl of enzyme sample was added. The cuvette was mixed by inversion and the increase in absorbance at 402nm was followed over a period of 10-20 min in a Cecil spectrophotometer. The amount of product formed was calculated using the value of $18.4\text{mM}^{-1}\text{cm}^{-1}$ for the extinction coefficient of PNP at 402nm.

M.22 Na/K ATPase Enzyme Assay

This enzyme was used as a marker for plasma membranes. It proved more difficult to assay than the alkaline phosphatase enzyme. The method used was described by Ribot *et al* (1983). A buffer was prepared containing 3mM

MgCl₂, 130mM NaCl, 20mM KCl, 3.3mM ATP, 50mM Hepes pH 7.2. 0.5ml of this buffer, with and without the addition of 1mM Ouabain was mixed with 50-100 μ l of enzyme solution diluted in the buffer. The mixture was incubated for 10 min at 37^oC, then assayed for the presence of inorganic phosphate as outlined below (Marsh, 1959).

Two solutions were prepared: an acid molybdate solution containing 50ml of concentrated hydrochloric acid and a solution of 20g sodium molybdate dihydrate in water mixed and diluted to 500ml, and a citrate solution containing 100g of sodium citrate dihydrate diluted to 500ml after adjustment of the pH to 7.0 with HCl. The enzyme mixture was transferred to a plastic universal tube. 10ml each of water and butanol were added followed by 1ml of acid molybdate solution. The tube was capped and shaken vigorously for 5-10 sec. 2ml of citrate solution was added along with a further 2ml of water. The flask was again capped and shaken for 5-10 sec. 3ml of the upper phase was removed and transferred to a 1cm silica cuvette. 20 μ l of methanol was added to remove turbidity and the absorbance at 310nm was measured against a blank prepared as above but containing no phosphate.

The amount of phosphate present was determined from the construction of a standard curve. The activity of the Na/K ATPase was determined from the difference in the phosphate production of the enzyme solution in the presence and absence of ouabain.

M.23 Succinate Dehydrogenase Enzyme Assay

This enzyme was used as a marker for mitochondrial

membranes. It was assayed by the method of King (1987). The following reagents were prepared: 200mM phosphate buffer, pH 7.4 (made 1mM in potassium cyanide to inhibit the action of cytochrome oxidase); 3mM EDTA, pH 7.4; 600mM succinic acid, pH 7.4 with NaOH; 0.53mM 2,6-Dichlorophenolindophenol (DCIP); and enzyme solution suitably diluted in 200mM phosphate buffer, pH 7.4.

1.5ml of phosphate buffer, 0.3ml EDTA, 0.3ml DCIP, 0.1ml succinate and 0.75ml of water were placed in a disposable 1ml cuvette. The reaction was started by the addition of 50 μ l of enzyme solution and the decrease in absorption at 600nm was followed for 5-10 min in a Cecil spectrophotometer. The amount of product formed was calculated using a value of 21mM⁻¹ cm⁻¹ for the extinction coefficient of DCIP at 600nm.

M.24 Mouse Immunisation by Injection

The normal protocol used for immunising mice was as follows: DAY 1, 30 μ g of protein in 300 μ l of 1:1 PBS/complete Freund's adjuvant was injected interperitoneally into each of 2 Balb/c mice. DAY 15, 30 μ g of protein in 300 μ l of 1:1 PBS/incomplete Freund's adjuvant injected interperitoneally into each mouse. DAY 24, a tail vein boost of approximately 30 μ g of protein in 100 μ l of PBS per mouse. Monoclonal antibody fusion was carried out 4 days following the tail vein boost.

M.25 Mouse Immunisation by Nitrocellulose Implantation

This method for mouse immunisation was used to

raise polyclonal sera against P37 and P47 proteins (Chapter 4). It was adapted from the method described by Smith et al (1988).

A Xenopus laevis stage 12 embryo homogenate was solubilised in SDS-page sample buffer and run on a 12% high bis acrylamide gel as normal. The gel was heavily loaded with protein, i.e 1/2 embryo was loaded onto each track of a 12 track gel. After running, one track of the gel was silver-stained and the remaining gel was blotted onto nitrocellulose using the normal Western blotting protocol (Method M.15). The nitrocellulose was stained with Ponceau-S (0.4g of Ponceau-S in 1 litre of dw) for 10 min to visualise the proteins. Through comparison with the silver-stained track and with previous autoradiographed iodine labelled gels the correct bands were determined and cut out of the nitrocellulose as a strip about 3mm wide. The nitrocellulose strips were rinsed thoroughly in sterile PBS to remove the Ponceau-S stain and were given a final rinse in ethanol. Strips of the nitrocellulose about 2cm long were rolled into a spiral and implanted subcutaneously into the neck region of 2 Balb/c mice. 5 weeks following immunisation the mice sera were for tested for activity on embryo sections and on Western blots.

M.26 Cell Fusion and Tissue Culture

Cell fusion was carried out using the methods of Galfre et al. (1977). The following solutions were obtained and placed in a 37°C water bath: RPMI-1640 serum free medium (Gibco); RPMI-1640 medium, comprising RPMI and glutamine, 10%

foetal calf serum, 0.01 mg/ml Penicillin, 0.01mg/ml Streptomycin; RPMI-1640 2xHAT medium (Littlefield, 1964); PEG solution comprising 50% w/v PEG.1000 in RPMI serum free medium.

The spleen from a previously immunised mouse was aseptically removed and rinsed briefly in alcohol. The spleen was cut open and the cells washed into 15mls of RPMI serum free medium. The cell suspension was mixed by pipetting up and down with a 10ml sterile pipette. The suspension was transferred to a sterile universal tube and NS1 myeloma cells were added (approximately 10^7 NS1 cells were used with each spleen which comprised about 10^8 cells). The universal tube was spun for 7 min at speed 3 in a bench-top centrifuge. The supernatant was discarded and the pellet gently resuspended and warmed to 37°C. 0.8ml of PEG solution was added slowly and the suspension was incubated for 1 min at 37°C. 1ml of serum free RPMI was added to the suspension followed by an additional 20ml gently added over a 5 min period to dilute the PEG. The universal was spun for 15 min at room temperature at speed 3 in the bench-top centrifuge.

The supernatant was poured off and the pellet gently resuspended in the liquid that remained. 50ml of HAT free medium was added very gently and the suspension plated out into five 96 well plates, 0.1ml per well using sterile pasteur pipettes. The plates were then placed in a 37°C 5% CO₂ incubator. The following day 0.1ml of 2xHAT medium was added to each well. The plates were replaced in the incubator and left for about 2 weeks until colonies started to appear in the base of the wells. The medium was tested from wells

with cell growth on Western blots or sections. Positive colonies were then grown up through 24 well and 12 well plates into small tissue culture flasks. Cells were cloned by the dilution method.

M.27 Antibody Screening on Acrylamide Sections

This method is adapted from that described in Hausen and Dreyer, (1981). *Xenopus* embryos were fixed overnight at 4°C in 2% TCA in Barth X. The embryos were transferred into embedding acrylamide (8.4% acrylamide, 0.0134% bis and 0.7% Temed in PBS) for 5 hr to allow time for the acrylamide to impregnate the embryonic tissues.

Acrylamide bases were prepared by adding 10 μ l of 10% APS to 10ml of embedding acrylamide in a 9cm plastic petri dish. These bases were left to set at 4°C until needed. 10 μ l of 10% APS was added to 10ml of embedding acrylamide and this was poured over a prepared base. Embryos or embedding tissue were oriented in this medium before it set. The petri dishes were then placed at 4°C overnight to allow the polymerisation of the acrylamide to complete. The position of the embryos in the acrylamide was marked on the base of the petri dishes with a marker pen before plunging the dishes into a beaker of isopentane cooled in liquid nitrogen for 3 min. The blocks were then taped up and stored in a dry container at -20°C.

10 μ m embryo sections were cut from the blocks on a cryostat and picked up on clean microscope slides subbed in a solution of 0.1% gelatin (300 Bloom, Sigma). The sections were post-fixed in acetone for 10 min then stored in a dry

slide box at -20°C . Embryo sections were outlined with a black marker pen. $25\mu\text{l}$ of 1% BSA in PBS was added to the sections and left for 30 min to prevent non-specific binding. The BSA was removed and $25\mu\text{l}$ of antibody was added to each section and left to incubate for 30 min. The slides were washed for 3×5 min in PBS and the sections were incubated in $25\mu\text{l}$ of second antibody. This was either FITC conjugated RAM-IgG, or Rhodamine conjugated RAM-IgG, both were used diluted 1/50 in PBS. The slides were left to incubate for 30 min, then washed for 3×5 min in PBS and mounted using a solution of 50% glycerol/PBS. The slides were examined using a Nikon epi-fluorescent microscope.

M.28 Antibody Screening of Western Blots

The nitrocellulose blot was blocked in 2% milk powder (Cadbury's Marvel) in PBS at 4°C overnight. The strips of nitrocellulose were incubated in monoclonal antibody supernatant for 2 hr at room temperature. The filters were rinsed briefly in distilled water before washing twice for 10 min each in PBS. They were then incubated for 1 hr at room temperature in HRP-RAM (horse-radish peroxidase linked rabbit anti-mouse IgG, Sigma) diluted 1/300 in PBS. The filters were rinsed briefly in distilled water then washed twice for a period of 10 min each in PBS containing 0.05% Tween 20 (BDH). The strips were rinsed briefly in TBS (0.5M NaCl, 20mM Tris pH 7.4-7.5) to eliminate the presence of phosphate which inhibits the staining reaction. The colour developing solution was prepared by mixing a solution of 30mg 4-chloro-1-naphthol (Sigma) in 10ml AR grade methanol with a

solution of 30 μ l hydrogen peroxide (30%, Sigma) in 50ml TBS. This solution was added immediately to the nitrocellulose filters. Bands were visible after 5 min and the strips could be left overnight at 4°C if necessary to enhance the signal.

M.29 Immunoprecipitation

Immunoprecipitation of proteins was carried out on ice using detergent mix buffer (100mM Tris.HCl, pH 7.95, 1% v/v Triton-X100, 0.5% SDS, 5mM MgCl₂, 100mM KCl, 0.05% sodium deoxycholate, 1mM PMSF). This buffer was prepared fresh each time before use.

The labelled protein sample was homogenised in buffer comprising 100mM NaCl, 20mM Tris.HCl pH 7.4, 1% Triton-X100, 1mM PMSF. The homogenate was then spun in an eppendorf centrifuge for 1 min and the pellet discarded. The supernatant was divided into 50 μ l or 100 μ l fractions. 450 μ l of detergent mix buffer and 10 μ l of suitably concentrated antibody solution was added to each of these aliquots. The mixture was left on ice for 30 min. 80 μ l of a 10% suspension of formalin fixed Staphylococcus aureus envelopes (prepared by S. Bhamra and D. Jackson) was then added. These envelopes were prewashed twice before use in detergent mix buffer and added to the mixture to absorb the antigen/antibody complex. The mixture was left overnight at 4°C on a rotating platform. It was then spun for 20 sec in an eppendorf centrifuge and the pellet was washed 3 times by resuspension in detergent mix buffer followed by centrifugation as before. The washed pellet was then resuspended in SDS-page sample and analysed on a 1-dimensional gel.

On some occasions 10 μ l of RAM-IgG was added after the first antibody and the mixture left on ice for a second incubation of 30 min. This amplifies the signal maximising the reaction of the antigen/antibody complex with the Staphylococcus A envelopes.

M.30 Treatment of Sections with Neuraminidase

This method was described in Slack (1985).

Acrylamide embryo sections are outlined with a marker pen as for normal antibody screening. The sections were incubated with 1mU of Neuraminidase (Sigma) per section overnight in a covered box at 37°C. The neuraminidase was diluted in 50mM Na-acetate pH 5.5, 80mM NaCl, 5mM CaCl₂ at a concentration of 1mU/25 μ l. Control sections incubated in the buffer alone and without any overnight incubation were used. The sections were then screened as normal for antibody binding (Method M.27).

M.31 Ouchterlony Immunodiffusion Test

1.875g of agarose was dissolved in distilled water by heating and allowed to cool slightly. 1g of NaCl and 0.125g of Na-azide was dissolved in distilled water separately and added to the agarose solution. The volume was made up to 125ml and this solution was stored in 8ml volumes at 4°C until needed.

The agarose solution was warmed until molten and 4ml volumes were poured onto glass microscope slide on a flat levelled surface and allowed to set. Holes were punched out of the agarose in a hexagonal design using the end of a pasteur pipette (6 holes around the vertices of the hexagon and one

hole in the centre). 8 μ l quantities of the antibody solutions were added to the wells and the slides were placed in a damp box for one day at 4°C to allow for diffusion to occur. At this stage cross-reaction is visible between some wells. To preserve the slides they were washed for 1 hr in PBS then rinsed in distilled water overnight. The slides were covered with Whatman No. 50 filter paper and placed in a 60°C oven for 2 hr to dry. The paper was gently peeled off and the slides stained in 5.4% acetic acid, 0.72% Na-acetate, 10% glycerol, 0.1% Amido Schwartz stain, for 10 min. The slides were then destained for 2 hr in 5% acetic acid and photographed as necessary.

M.32 Wax Embedding and Sectioning of Embryos

Embryos were fixed in Bouins fixative (71% picric acid, 24% formaldehyde, 5% glacial acetic acid) for 15 min at 4°C (Capco and Jeffery, 1981). The embryos were then dehydrated through a series of ethanol washes (30%, 50%, 70%, 80%, 90%, 95% and absolute ethanol) at 4°C for 15 min each, then warmed to room temperature in fresh absolute ethanol for 20 min. The embryos were then subjected to the following series of incubations: 1:1 ethanol:toluene for 20 min at room temperature, toluene twice for 20 min each at room temperature, paraplast saturated toluene for 40 min at 60°C and 3 changes of paraplast at 60°C for 1 hr each.

The embryos were embedded in paraplast (a paraffin wax mp. 58°C) in a watch glass. Embryo sections were cut using a microtome at 8 μ m thickness. The sections were floated on distilled water on subbed microscope slides and dried on a

hot plate at 40°C for two days. Slides were subbed (Gall and Pardue, 1970) by dipping them in 0.5% gelatin (300 Bloom), 0.05% chrome alum and allowing them to air dry, before baking at 80°C for 8 hr to ensure effective retention of the sections on the slides.

Sections were rehydrated by two changes of toluene (15 min each), two of absolute ethanol (5 min each) and then washing rapidly in 95%, 90%, 80%, 70%, 50%, 30% ethanol and two changes of sterile distilled water.

M.33 Histoautoradiography of Radioactive Wax Sections

Throughout this method it was important to ensure no activation of the photographic emulsion by light or background radiation. With the Ilford L4 emulsion the Ilford 902 safelight could be used.

Samples of photographic emulsion (Kodak NTB2 or Ilford L4) were prepared in the darkroom by diluting the stock 1:1 with distilled water, dividing into 10ml volumes and storing these in scintillation vials each wrapped separately in foil. These vials were placed in a box containing cotton wool and the box itself wrapped in foil. The emulsion was then stored at 4°C until use.

To dip the slides 1 bottle of emulsion was removed and warmed to 46°C in a water bath for 15-20 min. The dipping chamber used was a slide storage container of volume about 15ml. The level of the emulsion in this container could be elevated using blank slides. The dipping chamber was also warmed in the waterbath and the rehydrated slides were placed on a slide warming tray at 40°C. The dipping chamber was

filled with the warmed emulsion and the slides were dipped for 10 sec each, allowed to drain for 10 sec each then placed vertically in slide drying racks for 2 hr to dry. The slides were placed in black slide boxes with molecular sieve dessicant and the boxes were sealed carefully with black tape and stored at 4°C.

Control slides were developed at intervals of 1 day, 2 days, 4 days and a week to check for correct exposure of the emulsion. The slides were developed in copling jars using Kodak D19 developer for 5 min, washed briefly in deionised water and fixed in Kodak Unifix diluted 1+3 for 5 min. The slides were then washed under running water for 30 min and rinsed in distilled water. The sections were dehydrated and stained using the following series of incubations: 70% ethanol for 5 min, 70% ethanol, 0.1% light green, for 5 min, 70% ethanol for 1 min, 80% ethanol for 5 min and toluene for 5 min. The slides were then mounted using a mixture of DPX mountant and toluene.

CHAPTER 3

RESULTS: CHARACTERISATION OF PURIFIED MEMBRANE PROTEINS

3.1 Introduction

One of the aims of this thesis is to investigate the role of plasma membrane proteins in the development of *Xenopus laevis* embryos. The initial approach adopted was to look for membrane proteins that showed variation in their level of expression in the membrane at different stages of *Xenopus* development. Membrane proteins that undergo temporal variation are thought more likely to be directly involved in the processes of development. In the work described in this chapter, different purification methods were used to isolate membrane proteins from *Xenopus laevis* embryos. Membrane proteins obtained from different stages of *Xenopus* embryos were then compared on SDS-page and IEF gels.

Very little previous analysis of membrane protein composition in *Xenopus laevis* eggs and embryos has been carried out. Richter used SDS-page to analyse manually dissected cortices of *Xenopus laevis* eggs, embryos and oocytes (Richter, 1980; Richter and Tintschl, 1983). Wolf *et al* (1978) analysed the vitelline membranes from *Xenopus laevis* eggs using SDS-page and Yurewicz *et al* (1975) used agarose gel and cellulose acetate electrophoresis to analyse the composition of the *Xenopus laevis* egg jelly coat. The work described in this chapter extends these preliminary studies and enables a more thorough analysis to be made of the range of membrane proteins present, and of membrane

protein variation which occurs during *Xenopus* early development.

3.2 Purification of Crude Membrane from *Xenopus laevis* Embryos

The first method used to purify membrane proteins from *Xenopus* embryos involved a crude extraction of embryo membranes on a discontinuous sucrose gradient (Method M.3). This method had the advantage of being quick and straightforward to carry out. A crude membrane extraction was carried out on 5 stages of *Xenopus laevis* embryos from unfertilised eggs through to stage 14 neurulae. The membranes were run on a 1-dimensional SDS-page gel which was silver-stained (Methods M.12 and M.17). The results are shown in Figure 1.

One of the major difficulties in purifying membranes from *Xenopus* embryos is the problem of contamination by yolk. This is evident in these samples by the presence of the major yolk protein lipovitellin at 120,000 Mr (A), (see Richter, 1980). The second major yolk protein, phosvitin (Mr 34,000) does not appear to stain strongly with silver-stain and appears on this gel as a negatively stained region (V). Later attempts to eliminate this residual yolk contamination failed because increasing the length of the initial centrifugation step to remove the last traces of yolk also caused the membrane to pellet resulting in very low yields. It was therefore necessary to balance the recovery of a reasonable amount of membrane against a small amount of residual yolk contamination using

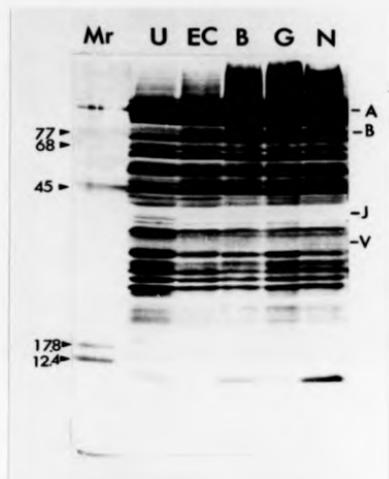


Figure 1 1-dimensional SDS-page of Crude Membrane from
Xenopus laevis Embryos at Different Stages of Development

A crude membrane preparation was carried out on 300 *Xenopus laevis* embryos at 5 stages of early development. The crude membrane pellets were solubilised in SDS-page sample buffer, and 50 μ l samples (about 50 μ g protein) were run on a 15% low bis SDS-page gel. The gel was silver-stained.

The tracks are as follows: Mr, molecular weight markers; U, unfertilised eggs; EC, stage 3-4 early cleavage embryos; B, stage 8 blastula embryos; G, stage 10 gastrula embryos; N, stage 14-15 neurula embryos.

the crude membrane technique.

Analysis of Figure 1 indicates two proteins showing possible temporal variation. The first is protein B (77,000 Mr) which is more abundant in the neurula track than for the four earlier stages. It is not possible to determine whether protein B is present in the earlier stage fractions because of the limited resolution of the gel. The second protein (J) has a molecular weight of around 40,000 and is present in the unfertilised track but is absent at later stages. Overall, the crude membrane protein profile is similar over all the 5 stages studied with approximately 40 protein bands being resolved.

Crude membrane fractions prepared from *Xenopus* embryos as described above for Figure 1 were analysed using 2-dimensional IEF gels (see Method M.13). The gels were silver-stained and the results are shown in Figures 2 and 3. Figure 2 shows the early cleavage embryo stage gel. The pH range on this gel varies between 4.9 and 6.5, but the majority of the spots lie in the region from pH 5.0 to 6.1 and from 25,000 to 140,000 Mr. For clarity only this region from the gels in this experiment has been shown in Figure 3.

Crude membrane proteins from 5 stages of *Xenopus* embryos are shown on IEF gels in Figure 3. The gels are presented with the same pH gradient scale and the figure gives an indication of the variation seen on 2-dimensional gels when running similar samples. Analysis of the gels is possible by lining up repetitive marker spots. Comparison with published data (Brock and Reeves, 1978; Bravo and Knowland, 1979; Ballantine et al. 1979) indicates that

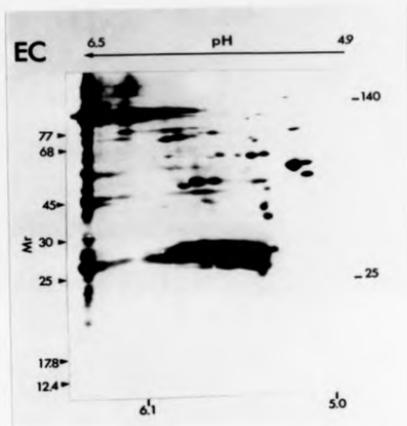


Figure 2 2-dimensional IEF Gel of Crude Membrane Extracts
from Stage 3-4 Xenopus laevis Embryos

A crude membrane preparation was carried out on 300 stage 3-4 *Xenopus laevis* embryos. The crude membrane pellet was solubilized in IEF lysis buffer and a 50 μ l sample (approximately 150 μ g of protein) was loaded onto an IEF tube gel. A control gel was run for pH determination, and molecular weight markers were run in a slot beside the tube gel in the second dimension. The gel was silver-stained.

protein 4 (55,000 Mr) is tubulin, a cytoskeletal protein forming the main component of microtubules. It is less straightforward to identify the spot representing actin (42,000 Mr) which is present in the embryo as a constituent of microfilaments. Actin is known to be synthesised in *Xenopus* at all stages from oocyte to mature frog (Bravo and Knowland, 1979). Both actin and tubulin are thought to be present in these membrane preparations as contaminants through cytoskeletal/membrane interactions. Comparison with published data (Brock and Reeves, 1978; Bravo and Knowland, 1979; Ballantine *et al.*, 1979) indicates that the pI of actin is around 5.4. This would suggest that spot 8 is the actin spot. However, comparison of the gels in Figure 3 with a later gel showing the total embryo homogenate (TH gel in Figure 7) shows that spot 8 appears as a very minor protein whereas actin is known to be a major protein in the embryo (Ballantine *et al.*, 1979). The other possible actin spot is 20 which is a major spot in the TH gel in Figure 7. This protein has an apparent molecular weight on these gels of 46,000 which is slightly higher than the molecular weight of actin. Confirmation of the true actin spot could be obtained by running purified actin in a sample of the embryo homogenate.

The contaminating yolk protein lipovitellin is seen in these gels as a heavily stained region (50) at 120,000 Mr and pI around 8.0. The unfertilised stage was not analysed in this experiment so it is not possible to look for protein I found on the 1-dimensional gel in Figure 1. The other protein showing variation in intensity in Figure 1 was protein B of molecular weight 77,000. Protein 5 has this molecular weight

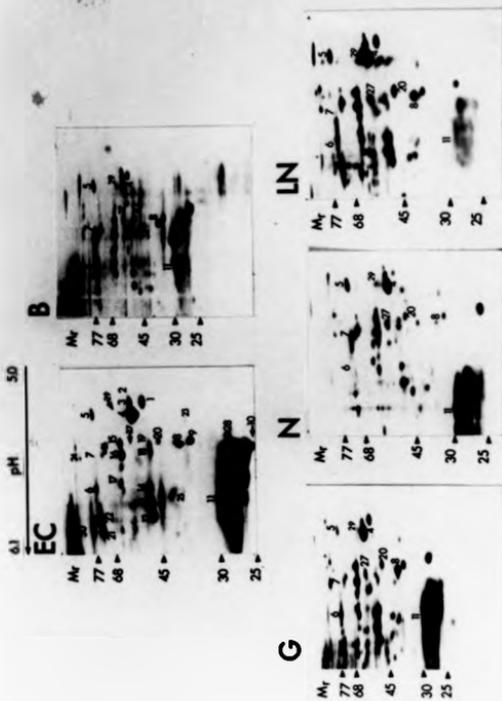


Figure 3 2-dimensional IEF Gels of Crude Membrane Extracts
from Xenopus laevis Embryos at Different Stages of
Development

A crude membrane preparation was carried out on 300 Xenopus laevis embryos at 5 stages of early development. The crude membrane pellets were solubilised in IEF lysis buffer and 50 μ l samples (approximately 150 μ g of protein) were loaded onto IEF tube gels. A control gel was run for pH determination, and molecular weight markers were run in slots beside the tube gels in the second dimension. The gels were silver-stained. For clarity only the central region of the gels is shown.

The gels are as follows: EC, stage 3-4 early cleavage embryos; B, stage 8 blastula embryos; G, stage 10 gastrula embryos; N, stage 14 early neurula embryos; LN, stage 22-23 late neurula embryos.

but is present in all the stages studied here. This protein does, however, appear to increase in abundance with developmental stage. Without further experimentation it is not possible to confirm that proteins B and 5 are the same. The other proteins of molecular weight around 77,000 are 6 and 7, these also show an unusual pattern of expression. 6 is present at early cleavage, is very minor in the blastula, reappears as a major spot in the gastrula, is absent in the early neurula and is again present as a major spot in the late neurula sample. 7 shows an approximately opposite pattern of expression, i.e when 6 is present 7 is absent and vice versa. Comparison with other gels shows that these spots can appear as up to 8 distinct spots of similar pI, but with the same molecular weight (see Figure 7). One explanation for this is that the spots indicate the same protein that carries a chemical group with a varying pI value causing it to migrate a different distance in the first dimension of the gel. For example the spots could represent a single glycoprotein species carrying a variable carbohydrate domain. Which of these spots are present does not seem to be developmentally determined and can be different in, for example, two early cleavage stage crude membrane gels. It is difficult to be sure of the explanation for the pattern of expression seen with these spots. No other protein of molecular weight 77,000 is present showing the same temporal variation of protein B. One explanation for this absence is that the pI of protein B lies outside the pH range obtained in the tube gel and hence that B was lost, or was not resolved in the first dimension gel. It was for this reason

that Nephge gels were run (described later in this chapter).

The heavily stained region 11 varies in its horizontal position across the gel. This region stains yellow with the silver-stain compared with the normal brown colour of most of the spots and this most likely to indicate lipids present in the gel (Dzandu *et al.* 1984, 1985). Further analysis of these and other crude membrane gels (not shown) revealed the presence of only one protein which shows consistent temporal variation in its expression on gels of crude membrane samples. This is protein 27 of molecular weight 55,000 which is absent in the early cleavage gels, becomes faintly visible in the blastula, and thereafter is present in the gels as a major spot. This pattern of expression is consistent with the protein being expressed for the first time following the mid-blastula transition. Protein 27 is of approximately the same molecular weight as tubulin, spot 4. This could explain why no variation in expression of protein 27 is seen on the 1-dimensional gel in Figure 1 since both proteins would collocate to the same band.

3.3 Comparison of Nephge and IEF 2-dimensional Gels

The pH range obtained in the isoelectric focussing gels shown in Figure 2 was 4.9-6.5. This is normal for the concentration of ampholines used in the IEF gel. Many membrane proteins have an overall neutral or basic charge which enables them to span the lipid bilayer. Such proteins are of interest but are lost outside the pH range obtained in the IEF gels. Nephge gels are similar to IEF gels but differ in two major respects: firstly a wider range of ampholines is

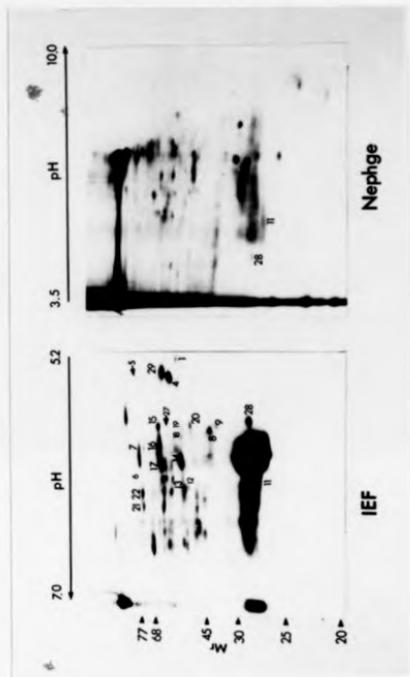


Figure 4 Comparison of IEF and Nephge 2-dimensional Gels

A crude membrane purification was carried out on 300 stage 4 *Xenopus laevis* embryos. The crude membrane pellets were solubilised either in IEF lysis buffer, or in Nephge gel lysis buffer and stored at -20°C until use. 50 μl samples (about 150 μg protein) were loaded onto the corresponding tube gels. The pH range of the IEF gel was determined from a control gel, and marker proteins were run in slots alongside both tube gels in the second dimension. The gels were silver-stained.

used in the tube gel thus extending the pH gradient in the first dimension, and secondly, the tube gels are not run to equilibrium which prevents the loss of basic proteins outside the pH range of ampholines used. The overall effect of using Nephge gels should therefore be to increase the number of proteins that can be detected using the 2-dimensional gel technique.

A comparison of Nephge gels (Method M.14) and IEF gels was carried out using crude membrane samples from *Xenopus* early cleavage embryos. The gels were silver-stained and are shown in Figure 4. The IEF gel is less highly resolved than the gels shown in Figure 3 but the pH gradient is wider. However, the overall resolution of proteins in the IEF gel is still preferable to that in the Nephge gel. Direct comparison between the two gels is difficult. In Figure 4 the position of spot 28 has been marked on the Nephge gel. Apart from the large heavily stained area 11, spot 28 is the only spot that could be identified with certainty on both gels. The IEF gel confirms that spot 27 is absent (which agrees with the findings from Figure 3), spot 7 is present and 6 is absent.

Nephge gels have been used previously to study ³⁵S-methionine labelled proteins in *Xenopus* embryos (Smith and Knowland, 1984). Their data shows Nephge gels which are more highly resolved than the gel in Figure 4. However, in comparison to their IEF gels run with the same protein samples, the Nephge gels are again less highly resolved. Further attempts to improve on the Nephge gel technique were no more successful. It was concluded that the Nephge gel

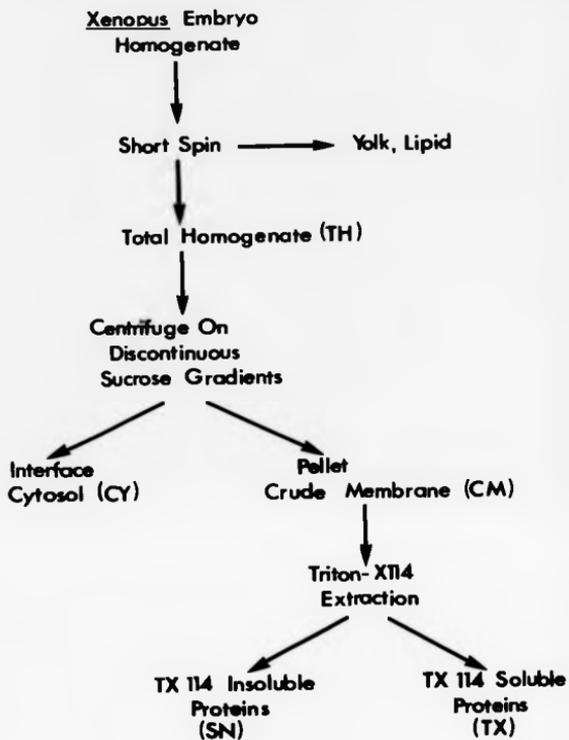


Figure 5 Experimental Protocol for the Crude Membrane
Purification and Triton-X114 Extraction

See Methods M.3 and M.4.

technique resulted in low resolution gels which were difficult to compare with the IEF gels. For this reasons all subsequent 2-dimensional gel analysis was carried out using the original IEF technique.

3.4 Crude Membrane Separation and TX114 Extraction

The crude membrane purification method separated membranes on a discontinuous sucrose gradient following an initial slow spin to remove yolk. Proteins seen on gels from the crude membrane fractions comprised a mixture of integral membrane proteins, surface bound membrane proteins and membrane associated proteins. It is possible to fractionate crude membrane proteins further into integral and non integral membrane proteins using the non-ionic detergent Triton-X114 (Bordier, 1981; see Method M.4). A summary of the separation of proteins using the crude membrane and TX114 methods is shown in Figure 5.

A crude membrane purification was carried out on stage 8 *Xenopus laevis* embryos. This was followed by a TX114 extraction on the crude membrane pellets. Samples from each stage of the fractionation were collected and analysed on an SDS-page gel (Figure 6) and on 2-dimensional IEF gels (Figure 7). The crude membrane track in Figure 6 shows the same protein profile as that seen in the tracks in Figure 1. Proteins B and J are absent or very faint, and the large yolk band A is present as before. The total homogenate (TH) is fractionated into cytosol (CY) and crude membrane (CM, see Figure 5). Of the proteins A, C, D, E, H, L and N present in the total homogenate, H and L fractionate mainly into the

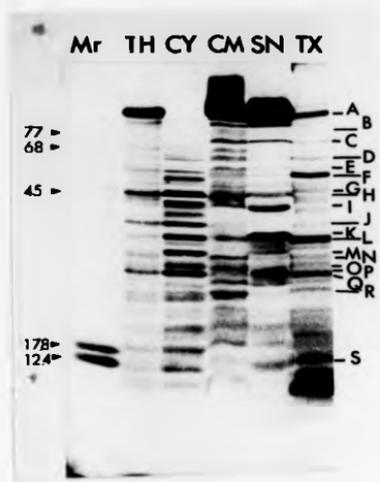


Figure 6 Crude Membrane and TX114 Purification Samples
Analysed on 1-dimensional Gels

A crude membrane purification and Triton-X114 extraction were carried out on 1000 stage 10 Xenopus laevis embryos (see Figure 5). Samples from 5 stages in the purification were taken as follows: TH, total embryo homogenate after a 2 sec spin to remove yolk; CY, cytosolic proteins collected at the interface of the sucrose layers in the crude membrane purification; CM, crude membrane pellet; SN, TX114 insoluble proteins; TX, TX114 soluble proteins. The CY, SN and TX fractions were acetone precipitated. 50 μ g samples were solubilised in SDS-sample buffer and run on a 15% low bis SDS-page gel. The gel was silver-stained.

cytosol, whereas proteins A, C, D, E and N are seen in the crude membrane fraction. Very few protein bands are seen in both the cytosol and the crude membrane tracks. Those that are could represent more than one protein species colocalizing in a single band. This indicates that a good separation of embryo proteins is obtained using the crude membrane technique.

The TX114 method separates crude membrane proteins into Triton insoluble proteins (also called Triton supernatant, SN) and Triton soluble proteins (TX). The major proteins in the SN track are A, C, H, I, K and O. Of these proteins A and C are also present in the TX track but to a far lesser extent, and proteins H, I and K appear specific to the SN track. The major bands in the TX track are A, F, J, L and P. Of these A, F and P are also seen in the SN track whereas J and L are specific to the SN track. These results indicate that crude membrane proteins are being fractionated using the TX114 technique.

Figure 7 shows samples from the crude membrane fractionation and TX114 purification analysed on 2-dimensional IEF gels. Comparison of the CM gel and the CY gel indicates substantial differences in the proteins present on each. The CY gel is difficult to analyse and the overall resolution of the gel is low. This was found for all examples of cytosol preparations run on IEF gels and the reasons for this remain unclear. Spot 4 (tubulin) and spot 57 (which may be actin) are present as major proteins. The other major proteins 37, 51 and 52 are more difficult to relate to spots in the TH and CM gels. 37 is possibly 31, although it appears

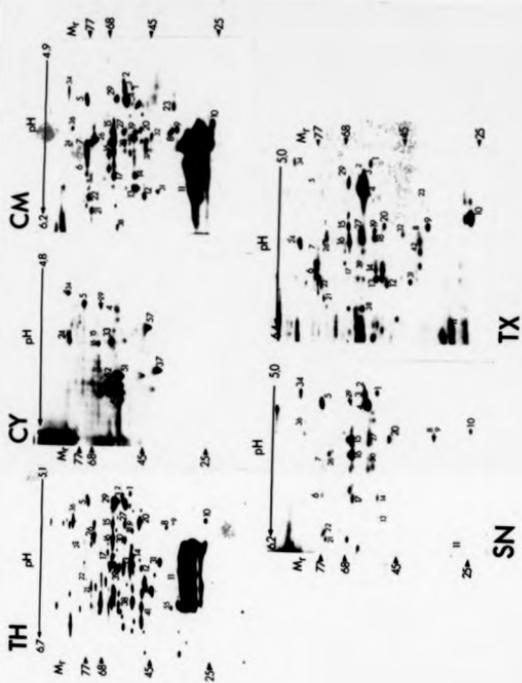


Figure 7 Crude Membrane and TX114 Purification Samples
Analysed on IEF Gels

Samples from a crude membrane and TX114 fractionation of stage 10 *Xenopus laevis* embryos were prepared as described in the legend to Figure 6 (see also Figure 5). The samples were solubilised in IEF gel lysis buffer and 50 μ l volumes were run on IEF tube gels. A control tube gel was run for pH measurement, and molecular weight markers were run alongside the IEF gels in the second dimension. The gels were silver-stained. The gels are as follows: TH, total embryo homogenate; CY, cytosolic proteins; CM, crude membrane pellet; SN, TX114 insoluble proteins; TX, TX114 soluble proteins.

at slightly higher molecular weight. 51 and 52 are in the region of the 39 group of spots in the TH gel but it is not possible to be certain which spots are which. Spot 33 in the CY gel does not appear to relate to any spot in the TH gel. However, the 15, 16, 17 group of spots is present in the CY gel as are spots 5 and 24. These are all also seen in the CM gel.

Protein 4 (tubulin) is present in the TH and CM gels as a major spot but appears much fainter in the CY gel. It is also present as a major protein in the SN and TX gels. Several spots seen in the TH and CM gels are absent in the CY gel, examples include 8, 9, 10, 11, 21, 22 and 29. In the CY gel spot 24 is much stronger than that seen in the CM gel. Some spots e.g. 5, the 15, 16, 17 group, and 34 are seen in both the cytosol and crude membrane gels. These are likely either to be contaminants picked up during the running of the gels, or proteins which are weakly associated with the membrane. Examples would include cytoskeletal components and indeed tubulin (spot 4) is seen in both the CM and CY gels. These results are consistent with the suggestion that the crude membrane separation results in a substantial, but not total, fractionation of embryo proteins.

In comparison with the CM and CY gels, the SN and TX gels show far greater similarities. Spots 8, 9, 12 and 29 present in the CM and TX gels are absent in the SN gel. Similarly spots 30 and 36 are present in the CM and SN gels but not the TX gel. A large number of spots however, are found in both the TX and SN gels, e.g. 1, 2, 3, 4, 10, 13, 14, the 15, 16, 17 group, 20 and others. The TX114 extraction

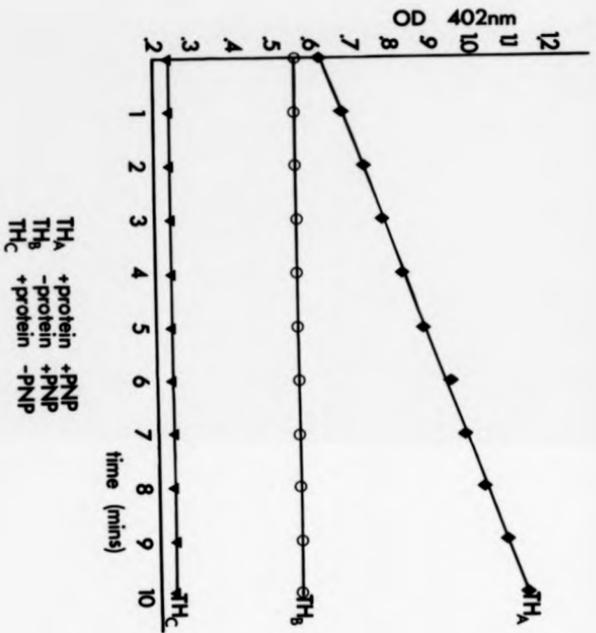


Figure 8 Illustration of Enzyme Marker Assays

Enzyme assays for alkaline phosphatase and succinate dehydrogenase were carried out on protein samples as described in Methods M.21 and M.23. This figure illustrates possible results obtained for the alkaline phosphatase enzyme. The procedure used for the succinate dehydrogenase enzyme is similar.

Sample A is analysed in the presence of the chromophore (PNP) and the protein mixture (TH; Total embryo homogenate). Samples B and C are controls for background variation. B is analysed in the absence of protein and C in the absence of the chromophore. Any variation in the optical density readings from samples B and C is taken into account when calculating the specific activity of sample A. The specific activity of the protein sample is calculated using the formula:

$$SA = OD' / (E \times C)$$

where SA is the specific activity of the protein sample in nMoles min⁻¹ mg⁻¹; OD' is the change in OD per minute in sample A after taking into account variation in samples B and C; E is the extinction coefficient of the chromophore at the wavelength used in mM⁻¹ cm⁻¹; and C is the concentration of protein in the sample assayed, in mg⁻¹ ml⁻¹.

appears to result in an effective fractionation of crude membrane proteins. However this fractionation is not total and some proteins are present in both the Triton-X114 soluble and insoluble phases.

3.5 Enzyme Marker Assays

The experiments resulting in Figures 6 and 7 confirmed that the crude membrane purification and Triton-X114 extraction techniques were effective at fractionating Xenopus embryo proteins. These experiments do not show whether the methods were effective at separating membrane from other cellular constituents. It is possible to look more closely at the fractionation of the embryo proteins by following the separation of sub-cellular markers. The markers used for this study were the plasma membrane enzymes alkaline phosphatase and Na/K ATPase, and the mitochondrial membrane enzyme succinate dehydrogenase. These marker enzymes were chosen in preference to all others because they had previously been used to assay membrane preparations from Xenopus laevis embryos (Bretzel *et al.* 1986). It was therefore thought that these enzymes should be active in Xenopus laevis embryo homogenates. The Na/K ATPase proved a very difficult enzyme to assay. Despite the fact that the enzyme had previously been detected in Xenopus laevis embryos (Bretzel *et al.* 1986), no activity of this enzyme in any of the samples could be found.

Figure 8 shows an example of the results obtained when assaying the alkaline phosphatase enzyme (see Methods M.21 and M.23). The enzyme assays were carried out in a

SUCCINATE DEHYDROGENASE

	Specific Activity †	Percentage of Max *	Purification Factor †
TH	18.9	11.3	1
CY	16.9	10.1	0.9
CM	11.5	6.9	0.6
SN	0.44	0.3	0.02
TX	167	100	8.8

Table 1 Succinate Dehydrogenase Assays on Samples from a
Crude Membrane and Triton-X114 Purification

A crude membrane purification and Triton-X114 extraction were carried out on stage 14 *Xenopus laevis* embryos. Samples from the fractionation were collected at 5 stages (see Figure 6), and Succinate Dehydrogenase assays were carried out as described in Method M.23. The samples are as described in the legend to Figure 6.

The first column (↓) indicates the specific activity of the sample in nMoles of product formed per mg protein present per min; the second column (↓) indicates the value of the specific activity as a percentage of the maximum; the third column (▼) indicates the degree of purification over the total homogenate starting material.

ALKALINE PHOSPHATASE

	Specific Activity †	Percentage of Max *	Purification Factor ‡
TH	2.8	0.6	1
CY	2.4	0.5	0.9
CM	24.9	5.2	8.9
SN	2.1	0.4	0.7
TX	480	100	171

Table 2 Alkaline Phosphatase Assays on Samples from a Crude Membrane and Triton-X114 Purification

A crude membrane purification and Triton-X114 extraction were carried out on stage 14 *Xenopus laevis* embryos. Samples from the fractionation were collected at 5 stages and Alkaline Phosphatase assays were carried out as described in Method M.21. The samples are as described in the legend to Figure 6.

The first column (\dagger) indicates the specific activity of the sample in nMoles of product formed per mg protein present per min; the second column (\dagger) indicates the value of the specific activity as a percentage of the maximum; the third column (\heartsuit) indicates the degree of purification over the total homogenate starting material.

similar manner for both alkaline phosphatase and succinate dehydrogenase and the calculation of specific activity as shown in Figure 8 is the same for both. Crude membrane preparations and TX114 extractions were carried out on stage 14 Xenopus laevis embryos. Samples from the fractionations were collected at each stage (see Figure 5) and were assayed for succinate dehydrogenase and alkaline phosphatase activity as shown in Figure 8. Table 1 shows a summary of the results obtained for succinate dehydrogenase. A 0.9 fold purification of the enzyme is seen in the cytosol compared with a 0.6 fold purification in the crude membrane. This suggests that mitochondria are fractionating mainly into the cytosol, but that there is a substantial contamination of mitochondria in the crude membrane fraction. A 0.02 fold purification for the enzyme over the embryo homogenate is seen in the SN fraction which is very low compared with the 8.8 fold purification of the enzyme in the TX fraction. This indicates that the succinate dehydrogenase enzyme, which is an integral protein of the inner mitochondrial membrane is highly soluble in the Triton-X114 detergent.

Table 2 shows the results obtained for the alkaline phosphatase enzyme assays. Nearly a 9 fold purification of this enzyme is seen in the crude membrane compared with 0.9 fold purification for the cytosol fraction. This suggests that the fractionation of the plasma membrane enzyme is mainly into the crude membrane. The activity of the enzyme in the TX fraction is very high, showing a 171 fold purification over the embryo homogenate compared with the 0.7 fold purification of the TX114 insoluble proteins. If the

Table 3 Comparison of Data from Enzyme Marker Assays

A summary of the results of the enzyme assays carried out on crude membrane and Triton-X114 soluble proteins from Xenopus embryos is shown alongside results using alternative membrane purification methods. TH indicates the specific activity of the enzyme in the initial sample homogenate; Mem indicates the specific activity of the enzyme in the purified membrane; and P_i indicates the purification factor and is the ratio of the specific activity in the membrane to the specific activity in the total homogenate. Specific activity is given in nMoles of product formed per mg protein per min. The samples are as follows:

1. Crude membrane proteins from Xenopus stage 14 embryos. Results from Tables 1 and 2.
2. Triton-X114 soluble proteins. Results from Tables 1 and 2.
3. Membrane purified from fertilised sea urchin eggs (Ribot et al. 1983) using sucrose gradient fractionation.
4. Membrane purified from sea urchin embryos by isolating cell surface membranes on polycationic beads (Helmly and Brown, 1987).
5. Plasma membrane purified from Xenopus laevis gastrula embryos using sucrose gradient fractionation (Bretzel et al. 1986).
6. Plasma membrane purified from Xenopus laevis neurulae (stage 14) using the method of Bretzel et al. (1986).
7. Purified succinate dehydrogenase enzyme from heart muscle (King, 1967)

purification of the alkaline phosphatase enzyme in the TX fraction over the CM fraction (19 fold) is compared with that of succinate dehydrogenase (15 fold) then clearly the values are fairly similar. This indicates that the Triton-X114 extraction is effective at fractionating both these integral membrane proteins enabling an approximately 15-20 fold purification of the proteins over the crude membrane samples.

Table 3 shows a summary of published data on the levels of these two enzymes in membrane samples. The values obtained for the membrane samples (shown in Tables 1 and 2) are in good agreement with other membrane purification methods. The exception is the data from Ribot *et al* (1983) which is generally lower than all the other data by a factor of around 50. This suggests a possible confusion with units.

3.6 Temporal Variation in TX114 Soluble and Insoluble Proteins

A crude membrane fractionation and Triton-X114 extraction was carried out on five stages of *Xenopus laevis* embryos. Samples from the purification were run on SDS-page and IEF gels. Figure 9 shows TX114 insoluble proteins (SN) run on a 1-dimensional SDS-page gel which was silver-stained. The overall profile of bands seen is similar for all stages studied. Band J (Mr 40,000) is present in the unfertilised track and is absent at all later stages. This band also showed this variation in the 1-dimensional gel of crude membrane samples (Figure 1). Band G (45,000 Mr) appears in the early cleavage track and becomes stronger at later stages and band H (48,000 Mr) is very strong in the unfertilised

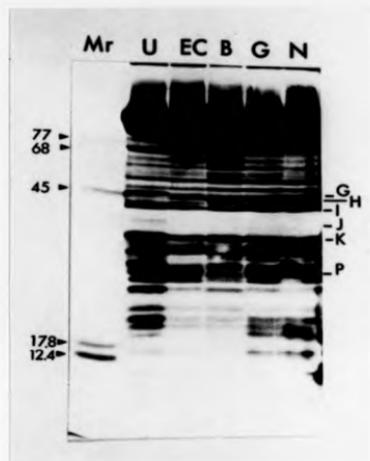


Figure 9 1-dimensional SDS-page of TX114 Insoluble Protein
Samples from Xenopus laevis Embryos at Different Stages of
Development

A crude membrane purification and TX114 extraction were carried out on 500 *Xenopus laevis* embryos at 5 stages of early development. The TX114 insoluble protein fractions were acetone precipitated and solubilised in SDS-page sample buffer. 50 μ l samples (approximately 50 μ g protein) were run on a 15% low bis gel. The gel was silver-stained.

The tracks are as follows: Mr, molecular weight markers; U, unfertilised eggs; EC, stage 4 early cleavage embryos; B, stage 8 blastula embryos; G, stage 10 gastrula embryos; N, stage 14 neurula embryos.

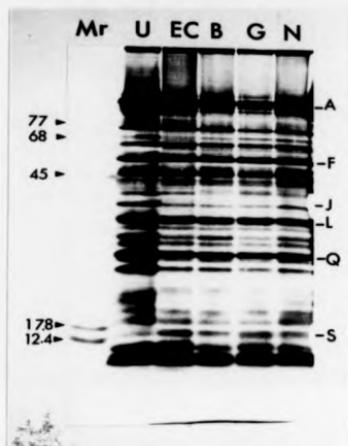


Figure 10 1-dimensional SDS-page of TX114 Soluble Protein
Samples from Xenopus laevis Embryos at Different Stages of
Development

A crude membrane purification and TX114 extraction were carried out on 500 *Xenopus laevis* embryos at 5 stages of early development. The TX114 soluble proteins were acetone precipitated and solubilised in SDS-page sample buffer. 50 μ l volumes (approximately 50 μ g protein) of the samples were run on a 15% low bis gel. The gel was silver-stained.

The tracks are as follows: Mr, molecular weight markers; U, unfertilised eggs; EC, stage 4 early cleavage embryos; B, stage 8 blastula embryos; G, stage 10 gastrula embryos; N, stage 14 neurula embryos.

track and very weak thereafter. Band P (around 30,000 Mr) shows considerable variation in the strength of signal in the different stages and appears much stronger in the gastrula and neurula stages. This could represent an enhancement of the protein level from the low maternal level to a higher zygotic level. Figure 10 shows the corresponding stages of TX114 soluble proteins run on a 1-dimensional SDS-page gel. The unfertilised egg track in this gel shows a slightly higher protein loading than the other tracks. Apart from this there are no detectable differences in the protein profiles of the TX114 soluble proteins over the 5 stages shown.

Figure 11 shows TX114 soluble proteins from 5 stages of Xenopus early development run on 2-dimensional IEF gels. The protein profiles of these gels are highly resolved and are very similar. Close analysis of the gels has indicated the presence of only two differences between the stages which are reproducible in similar experiments. These are the proteins marked 45 (45A and 45B, Mr 24,000) which are seen in the unfertilised gel and are present very faintly in the early cleavage gel but are absent thereafter. Protein 27 (which was seen in the 2-dimensional gels of the crude membrane samples as absent in the early cleavage stage), is present in all of these TX114 gels (see gel B) including the unfertilised egg stage. The reason for this discrepancy remains unclear.

3.7 Purification of Plasma Membrane from Xenopus Embryos using Discontinuous Sucrose Gradients

The crude membrane purification method resulted in

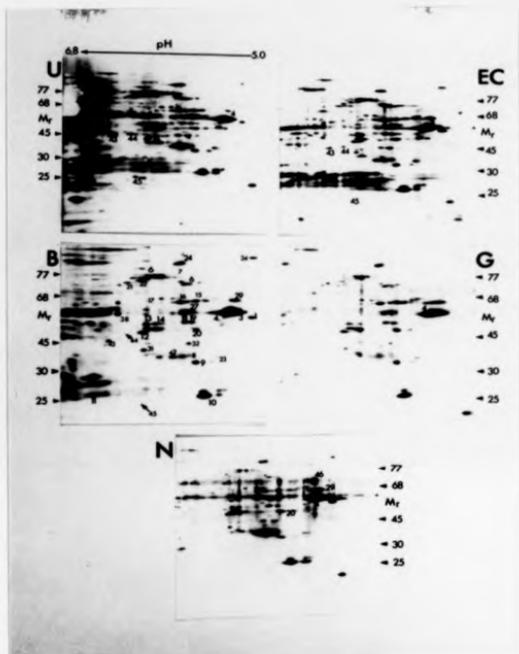


Figure 11 2-dimensional IEF Gels of TX114 Soluble Protein
Samples from Xenopus laevis Embryos

A crude membrane purification and TX114 extraction were carried out on 1000 *Xenopus laevis* embryos at 5 stages of early development. The TX114 soluble proteins were acetone precipitated and solubilised in IEF gel lysis buffer. 50 μ l volumes (approximately 150 μ g protein) were loaded onto IEF tube gels. A control gel was run for pH measurement, and molecular weight markers were run alongside the tube gels in the second dimension. The gels were silver-stained.

The gels are as follows: U, unfertilised eggs; EC, stage 4 early cleavage embryos; B, stage 8 blastula embryos; G, stage 10 gastrula embryos; N, stage 14 neurula embryos.

a mixture of all cellular membrane material. This was confirmed by the results of the succinate dehydrogenase assay which indicated that mitochondria were present in the membrane fractions to a significant extent. More information could be obtained on the nature of the *Xenopus* embryo cell surface during development using a membrane purification method which isolated plasma membrane from other cellular constituents. The method chosen to purify plasma membranes from *Xenopus laevis* embryos was that described by Bretzel *et al* (1986). This method is described fully elsewhere (see Method M.5) and a summary of the technique is shown in Figure 12.

A plasma membrane preparation was carried out on 5 stages of *Xenopus laevis* embryos. The membrane samples were analysed on a 1-dimensional SDS-page gel which is shown in Figure 13. There is a substantial amount of yolk contamination in the plasma membrane samples shown by the presence of lipovitellin (Band A). The resolution of this gel is only fair and the protein loading in the neurula track is lower than that in the other tracks which makes a detailed analysis of the gel more difficult. However two proteins showing variable expression are seen. These are proteins T and U. Protein T of molecular weight 72,000 is absent, or very weak in the unfertilised track but is much stronger in the fertilised embryo stages. Protein U (47,000 Mr) appears as a major band in the 4 earlier stages but the band is absent in the neurula track.

Samples from the plasma membrane purification were also analysed on 2-dimensional IEF gels shown in Figure 14.

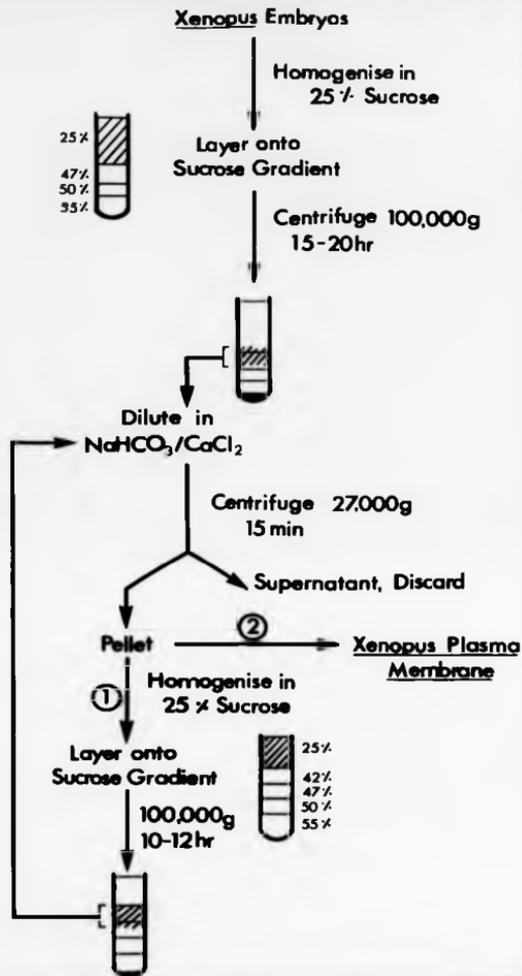


Figure 12 Experimental Protocol for the Purification of
Plasma Membrane from Xenopus laevis Embryos

See Method M. 5.

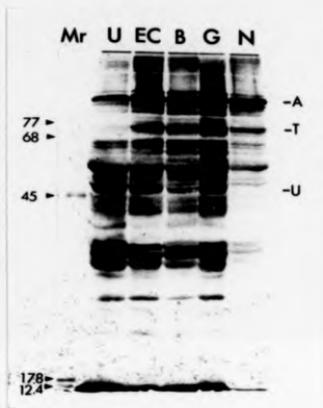


Figure 13 1-dimensional SDS-page of Plasma Membrane Samples
from Xenopus laevis Embryos

A Plasma membrane purification was carried out on 1000 Xenopus laevis embryos at 5 stages of early development. Extracts of the purified membrane were solubilised in SDS-page sample buffer to a concentration of $1\mu\text{g}/\mu\text{l}$. 50ul volumes were loaded onto a 15% low bis SDS-page gel. The gel was silver-stained.

The tracks are as follows: Mr, molecular weight markers; U, unfertilised eggs; EC, stage 3-4 early cleavage embryos; B, stage 8 blastula embryos; G, stage 14 gastrula embryos; N, stage 14 neurula embryos.

The initial appearance of these plasma membrane gels is similar to those previously shown for crude membrane and TX114 soluble proteins (Figures 3 and 11). In all of the plasma membrane gels in Figure 14 the lower 6 proteins in the 15, 16, 17 group are present but the upper 3 are absent. These are the first gels where these 3 spots have not been seen. Assuming that a purification of plasma membrane has been carried out, these missing proteins are likely to represent membrane proteins that are not present on the plasma membrane. The gels show that the 6-7 group of proteins are resolving here as at least 5 distinct spots with the same molecular weight. These proteins do not show any significant temporal variation. Spot 5 appears very weak in the unfertilised and early cleavage stage gel but is present as a strong spot in all the later stage gels. This spot was absent in the TX unfertilised egg IEF gel (see Figure 11). The group of spots around 48 and 49 of molecular weights around 40,000 is also seen to show some temporal variation. 48 and 49 are barely visible in the unfertilised and early cleavage stage gels but thereafter become progressively stronger. A second protein shows a similar pattern of expression on these gels. This is 47 (75,000 Mr) which is a major spot in the neurula gel, is visible in the gastrula, blastula and early cleavage gels but is absent in the unfertilised egg stage. The group of proteins around 28 (25,000-30,000 Mr) seen in the neurula gel as at least 9 distinct proteins, appears absent in the earlier stage gels. One more protein shows variation on these gels. This is 53 (55,000 Mr) which has an unpredictable pattern of expression. It is present as a very faint spot in

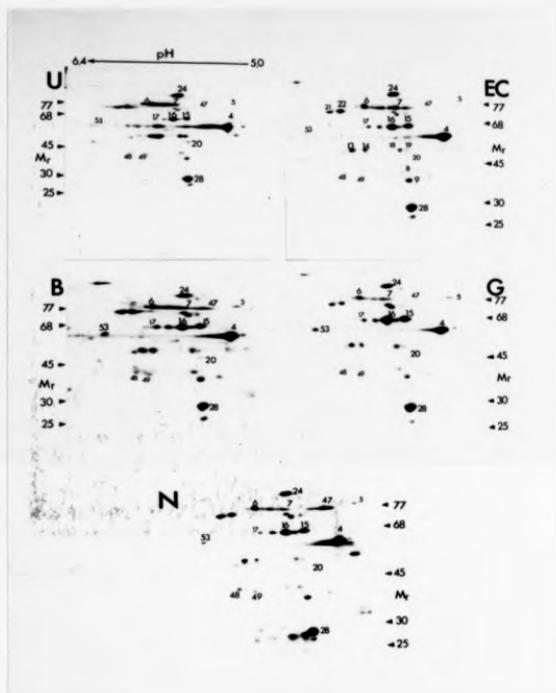


Figure 14 2-dimensional IEF Gels of Plasma Membrane Samples
from *Xenopus laevis* Embryos at Different Stages of
Development

A plasma membrane purification was carried out on 1000 *Xenopus laevis* embryos at 5 stages of early development. Extracts of the purified membrane were solubilised IEF lysis buffer to a concentration of 3 μ g/ μ l. 50 μ l volumes were loaded onto IEF tube gels. A control gel was run for pH determination, and molecular weight markers were run alongside the tube gels in the second dimension. The gels were silver-stained.

The gels are as follows: U, unfertilised eggs; EC, stage 3-4 early cleavage embryos; B, stage 8 blastula embryos; G, stage 10 gastrula embryos; N, stage 14 neurula embryos.

the unfertilised and early cleavage stage gels, is strong in the blastula and gastrula stage gels, and is weaker in the neurula stage gel.

3.8 Temporal Variation in Newly Synthesised Crude Membrane Proteins

In the work described so far biochemically purified embryo membranes analysed on gels were used to look for membrane proteins which show temporal variation during early development. An alternative approach was to look at membrane proteins in Xenopus embryos that are actively synthesised at each of the different stages of development (see Method M.10). Xenopus embryos were injected with ^{35}S -methionine and left for 2 hours to incorporate the label. A crude membrane preparation was then carried out and the samples were scintillation counted to determine the level of incorporation of the label. The samples were analysed on SDS-page and IEF gels. All embryo injections were carried out by Dr E.A. Jones. The results from this experiment are shown in Figures 15, 16 and 17.

Figure 15 shows the newly synthesised crude membrane proteins run on an SDS-page gel. Compared with the previous 1-dimensional gels (see Figures 1, 9 and 10) significant temporal variation is seen in the proteins in this figure. It is important to note that the lettering of the bands differs from that adopted previously. The bands d, g, h, j, k, l, m and n are all present to a greater extent in the late neurula track than in the earlier stage embryo tracks. Proteins a, c, e and o show an increase in signal

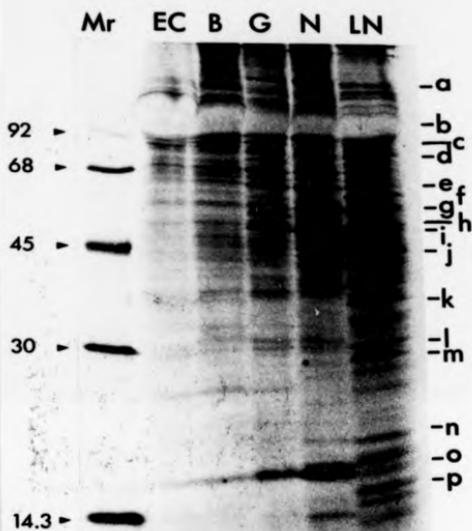


Figure 15 1-dimensional SDS-page of Newly Synthesised Crude Membrane Proteins from Xenopus laevis Embryos at Different Stages of Development

200 *Xenopus laevis* embryos for each of 5 stages in development were individually injected with ^{35}S -methionine. The embryos were left for 2 hours after injection then mixed with 200 unlabelled embryos and a crude membrane preparation was carried out. The crude membrane pellets were dissolved in SDS-page sample buffer, and the radioactivity in each sample was determined by TCA precipitation and scintillation counting. 150,000 cpm was loaded onto each track of a 15% low bis SDS-page gel. The gel was fluorographed, dried and autoradiographed.

The tracks are as follows: Mr, molecular weight markers; EC, early cleavage embryos labelled at stage 2, processed at stage 5; B, blastula embryos labelled at stage 6, processed at stage 8; G, gastrula embryos labelled at stage 10, processed at stage 11; N, neurula embryos labelled at stage 14, processed at stage 15; LN, late neurula embryos labelled at stage 22-23, processed at stage 23-24. Note that the lettering differs from the system used in previous figures.

Figure 16 Comparison of Total Crude Membrane Proteins and Newly Synthesised Crude Membrane Proteins from Xenopus laevis Embryos

³⁵S-methionine labelled crude membrane from stage 22 *Xenopus laevis* embryos was prepared as described in the legend to Figure 15. The crude membrane pellet was solubilised in IEF gel lysis buffer and the radioactivity in the sample determined by TCA precipitation and scintillation counting. A volume of the sample containing 280,000 cpm was diluted with lysis buffer to 50 μ l and loaded onto a IEF tube gel. A control gel was run for pH measurement, and ¹⁴C labelled molecular weight markers were run alongside the tube gel in the second dimension. The gel was silver-stained to show total protein, then fluorographed, dried and autoradiographed.

with stage up to stage 14 neurula, the signal falling again in the late neurula track. Protein i appears as a major band in the gastrula track and diminishes again in the two neurula tracks whereas proteins f and p are visible only in the late neurula track. A summary of the proteins showing temporal variation on this gel is given in Table 6.

Figures 16 and 17 show the newly synthesised crude membrane proteins run on 2-dimensional IEF gels. Note again that the numbering system used for the ^{35}S -autoradiographs differs from that adopted previously. Figure 16 shows the late neurula embryo stage gel and autoradiograph from this experiment. The crude membrane proteins being actively synthesised at this stage of development are very different to the overall range of proteins present in the crude membrane. The differences are so marked that there appears very little similarity between the gel and autoradiograph. The labelling system adopted for the autoradiographs has been used on the stained gel in this figure to allow comparison of the spots. Major spots in the autoradiograph appear often as minor or even absent in the stained gel, and vice-versa.

The autoradiographs in Figure 17 show crude membrane proteins from different stages of *Xenopus* embryos labelled with ^{35}S -methionine. The temporal variation seen in these labelled membrane proteins is significant, and a comparison of the early cleavage and late neurula stage gels shows little similarity. Only a small number of the spots remain constant over the 5 stages studied. The temporal variation of the proteins seen in these gels is extensive, and for clarity a summary has been produced in Table 7. It is

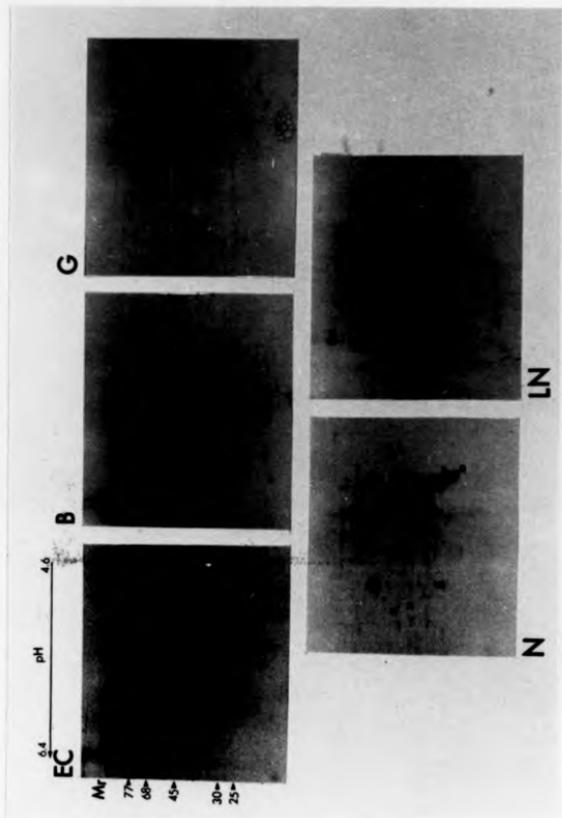


Figure 17 2-dimensional IEF gels of Newly Synthesised Crude Membrane Proteins from Xenopus laevis Embryos

³⁵S-methionine labelled crude membrane samples were prepared as described in the legend to Figure 15. The crude membrane pellets were solubilised in IEF lysis buffer and the radioactivity in the samples was determined by TCA precipitation and scintillation counting. Volumes of the samples containing 280,000 cpm were diluted with lysis buffer to 50 μ l and were loaded onto IEF tube gels. A control gel was run for pH measurement, and ¹⁴C labelled molecular weight markers were run alongside the tube gels in the second dimension. The gels were fluorographed then dried and autoradiographed.

The gels are as follows: EC, early cleavage embryos labelled at stage 2, processed at stage 5; B, blastula embryos labelled at stage 6, processed at stage 8; Gastrula embryos labelled at stage 10, processed at stage 11; N, neurula embryos labelled at stage 14, processed at stage 15; LN, late neurula embryo labelled at stage 22-23 processed at stage 23-24.

clear from both the 1-dimensional and 2-dimensional gel analyses that this last technique has proved the most successful at identifying membrane proteins showing temporal variation in Xenopus laevis embryos.

3.9 Conclusions

The aim of the work in this chapter was to look for plasma membrane proteins showing a reproducible pattern of temporal variation on 1-dimensional and 2-dimensional gels. Such proteins could be implicated as being important in the development of Xenopus laevis embryos. Proteins found to show temporal variation were summarised in Tables 4-7. Experiments carried out have also involved a study of the range of membrane proteins present in Xenopus embryos which enables comparison of the results with published data.

Richter (1980) used Coomassie Blue staining of 1-dimensional SDS-page gels to analyse his manually dissected cortices from Xenopus eggs and embryos. He noted up to 21 protein bands present in the cortex of the Xenopus unfertilised egg. The protein profile from this gel has been compared with that of the 1-dimensional gel of plasma membrane proteins from Xenopus eggs (Figure 13). The range of proteins detected using silver-staining is higher than that obtained using Coomassie Blue staining. Although there are similarities between the two protein tracks (e.g. Band T, 77,000 Mr appears as a major protein in both gels), a thorough analysis and comparison is difficult due to the different techniques used. No other previous work was found which analysed the composition of plasma membrane proteins in

Protein	M _r	Sample	U	EC	B	G	N
B	77	CM	○	○	○	○	S
		SN	○	○	○	○	○
		TX	○	○	○	○	○
		P	○	○	○	○	○
T	72	CM	S	S	S	S	S
		SN	S	S	S	S	S
		TX	S	S	S	S	S
		P	W	S	S	S	S
U	47	CM	S	S	S	S	S
		SN	S	S	S	S	S
		TX	S	S	S	S	S
		P	S	S	S	S	W
G	46	CM	S	S	S	S	S
		SN	S	W	W	W	W
		TX	S	W	W	W	W
		P	W	W	W	W	W
H	45	CM	S	S	S	S	S
		SN	○	W	S	S	S
		TX	W	W	W	W	W
		P	W	W	W	W	W
J	40	C	S	○	○	○	○
		SN	S	○	○	○	○
		TX	S	W	W	W	W
		P	S	W	W	W	○

Table 4 Membrane Proteins Showing Temporal Variation in Xenopus laevis Development Detected on 1-dimensional Stained Gels

This Table summarises results from Figures 1, 9, 10 and 13. Proteins are shown where inconsistent levels of expression were detected in membrane samples from different stages of Xenopus laevis embryos on SDS-page gels.

The abbreviations are as follows: Mr, protein molecular weight in 1000 Daltons; CM, crude membrane; SN, Triton-X114 insoluble proteins; TX, Triton-X114 soluble proteins; P, Plasma membrane. The embryo stages are as follows: U, Unfertilised eggs; EC, Early cleavage stage embryos; B, Blastulae embryos; G, Gastrulae embryos; N, Neurulae embryos; LN, Late neurulae.

o protein band absent

w protein band weak

s protein band strong

Protein	Mr		U	EC	B	G	N	LN
27	55000	TH	-	O	O	O	S	S
		CM	-	O	W	S	S	S
		TX	S	S	S	S	S	-
		PM	W	W	W	O	O	-
45 _{A+B}	23000	TH	-	O	O	O	O	O
		CM	-	O	O	O	O	O
		TX	S	W	O	O	O	-
		PM	O	O	O	O	O	-
5	77000	TH	-	W	W	W	S	S
		CM	-	S	S	S	S	S
		TX	O	W	O	O	W	-
		PM	W	W	S	S	S	-
48	40000	TH	-	O	O	O	O	O
		CM	-	O	O	O	O	O
		TX	O	O	O	O	O	-
		PM	W	W	S	S	S	-
47	76000	TH	-	O	O	O	O	O
		CM	-	O	O	O	O	O
		TX	O	O	O	O	O	-
		PM	O	W	W	W	S	-
53	56000	TH	-	O	O	O	O	O
		CM	-	O	O	O	O	O
		TX	O	O	S	S	S	-
		PM	O	O	S	S	W	-

Table 5 Membrane Proteins Showing Temporal Variation in
Xenopus laevis Development Detected on 2-dimensional Stained
Gels

This Table summarises results from Figures 3, 11, 14, 22 and 23. Proteins are shown where inconsistent levels of expression were detected in membrane samples from different stages of Xenopus laevis embryos on IEF gels. The abbreviations used are outlined in the legend to Table 4.

Protein	M _r	Stages Present
a	150	Signal Increases to N, Decreases in LN
b	120	Strong in EC, Decreases to LN
c	90	As a
d	80	Increasing Signal to LN
e	65	As a
f	60	Present Only in LN
g	55	As d
h	51	As d
i	50	Absent EC and B, Strong in G, Decreases in N and LN
j	42	As d
k	35	As f
l	30	As d
m	27	As d
n	20	Absent in EC, B and G ; increases N to LN
o	17	As a
p	11	As d

Table 6 Newly Synthesized Membrane Proteins Showing
Temporal Variation in Xenopus laevis Development Detected on
SDS-page Gels

This Table summarises results from Figure 15. Proteins are shown where inconsistent levels of expression were detected in ³⁵S-methionine labelled membrane samples from different stages of *Xenopus laevis* embryos analysed on SDS-page gels. The embryo stage abbreviations used are outlined in the legend to Table 4.

PROTEINS SHOWING INCREASED INTENSITY WITH INCREASING STAGE

PROTEIN	Mc	PROTEIN	Mc
4	69,000	31	45,000
13	71,000	32	88,000
15	78,000	33	44,000
19	83,000	35	50,000
21	27,000	39	53,000
22	25,000	40	44,000
23	33,000	42	83,000
30	40,000		

PROTEINS SHOWING DECREASE IN INTENSITY WITH INCREASING STAGE

PROTEIN	Mc	PROTEIN	Mc
5	41,000	18	44,000
6	48,000	28	36,000
9	81,000	36	31,000
10	45,000	37	42,000
11	46,000	38	30,000

PROTEINS SHOWING UNEVEN VARIATION IN INTENSITY WITH INCREASING STAGE

PROTEIN	Mc	EC	B	G	N	LN
7	35,000	w	w	s	vs	0
8	34,000	0	w	w	s	0
12	33,000	w	w	s	vs	0
26	45,000	0	0	s	w	s
29	32,000	0	0	0	s	0

Table 7 Newly Synthesised Membrane Proteins Showing
Temporal Variation in Xenopus laevis Development Detected on
IEF Gels

This Table summarises results from Figure 17. Proteins are shown where inconsistent levels of expression were detected in ^{35}S -methionine labelled membrane samples from different stages of Xenopus laevis embryos analysed on IEF gels. The embryo stage abbreviations used are outlined in the legend to Table 4.

Xenopus embryos using 1- or 2-dimensional gel techniques.

3.9a Membrane Protein Purification Techniques

Membrane proteins which are thought most likely to play an important role in the development of the Xenopus embryo are those on the plasma membrane. These proteins have the potential to react to external stimuli and to influence development through inter-cellular contact and communication. For this reason the technique used to purify crude membrane (see Figure 5) is of limited use in this study since the preparation results in a mixture of all cellular membranes. This was confirmed by the results of the enzyme assays. Results from the crude membrane technique are still of value provided it is realised that proteins showing temporal differences may not be cell surface proteins. The crude membrane technique should therefore be used in conjunction with other membrane purification techniques in the analysis of cellular membranes.

The detergent Triton-X114 was used to purify integral membrane proteins from crude membrane samples. The technique uses temperature changes to precipitate phase fractionation and membrane proteins are thus separated by their ability to form mixed micelles with TX114 (Bricker and Sherman, 1982). This technique has previously been shown to be effective at separating integral membrane proteins, and the nature of Triton-X114 as a 'mild' detergent is thought not to be overtly damaging to the proteins during the purification process (Bordier, 1981).

The integral membrane protein extraction relies on

the degree of solubility of each protein in TX114. It would not therefore be expected to provide a 100% fractionation of each protein between the Triton soluble and insoluble phases (TX and SN). This was clearly seen in the IEF gels of TX114 soluble and TX114 insoluble proteins (Figure 7) which showed that many proteins could be found in both samples. However the TX114 extraction was particularly effective for the enzyme markers succinate dehydrogenase and alkaline phosphatase which are both integral membrane proteins. An alternative method of separating integral and extrinsic membrane proteins from crude membrane samples uses carbonate at high pH to remove non-integral proteins. This method was attempted on several occasions and results were compared with those obtained using the Triton extraction. The TX114 method was deemed preferable in terms of resultant yield of protein, but the overall composition of protein in the two samples was very similar (results not shown).

Several techniques are available for the purification of plasma membrane from amphibian embryos. Grunz (1980) describes one such method which uses a heterobifunctional reagent (SPDP) to covalently link amino groups in membrane proteins to Thiopropyl-Sepharose 6B. Cell surface membrane can then be obtained after allowing for settling of the sepharose followed by washing and chemical reduction. This method was attempted for *Xenopus* embryos but very low membrane yields were obtained. There was also a problem with contamination of the membrane by cellular constituents if cell lysis occurred before the initial labelling step. For these reasons this method was not chosen

for the analysis described in this chapter.

A similar method in several respects to the SPDP method was described by Helmly and Brown (1987). Sea urchin embryo cell membranes can be separated using polycationic beads. In this method attachment of disaggregated embryo cells to the beads is followed by cell lysis, centrifugation and chemical reduction to reclaim the membrane. This method was not attempted due to the similarities with the SPDP method described above. Ribot *et al* (1983) describe a method for purifying plasma membrane from fertilised sea urchin eggs which uses discontinuous sucrose gradients to fractionate cellular constituents and to separate plasma membrane. This is an extension of a previous method described by Kinsey *et al* (1980) which was found to be ineffective for fertilised sea urchin eggs. This sucrose gradient fractionation was not attempted and the similar method described by Bretzel *et al* (1986) was chosen in preference primarily because it had been developed and tested on *Xenopus* embryos. It would be useful to compare the plasma membrane samples obtained from *Xenopus* embryos using these two techniques.

The plasma membrane purification method used in this chapter (Bretzel *et al*, 1986) proved more difficult to carry out than the crude membrane and TX114 extractions but is potentially more useful since it provides a sample of plasma membrane relatively free from contamination by other cellular membranes. This was confirmed by the results of enzyme assays on the plasma membrane samples (see Table 3) which showed the level of succinate dehydrogenase (a mitochondrial enzyme) in the membrane fraction was very low compared with that in the

embryo homogenate, whereas the level of alkaline phosphatase (a plasma membrane enzyme) was significantly higher.

Analysis of plasma membrane samples on gels showed the loss of certain spots present in the crude membrane and TX114 gels indicating increased purification of these samples. Proteins in the plasma membrane samples which show reproducible temporal variation are likely to be plasma membrane constituents, a fact which is not necessarily true for such proteins found in crude membrane and TX114 samples. The purification of plasma membrane proteins is clearly a valuable technique in comparing cell surface proteins present at different stages of *Xenopus* embryo development.

3.9b The Analysis of Newly Synthesised Membrane Proteins

By far the most significant results in terms of membrane proteins showing temporal variation were obtained from the analysis of newly synthesised membrane proteins using ³⁵S-methionine (Tables 6 and 7). A crude membrane purification was used to investigate newly synthesised membrane proteins since a significant level of the radioactive label in the plasma membrane would not be expected to be found after a period of only 2 hours. Leaving the embryos for a longer incorporation time has been shown to result in a discharge of the label from the embryo and a resulting low level of incorporation. The use of the crude membrane technique means, however, that there is no specificity for plasma membrane proteins in the samples.

Comparison of a silver-stained gel of crude membrane proteins with the corresponding ³⁵S autoradiograph

(see Figure 16) indicated significant variation in the total range of crude membrane proteins with those being actively synthesised. This analysis method is therefore more sensitive at detecting variation in the expression of membrane proteins at different stages of embryo development. This was confirmed by the large number of proteins seen to show temporal variation using this technique compared with the relatively small numbers detected using silver-staining. It is perhaps surprising that the majority of membrane proteins show temporal variation, and that very few proteins are synthesised at levels which remain constant over all the stages. Since the total analysis of ^{35}S -methionine labelled newly synthesised proteins has been carried out on only one occasion (although parts of the series have been duplicated) a complete repeat of this experiment is necessary to confirm that the temporal variation seen in these membrane proteins is reproducible.

3.9c Comparison of the Results with Published Data

No previous work has been found which used 1- and 2-dimensional gel techniques to investigate changes in the plasma membrane of Xenopus embryos during development.

^{125}I labelling has been used on other animal embryos to study cell surface changes during development, e.g. Drosophila (Woods et al., 1987) and Pleurodeles (Darribere et al., 1982). This will be discussed in more detail in Chapter 4 of this thesis.

^{35}S -methionine has been used on several occasions to study changes in total protein synthesis in Xenopus

embryos during development. Brock and Reeves (1978) investigated *de novo* protein synthesis in *Xenopus* embryos at six different stages of development from early cleavage to stage 28-32 tadpole. They described significant reproducible changes in the pattern of protein synthesis between each of the six stages studied. Bravo and Knowland (1979) used ^{35}S -methionine to study classes of proteins synthesized in oocytes, eggs, embryos and differentiated tissues of *Xenopus laevis*. They reported that the pattern of protein synthesis in *Xenopus* embryos changes quite substantially during early development at all stages from early cleavage to stage 28-32. Ballantine et al (1979) also analysed *de novo* protein synthesis in *Xenopus* embryos. They found relatively few sorts of abundant proteins that begin synthesis during early *Xenopus* development, and indicated that the bulk of the proteins made in the tailbud tadpole were also made in the oocyte. The first new proteins are seen at the gastrula stage and not before. These observations are consistent with those previously made for sea urchin embryos (Brandhorst, 1976) but are distinct from those of Brock and Reeves (1978) and Bravo and Knowland (1979) discussed above.

Analysis of the newly synthesised crude membrane results in Figures 15 and 17 indicate that there are few, if any, detectable proteins which appear in the blastula stage sample which were absent in the early cleavage stage sample. In contrast the gastrula stage sample shows several proteins not previously seen and the protein pattern changes again at neurula and late neurula. No analysis of newly synthesised crude membrane proteins in unfertilised eggs or oocytes was

carried out which would indicate which of the protein species are being synthesised for the first time following fertilisation. However, the results in this chapter appear consistent with the findings of Ballantine *et al* (1979). There are several reasons why different conclusions have been drawn from these experiments investigating protein synthesis in *Xenopus* embryos. One possibility are limitations in the reproducibility of IEF gels when analysing changes in protein expression. This will be discussed in more detail in Chapter 8.

CHAPTER 4

RESULTS: RADIOACTIVE LABELLING OF CELL SURFACE PROTEINS

4.1 Introduction

In the previous chapter, membrane protein purification methods and gel techniques were used to look for variation in membrane proteins present at different stages of Xenopus development. This approach was extended using 125 Iodine as a label for the Xenopus embryo cell surface.

Surface labelling has previously been used to study membrane protein distribution in embryos. Richter (1980) and Richter and Tintschl (1983) used labelling with 125 Iodine and with the non penetrating di-isocyanate derivative 3 H-DIDS to look at cell surface proteins in Xenopus oocyte and embryo membranes. No work was carried out to investigate changes in cell surface proteins at different stages of development. Darribere *et al* (1982) used surface labelling with 125 Iodine to look at cell surface changes in the early embryogenesis of Pleurodeles waltlii. They found that the detectable protein profile of membranes on the outside of the embryo did not change between early cleavage and late gastrula stages of development. Surface labelling of disaggregated embryos indicated seven proteins specific to internal cell membranes and the quantity of these proteins was found to vary during early development. The most significant differences in labelled proteins were detected at periods corresponding to extensive morphogenetic movement such as gastrulation. Surface labelling with 125 Iodine has also been used to

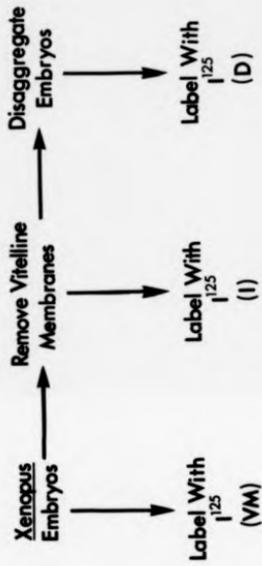


Figure 18 Experimental Protocol for the Surface labelling
of *Xenopus laevis* Embryos using ¹²⁵Iodine

Xenopus embryos were surface labelled with the vitelline membrane in place (VM), with the vitelline membrane removed (I) and after removal of the vitelline membrane and disaggregation of the embryo (D). See Method M. 9.

analyse variation in the cell surface proteins of Drosophila during development (Woods et al. 1987). Differences were found in surface proteins labelled in unevagnated and evagnated Drosophila imaginal discs. These proteins are thought to be involved in processes such as cell rearrangement which occur during disc morphogenesis.

The labelling of cell surface membranes using ¹²⁵Iodine is used in this chapter to look for temporal variation in cell surface proteins during Xenopus laevis development. A comparison will also be made between labelled proteins present on internal and external cell surfaces of Xenopus laevis embryos during development.

4.2 Analysis of ¹²⁵Iodine Labelled Cell Surface Proteins in Xenopus laevis Embryos

The ¹²⁵Iodine surface labelling technique was adapted from that described in Darribere et al (1982) (Method M.9). Samples of stage 22 Xenopus laevis embryos were surface labelled with ¹²⁵Iodine as outlined in Figure 18. Embryos were labelled with the vitelline membrane in place ('vitelline membrane embryos', VM); with the vitelline membrane removed ('intact embryos', I) and with the vitelline membrane removed and the embryo disaggregated (D). After labelling and washing, the embryos were homogenised in SDS-page sample buffer, centrifuged briefly to remove yolk, and samples were run on an SDS-page gel. The results of this experiment are shown in Figure 19.

The disaggregated embryo track (D) indicates around 15 labelled proteins that can be resolved on this gel.

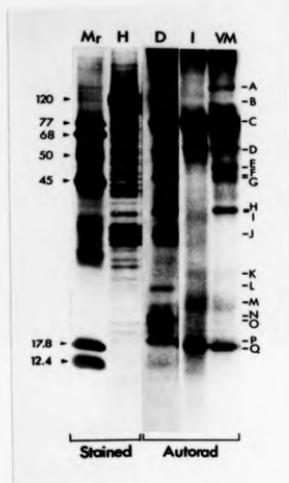


Figure 19 1-dimensional SDS-page of ¹²⁵Iodine Surface
Labelled Xenopus laevis Embryos

Ten stage 22 Xenopus laevis embryos were surface labelled with ¹²⁵Iodine. The embryos were homogenised in SDS-page sample buffer and the radioactivity in 1ul volumes of the samples was determined. Volumes containing 50,000cpm were diluted to 50ul and were run on a 15% low bis SDS-page gel. The gel was silver-stained then dried and autoradiographed. Tracks from the stained gel and autoradiograph are shown.

The tracks are as follows: Mr, molecular weight markers; H, total embryo homogenate silver-stained track; D, disaggregated embryo sample; I, intact embryo sample; VM, 'vitelline membrane' sample. The lettering of bands differs from that used in Chapter 3.

Comparison of the autoradiograph with the silver-stained track H shows that only a small number of the total proteins present in the embryo have been labelled. In addition, major proteins present in the stained gel are not necessarily seen in the autoradiograph tracks. An example is the major band C (80,000 Mr) present in all three tracks in the autoradiograph but seen as a minor band on the stained gel. In contrast the yolk protein B (lipovitellin, 120,000 Mr) seen in the stained tracks is not present in the labelled tracks. This observation suggests that the labelling technique is selective for the cell surface.

Differences are seen between the proteins labelled in the 3 different samples. The VM track shows 4 protein bands not seen in tracks I and D. These vitelline membrane specific bands are A (Mr 125,000); E (Mr 49,000); G (Mr 46,000) and H (Mr 37,000). Bands specific to the VM samples suggests that protein species are present in the vitelline membrane that are absent on the embryo surface. The vitelline membrane track (VM) has all the labelled protein bands present in the intact embryo track (I) (eg. C, D, K, M, Q). One explanation might be that that the vitelline membrane contains the same protein species as the cell surface. However, this is not supported by published data (Richter, 1980). An alternative explanation is that the vitelline membranes were ruptured at some point before or during labelling which left the cell surface accessible to the labelling mixture. Work carried out later in this chapter using the histo-autoradiographic technique shows the labelling of a 'vitelline membrane' embryo (see Figure 27).

No damage to the vitelline membrane is seen after labelling. This suggests that damage to the membrane did not occur in every VM embryo labelled, but of course, does not rule out the possibility that one or more embryos in the sample did suffer such damage. Another explanation is that the vitelline membrane is inherently permeable to the labelling mixture so enabling labelling of the embryo surface in addition to the labelling of the vitelline membrane.

The disaggregated track (D) shows several protein species that are not seen in the intact embryo track (I). These are F (Mr 47,000); I (Mr 37,000); J (Mr 28,000); L (Mr 21,000); N (Mr 19,000) and O (Mr 18,500). Later work (see Figure 21) indicates only two of these proteins, F and I, that are reproducibly labelled in disaggregated embryos and not in intact embryos. These proteins will also be referred as P47 and P37. Both of these proteins are similar in molecular weight to proteins labelled specifically in the vitelline membrane sample (proteins G and H). It could be suggested that the presence of F and I in track D represents contamination in the sample by vitelline membrane. This can be discounted for the following reasons:

1. Close analysis of the gel shows that the molecular weight of the vitelline membrane protein G, is slightly lower than the disaggregated protein F. Similarly the molecular weight of the vitelline membrane protein H is slightly higher than that of the disaggregated protein I. These differences have been observed on gels run on several occasions and they suggest that the protein species G and F, and also H and I are distinct.

2. If contamination by the vitelline membrane is the explanation of proteins I and F in track D, then one would also expect to see the other vitelline membrane specific bands A and E. These are absent in track D.

3. If contamination by vitelline membrane occurs in the disaggregated embryo samples then one would also expect it to occur in the intact embryo sample. This is not seen.

It seems clear that the labelled bands specific to the disaggregated embryo samples do not indicate contamination by vitelline membrane. It is therefore suggested that the protein species P37 and P47 (I and F) labelled specifically in the disaggregated embryos samples represent proteins that are present on inter-cellular membranes and which are not exposed to the labelling medium in normal intact embryos.

Stage 22 *Xenopus laevis* embryos were surface labelled with 125 Iodine as shown in Figure 18. These were analysed on 2-dimensional IEF gels which are shown in Figure 20. Note that the numerical labelling of spots in these gels uses the system adopted for the silver-stained IEF gels in Chapter 3 (e.g. see Figures 3 and 7).

The resolution of these autoradiographs is poor. This is due mainly to the nature of 125 Iodine as a radioactive source which is known to give poor quality autoradiographs (see results in Darriberre et al. 1982). However, analysis of these autoradiographs is possible despite the poor resolution, and a repetitive pattern of spots is seen in the comparison of similar gels.

In the VM gel, the major spots V1 (around 200,000

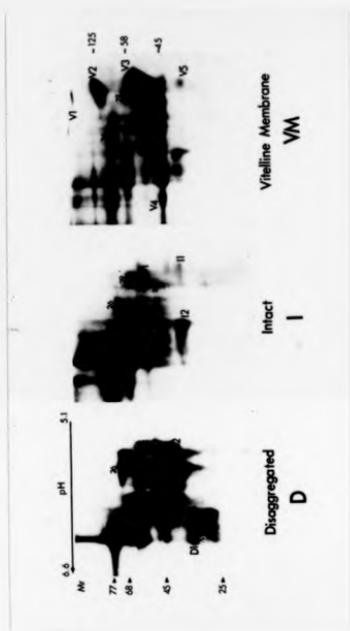


Figure 20 2-dimensional IEF Gels of ¹²⁵Iodine Surface
Labelled *Xenopus laevis* Embryos

Ten stage 22 *Xenopus laevis* embryos were surface labelled with ¹²⁵Iodine as described in the legend to Figure 19. The embryos were homogenised in IEF lysis buffer and the radioactivity in 1µl volumes of the samples was determined. Volumes containing 300,000cpm were diluted to 50µl and were loaded onto IEF tube gels. A control gel was run for pH determination, and molecular weight markers were run beside the tube gels in the second dimension. Autoradiographs of the gels are shown.

The gels are as follows: D, disaggregated embryo sample; I, intact embryo sample; VM, vitelline membrane embryo sample.

Mr); V2 (125,000 Mr); V3 (47,000-58,000 Mr); V4 (46,000 Mr) and V5 (37,000 Mr) are present which are not seen in the intact or disaggregated embryo gels. If we compare this with the vitelline membrane specific proteins seen in Figure 19: A (125,000 Mr); E (49,000 Mr); G (46,000 Mr) and H (37,000 Mr) then we see that spot V2 and A are likely to represent the same protein, as are the line of spots V4 and band G, and also V5 and band H. Note that V5 appears at a higher molecular weight than protein I1 seen in the intact gel, and hence these proteins are distinct. Spot V3, which is very strong appears in the same position as spots 1, 3 and 4 seen in gel D. V3 could include the VM specific protein E detected in Figure 19. However E is a distinct band whereas V3 is a very large spot. It is therefore likely that more than one protein is present in the region V3, or that the protein present is a major one and is not E. The spot V1 appears to represent a vitelline membrane specific protein not seen previously in Figure 19. V1 has also been observed in other 2-dimensional gels of VM samples (data not shown).

The analysis of the proteins specific to the disaggregated embryo gel is more complex. Protein D2 (Spot 20, Mr 47,000) in gel D is the same molecular weight as protein F (P47) in Figure 19. A small amount of this spot is seen in the intact embryo gel (lying above I1) but it is not possible to determine whether this spot is present in the VM gel. Note that the appearance of D2 (47,000 Mr) as a spot is very different to that of V4 (46,000 Mr) which appears as a band of spots. This supports the evidence that the proteins G and F seen in Figure 19 are distinct. Comparison with other

intact and disaggregated gels (see Figures 22 and 23) suggests that spot D2 is likely to represent band F (P47) seen in the 1-dimensional autoradiograph in Figure 19, and is specific to labelling of the disaggregated embryo.

Spot D1 (37,000 Mr) is thought to represent spot J (P37) seen in Figure 19 as specific to the disaggregated track. Comparison with other intact and disaggregated gels (see Figures 22 and 23) supports this suggestion. However it is not possible to be certain for the following reasons: spot D1 appears as a very minor spot on some 2-dimensional autoradiographs of labelled disaggregated embryos, but on 1-dimensional gels it appears as a major band. It also is possible that P37 has a pI value outside the pH range obtained on these IEF gels. This would mean that a spot representing this protein would not be present on these IEF gels.

The spots I1 and I2 (both around 34,000 Mr) appear in the intact embryo gel but are absent in the disaggregated embryo gel. Other labelled spots (eg. 26, 29, 4, 1) are present in both the disaggregated and intact embryo samples. This could be explained by some protein species being washed from the the embryo surface in the disaggregating medium and hence not appearing in gels of the disaggregated embryo samples. Attempts to control for this possibility were inconclusive (data not shown).

4.3 Comparison of Cell Surface Proteins from Different Stages of *Xenopus laevis* Development

Xenopus laevis embryos from 5 stages of development

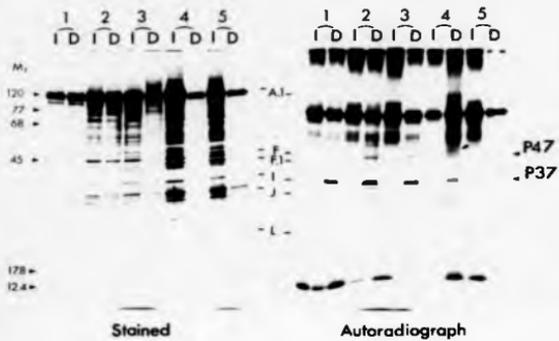


Figure 21 1-dimensional SDS-page of ¹²⁵Iodine Surface
Labelled Xenopus laevis Embryos at Different Stages of
Development

Twenty Xenopus laevis embryos at five stages of development were surface labelled with ¹²⁵Iodine. Ten embryos from each stage were labelled intact (I) and ten embryos were labelled after disaggregation (D). The embryos were homogenised in SDS-page sample buffer and the radioactivity in 1 μ l volumes of the samples was determined. Volumes containing 50,000cpm were diluted to 50 μ l and were run on a 15% low bis SDS-page gel. The gel was silver-stained then dried and autoradiographed. Both the stained gel and autoradiograph are shown.

The tracks are as follows: I, intact embryos; D, disaggregated embryos; 1, stage 3-4 early cleavage embryos; 2, stage 8 blastula embryos; 3, stage 11 gastrula embryos; 4, stage 14 early neurula embryos; 5, stage 21 late neurula embryos.

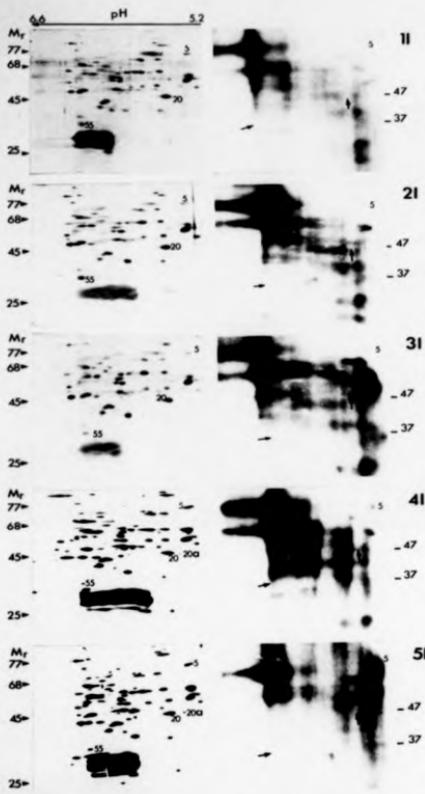
were surface labelled with ¹²⁵Iodine as described in Method M.9. Both intact and disaggregated embryos from each stage were labelled and the 10 samples were analysed on 1-dimensional and 2-dimensional gels. Figure 21 shows the samples analysed on an SDS-page gel and compares the autoradiograph with the silver-stained gel.

On the autoradiograph the pattern of labelled proteins is similar to that seen in Figure 19. The disaggregated tracks shows clearly the 2 prominent bands F and I (P47 and P37), that are absent in the intact embryo tracks. Note that the other major D specific bands seen in Figure 19 (J, 26,000 Mr; L, 21,000 Mr; N, 20,000 Mr and O, 19,500 Mr) are very faint or absent in this gel. The alternating pattern of bands F and I in the intact and disaggregated tracks across the autoradiograph is very clear. A small amount of labelled protein I is seen in the track 2I but band F is absent. It is difficult to explain why one of these disaggregated embryo specific bands should be present in the intact embryo track and not the other. In addition very little of proteins F and I are seen in track 5D.

An additional protein (F.1, 44,000 Mr) is seen in both intact and disaggregated tracks that was absent in Figure 19. Track 4D shows a band (A.1) at around 120,000 which is absent in all other tracks. This band is close to the molecular weight of the vitelline membrane specific band A (125,000 Mr) seen in Figure 19, and it is possible that this sample is contaminated with vitelline membrane. If this is so then the other VM specific bands (E, G, H) should also be present in this track. Bands G and H are very close in

molecular weight to the disaggregated embryo specific bands I and F (discussed above) and it would be difficult to distinguish between these on the same track of the gel. The VM specific band E (49,000 Mr) should appear at a slightly higher molecular weight than band G/F. A faint band at this molecular weight is present in track 4D (indicated in the figure by an arrow). This indicates that contamination with vitelline membranes probably is occurring in this sample, and therefore that bands A.1 and A represent the same protein. Note the absence of bands A and E in any other sample suggesting that these samples are free of contamination by vitelline membrane. No temporal variation in the pattern of labelled proteins on the autoradiograph in Figure 21 can be detected. It is clear that the resolution of the ¹²⁵Iodine autoradiographs is not sufficient to detect minor changes in the pattern of labelled cell surface proteins that may occur during development.

The same 10 samples of disaggregated and intact labelled embryos shown in Figure 21 were also analysed on 2-dimensional IEF gels. These are shown in Figures 22 and 23. The resolution of the 2-dimensional autoradiographs is again very poor but comparison with the stained gels confirms that the problem does not lie with the electrophoresis technique since the silver-stained gels are highly resolved. The positions of spots D1 and D2 which are thought to represent the disaggregated embryo specific proteins P37 and P47 are shown with arrows in the autoradiographs in Figure 23. Note that the position of spot D1 coincides with spot 55 on the stained gels. D2 appears more as a short band than as a



Stained

Autoradiographs

INTACT

Figure 22 2-dimensional IEF Gels of ¹²⁵Iodine Surface
Labelled Intact Xenopus laevis Embryos at Different Stages
of Development

Ten intact Xenopus laevis embryos at five stages of development were surface labelled with ¹²⁵Iodine. The embryos were homogenised in IEF lysis buffer and the radioactivity in 1 μ l volumes of the samples was determined. Volumes containing 300,000cpm were diluted to 50 μ l and were loaded onto IEF tube gels. A control gel was run for pH determination, and molecular weight markers were run beside the tube gels in the second dimension. The gels were silver-stained then dried and autoradiographed. Stained gels and autoradiographs are shown.

The abbreviations are as follows: Mr, Molecular weight markers; I, intact embryos; 1, stage 3-4 early cleavage embryos; 2, stage 8 blastula embryos; 3, stage 11 gastrula embryos; 4, stage 14 early neurula embryos; 5, stage 21 late neurula embryos.

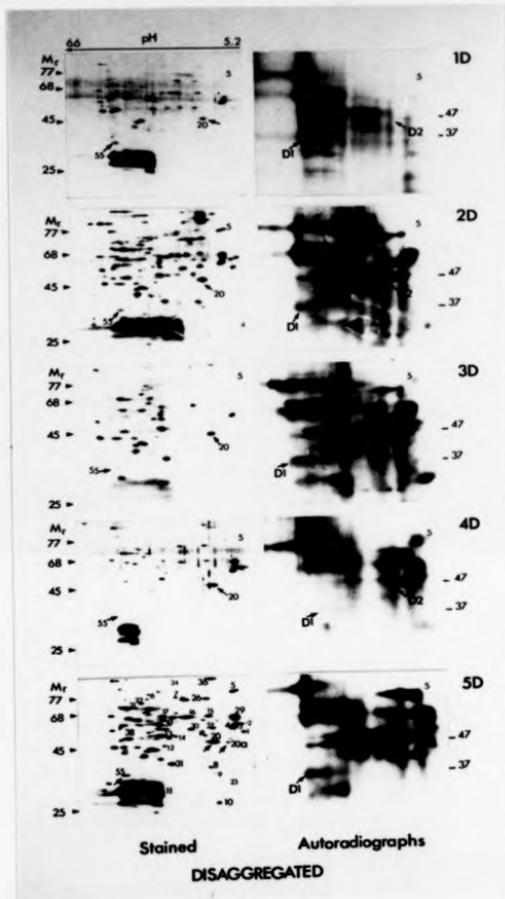


Figure 23 2-dimensional IEF Gels of 125 Iodine Surface
Labelled Disaggregated *Xenopus laevis* Embryos at Different
Stages of Development.

Ten disaggregated *Xenopus laevis* embryos at five stages of development were surface labelled with 125 Iodine. The embryos were homogenised in IEF lysis buffer and the radioactivity in 1 μ l volumes of the samples was determined. Volumes containing 300,000cpm were diluted to 50 μ l and were loaded onto IEF tube gels. A control gel was run for pH determination, and molecular weight markers were run beside the tube gels in the second dimension. The gels were silver-stained then dried and autoradiographed. Stained gels and autoradiographs are shown.

The abbreviations are as follows: Mr, Molecular weight markers; D, disaggregated embryos; 1, stage 3-4 early cleavage embryos; 2, stage 8 blastula embryos; 3, stage 11 gastrula embryos; 4, stage 14 early neurula embryos; 5, stage 21 late neurula embryos.

resolved spot. One end of this band coincides with the position of spot 20 on the stained gels, but the other end of the band (which lies directly below the tubulin spot 4) appears to coincide in position with the stained spot 20a which is only seen in the neurula stage gels (see Figures 22 and 23, gels 4I, 5I, 4D and 5D). Spot D2 does not show any temporal variation. D2 may be present in all the stained gels but at a level too low to be detected by silver-staining. Alternatively D2 may represent a protein distinct from 20/20a which is not detected at all by the silver-stain.

D1 and D2 are present in all five disaggregated embryo gels, but are weak or absent in the intact embryo gels (shown by arrows in Figure 22). A small amount of these spots are seen, for example, in the autoradiographs of gels 2I and 3I, but comparison with the disaggregated gels shows that these are very weak. This finding also agrees to some extent with the results of the SDS-page gel shown in Figure 21 where small amounts of these otherwise D specific bands can be detected in these intact embryo tracks.

The stained gel 5D in Figure 23 has been numerically labelled as previously in Figure 7. Comparison of the stained gel with the autoradiograph enables determination of proteins present in the embryo homogenate which have been surface labelled with 125 Iodine. This comparison can indicate which embryo proteins are present on the cell surface. This is useful with respect to the work presented in the last chapter where some membrane preparations (eg. the crude membrane purification) did not separate plasma membranes from other cellular membranes such as mitochondrial membranes and

Protein	M _r	Present	Protein	M _r	Present
1	54	Y	2	56	N
3	55	Y	6	55	N
4	55	Y	7	55	N
5	77	Y	8	40	N
10	26	Y	9	38	N
13	50	Y	11	26-30	N
14	50	Y	12	45	N
18	49	Y	15	63	N
20	47	Y	16	63	N
20 _a	47	Y	17	63	N
26	75	Y	19	49	N
27	55	Y	21	70	N
29	60	Y	22	72	N
39	58	Y	23	32	N
41	45	Y	24	115	N
55	37	Y	30	55	N
			31	41	N
			34	120	N
			36	117	N
			38	49	N

Table 8 Embryo Membrane Proteins Surface Labelled with
¹²⁵Iodine

This table indicates which of the Xenopus laevis embryo membrane proteins detected on IEF gels in Chapter 3 are also detected on IEF autoradiographs of embryos surface labelled with ¹²⁵Iodine. The numbering system refers to that used in the majority of IEF gels in Chapter 3.

Y, the protein is detected on autoradiographs of surface labelled embryos; N, the protein is not detected on autoradiographs of surface labelled embryos

endoplasmic reticulum. A summary of those embryo proteins which were surface labelled with ^{125}I Iodine is shown in Table 8. This table analyses only those proteins studied on IEF gels in Chapter 3.

A thorough comparison of the autoradiographs is difficult because of the poor resolution. No clear differences in labelled proteins were detected in different stages on an initial analysis of the gels. Table 5 summarised those proteins showing temporal variation on silver-stained IEF gels in Chapter 3. This can be used to confirm whether these proteins are surface labelled with ^{125}I Iodine and whether the labelled protein varies over the stages shown. Using this method only the labelled protein 5 (77,000 Mr) was found to vary over the developmental stages studied. This spot resolves in some of the stained gels as two separate proteins (5a and 5b) which are either both present or both absent on the gels. In the autoradiographs in Figures 22 and 23, spot 5 is absent in the early cleavage gels, weak in the blastula and gastrula gels, and weak or strong in the neurula and late neurula gels. Table 9 gives a summary of the temporal variation of proteins 5a and 5b in the samples studied so far.

The data shown in Table 9 suggests that proteins 5a and 5b are membrane proteins but, since they are not present in the TX fractions they are not likely to be integral membrane proteins. Spot 5 is seen in cytosol samples as well as in the crude membrane suggesting that it may be a weakly associated membrane protein, for example a cytoskeletal protein or extracellular matrix protein. The variation in

	Sample	U	EC	B	G	N	LN
1	Autorads I	-	○	w	w	w	s
2	Autorads D	-	○	w	w	s	w
3	Stained gels I	-	w	w	w	s	s
4	Stained gels D	-	w	s	w	w	s
5	CM	-	s	s	s	s	s
6	TX	○	○	○	○	○	-
7	PM	w	w	s	s	s	-
		TH	CY	CM	SN	TX	
8	Purification	s	s	s	s	w	

Table 9 Temporal Variation in the Xenopus laevis Embryo
Proteins 5a and 5b

Rows 1-4 indicate results from Figures 22 and 23; I, intact embryos; D, disaggregated embryos. Lines 5-8 indicate results from Figures 3, 11, 14 and 7. Abbreviations used are outlined in the legend to Table 5.

intensity of spots 5a and 5b at different embryo stages is unclear and in the crude membrane gels they appear at a constant level in all the stages studied. Further investigation is needed to confirm the variation in expression of proteins 5a and 5b during the early development of *Xenopus laevis*. No other labelled proteins showing temporal variation were detected on these gels.

4.4 Controls for Cell Lysis and Penetration of the Iodine Label

The cell surface labelling technique has so far been assumed to be specific for the cell surface. This assumption was based on the following arguments:

1. Figure 19 showed that only a small proportion of the total number of embryo proteins present are being labelled, and that the profile of labelled proteins is very different to the profile of stained proteins.

2. Previous experiments using the technique have shown that the labelling mixture does not penetrate the cell surface (Darribere et al, 1982).

3. The major yolk protein lipovitellin (120,000 Mr) which is abundant in the embryo, remains unlabelled.

To confirm that the iodine labelling mixture does not penetrate the cell surface, histo-autoradiography was carried out on intact, disaggregated and VM embryos using the technique described in Method M.33. This histo-autoradiographic technique will be discussed in more detail in Chapter 5, but a summary of the technique is shown in Figure 24.

Xenopus laevis
embryos

Remove vitelline membranes
Disaggregate embryos if required

Surface label with iodine-125
Wash

Allow embryos to
develop as required

Fix embryos in
Bouin's fixative

Embed in wax

Section embryos
on microtome

Rehydrate and
mount sections

Dehydrate and
dip slides in
photographic
emulsion

Leave slides
to expose

Develop slides

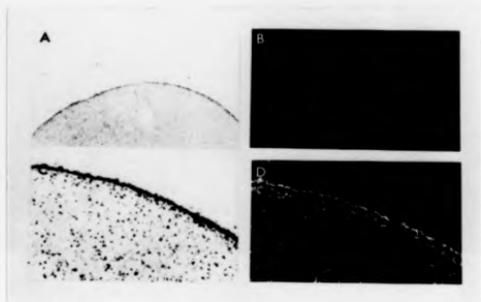
Stain sections with
light green
mount

Figure 24 Experimental Protocol for the
Histo-autoradiography of Xenopus laevis Embryos using
 125 Iodine

See Methods M.9 and M.33.

Figure 25 shows the histo-autoradiography of stage 14 intact embryos. The majority of the ^{125}I label is restricted to the cell surface. Some background labelling is seen and the level of this is higher over the embryo than around the outside of the section. The explanation for this is that during the cutting of the embryos, the radioactive sections are floated on water to stretch the wax. Small amounts of label are leached into the water which, if collected later, can be shown to contain a low level of radiation. This suggests that for a period of around 10 min the embryo sections are lying in a pool of radioactive water, and this label appears to stick more readily to the embryo tissue than to the surrounding wax and the subbed slide. The level of this background radiation over the sections is, however, low compared with that around the embryo surface. The experiment shown in this figure along with similar experiments involving the histo-autoradiography of intact embryos (see Chapter 5) shows that the ^{125}I label does not penetrate the embryo surface.

One explanation for the detection of additional proteins labelled in disaggregated embryos to those in intact embryos is that cell lysis or penetration of the label is occurring specifically in the disaggregated embryos. This would appear unlikely for two reasons, firstly the disaggregation medium is very mild and there appears to be no reason why incubation of embryos in the medium lacking calcium and magnesium should result in significant cell lysis. Secondly, the labelled disaggregated and intact embryo samples differ in their protein profile by a maximum of three



**Figure 25 Histo-autoradiography of Xenopus laevis Intact
Embryos using ¹²⁵Iodine**

Histo-autoradiography of stage 14 intact Xenopus laevis embryos was carried out as shown in Figure 24 (see Methods M.9 and M.33). The slides were left for four days before developing.

A, x10 magnification, bright field illumination; B, x10 magnification, dark field illumination; C, x40 magnification, bright field illumination; D, x40 magnification, dark field illumination.

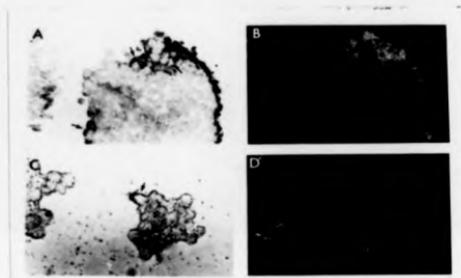


Figure 26 Histoautoradiography of Xenopus laevis
Disaggregated Embryos using 125 Iodine

Histo-autoradiography of stage 14 disaggregated *Xenopus laevis* embryos was carried out as shown in Figure 24 (see Methods M.9 and M.33). The slides were left for four days before developing.

A, x40 magnification, bright field illumination; B, x40 magnification, dark field illumination; C, x10 magnification, bright field illumination; D, x10 magnification, dark field illumination.

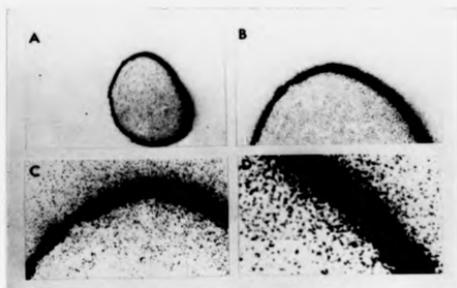


Figure 27 Histo-autoradiography of *Xenopus laevis*
'Vitelline Membrane' Embryos using 125 Iodine

Histo-autoradiography of stage 14 *Xenopus laevis* embryos was carried out as shown in Figure 24 (see Methods M.9 and M.33). The embryos were intact and the vitelline membranes had not been removed. The slides were left for four days before developing.

A, x10 magnification; B, x20 magnification; C, x40 magnification; D, x100 magnification.

or four bands. If lysis was occurring in the disaggregated samples, one should expect to see a large number of additional proteins labelled. Despite these arguments, it is still necessary to show that cell lysis, or penetration of the label does not occur when disaggregated embryos are labelled.

Histo-autoradiography was carried out on disaggregated stage 14 embryos and examples of this are shown in Figure 26. It is difficult to show the labelling of individual cells because on disaggregation of the embryo the cells settle to the bottom of the labelling well and only the accessible surfaces are labelled. This figure shows the aggregates of embryo cells obtained. These are more heavily labelled on one side than on the other depending on the orientation of the material in the labelling medium. Figure 26 reveals the presence of individual cells which are externally labelled but which show no internal labelling. These are indicated with arrows. This experiment can only reveal that a fraction of the disaggregated embryo cells were surface labelled and did not undergo cell lysis. It is not possible to prove that this is true for every cell present in the embryo.

Figure 27 shows the histo-autoradiography of a Xenopus laevis embryos iodinated with the vitelline membrane in place. This experiment was carried out under the same conditions and exposure times as for the intact and disaggregated embryos, but the resulting level of incorporation of the label is very much higher. Figure 27 shows that the vitelline membrane has remained intact during

the labelling procedure. There are several possible reasons why the degree of incorporation of the label in VM embryos is so high:

1. The vitelline membrane contains a large number of proteins with the accessible tyrosine residues necessary for attachment of ^{125}I iodine.

2. Labelling of lipid or other non-protein species is occurring in the vitelline membrane which was not occurring on the cell surface membranes. The use of lactoperoxidase/glucose oxidase labelling using ^{125}I iodine is known to give low level incorporation of label into lipid (Darribere et al. 1982).

3. The vitelline membrane contains glycoconjugate species which are inherently 'sticky' to the label.

4. The vitelline membrane may be permeable to the labelling mixture (as discussed previously). This would mean that the embryo surface was labelled in addition to the vitelline membrane.

Suggestion 4 is thought to be correct from the data shown in Figure 19. The results from these histo-autoradiographic experiments suggest that the labelling procedure is specific for the cell or embryo surface in the intact and VM embryos, and that cell lysis or penetration of the label does not occur during the labelling procedure. This conclusion is implied for the disaggregated embryos but cannot be shown conclusively.

4.5 Attempted Production of Antisera against F37 and P47

The results from histo-autoradiographic experiments

suggested that the 125 Iodine labelling technique was specific for the cell surface. This supports the suggestion that the proteins P37 and P47 are present on internal embryo cell surfaces and are not seen on autoradiographs as a result of cell lysis or penetration of the label. To characterize P37 and P47 further and to confirm, or otherwise, the suggestion that they are internal cell surface proteins, an attempt was made to raise polyclonal antibody sera against these proteins using the nitrocellulose implantation method described in Smith *et al* (1986) (see Method M.25). A summary of the method used is shown in Figure 28.

Two Balb/c mice were implanted with nitrocellulose for each of the P37 and P47 proteins. Five weeks following this implantation, bleeds were taken from the mice and were tested against a Western blot of *Xenopus laevis* embryo homogenates shown in Figure 29. The nitrocellulose tracks are shown alongside a portion of the stained gel containing a marker track and the embryo homogenate. Only one of the seven tracks shows the presence of a positive band. This is track 6, which was screened with sera from the first mouse immunised with P37 protein (mouse 37.1). Two bands are seen in this track, a major band at 37,000 Mr and a minor band at around 55,000 Mr. The major band could indicate the presence of antibodies in the mouse sera against P37. However, other proteins at 37,000 Mr would have been present on the nitrocellulose implanted into the mouse and it is not possible to confirm that antibodies are present against P37 without further investigation. No signal above background was detected in any of the control tracks, nor in the other mouse

1-DIMENSIONAL SDS-PAGE
GEL OF XENOPUS
EMBRYO PROTEIN



↓
STAIN GEL



↓
CUT GEL FOR
COMPARISON LATER



↓
ELECTROBLOT ONTO
NITROCELLULOSE

↓
CUT REQUIRED BAND
FROM NITROCELLULOSE



↓
IMPLANT NITROCELLULOSE
SUBCUTANEOUSLY INTO MOUSE

↓
LATER: SCREEN POLYCLONAL
SERUM

↙
IMMUNOFLUORESCENCE ON
XENOPUS SECTIONS

↘
WESTERN
BLOTS

Figure 28 Experimental Protocol for the Immunisation of
Mice by Subcutaneous Implantation of Nitrocellulose

See Method M. 25.

sera tracks.

The sera from the 4 immunised mice were also tested on stage 6 TCA fixed sections. The serum from mouse 37.1 was further screened on embryo sections which had been fixed using paraformaldehyde, freeze substitution and ethanol. Stage 6 embryo blocks for this experiment were provided by Dr. L. Dale. The results from the screening of the 37.1 sera are shown in Figure 30. No detectable signal was observed in any of the screenings of the mouse sera on the sections. There are several possibilities to explain the failure of the screening experiment of the 37.1 serum on stage 6 embryo sections:

1. The antigen is not present at this stage in the embryo development. This is clearly false as is shown in Figure 21 where the P37 protein is seen to occur in all stages studied from early cleavage through to stage 22.

2. The antigen is being lost or otherwise destroyed during the fixation of the stage 6 embryos prior to cryosectioning. This is possible but unlikely since a variety of embryo fixation techniques were used and in all cases the sections were negative.

3. The antibody shown to give the positive signal on the Western blot in Figure 29, recognised a protein of molecular weight around 37,000 that is distinct from the P37 protein thought to be present on internal cell membranes. This is plausible since the mouse was immunised with nitrocellulose cut from the region of the blot containing the P37 protein. IEF gels confirm that several protein species are present at 37,000 Mr (see the silver-stained gels in

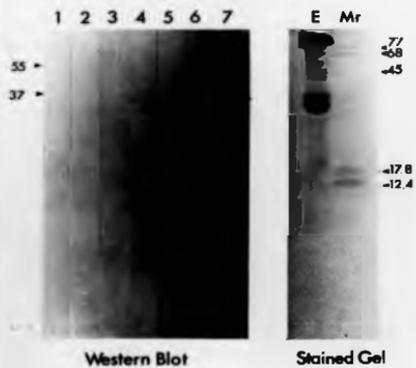


Figure 28 Western Blot of Xenopus laevis Embryo Homogenate
Screened with Sera from Mice Immunised with P37 and P47

Ten stage 11 *Xenopus laevis* embryos were homogenised in 500ul of SDS-page sample buffer. 50ul volumes were run in eight tracks of a 12% high bis SDS-page gel. After running, a portion of the gel was cut and stained in Coomassie Blue. The remaining gel was electroblotted (See Method M.15) and screened as follows: 1, control- PBS in place of mouse serum; 2, control- PBS in place of RAM-HRP; 3, control- serum from uninjected mouse; 4, serum from first mouse immunised with P47; 5, serum from second mouse immunised with P47; 6, serum from first mouse immunised with P37; 7, serum from second mouse immunised with P37.

E, stained track of embryo homogenate; Mr, stained track of molecular weight markers.

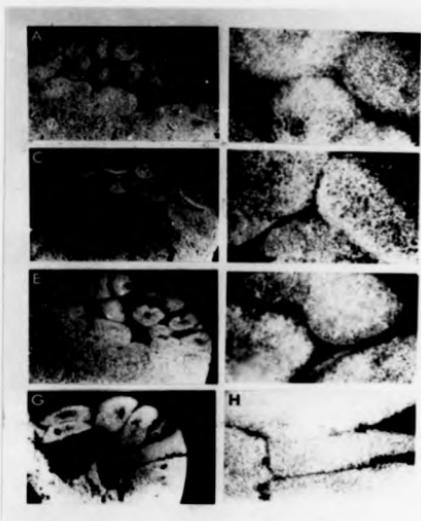


Figure 30 Xenopus laevis Embryo Sections Screened with Serum from the First Mouse Immunized with P37

Stage 6 *Xenopus* embryo sections were screened using indirect immunofluorescence with serum from the first mouse immunized with P37 (see Method M.27).

The sections are as follows: A, TCA fixed embryo, x10 magnification; B, TCA fixed embryo, x40 magnification; C, Paraformaldehyde fixed embryo, x10 magnification; D, Paraformaldehyde fixed embryo, x40 magnification; E, Embryo fixed using freeze substitution, x10 magnification; F, Embryo fixed using freeze substitution, x40 magnification; G, Ethanol fixed embryo, x10 magnification; H, Ethanol fixed embryo, x40 magnification.

Embryos fixed using paraformaldehyde, ethanol or by freeze substitution were provided by Dr. L. Dale. All fluorescence seen in this figure is background, and no staining of membranes was observed.

Figures 22 and 23).

4. The Western blot shown in Figure 29 represented a false positive signal, and no antibodies were raised against any of the immunised proteins. This possibility seems unlikely since the molecular weight of the band detected was 37,000 Mr. However, the result should be confirmed by repetition of the Western blot experiment.

4.6 Conclusions

The objective of the work described in this chapter was to use the ^{125}I -Iodine surface labelling technique to extend the characterisation of the *Xenopus* embryo cell membrane carried out in Chapter 3. The labelling technique offered the advantage of enabling study of the cell surface without the problems of contamination by other cellular membranes. It was proposed to use SDS-page and IEF gel techniques to look first for temporal variation in labelled proteins and secondly, through comparison of the proteins labelled in intact and disaggregated embryos, to look for proteins specific to internal cell surface membranes. The work has been only partially successful in achieving these aims. Proteins thought to be specific to internal cell surface membranes have been detected, but analysis of proteins showing temporal variation was difficult because of the poor resolution of the ^{125}I -Iodine autoradiographs.

4.6a The use of ^{125}I -Iodine in Electrophoresis Techniques

The resolution of the ^{125}I -Iodine autoradiographs was poor and this prevented in-depth analysis of the experiments.

Comparison of the autoradiographs with the corresponding stained gels confirmed that the low resolution was not caused by any failure of the gel technique (see Figures 22 and 23). Much of the published data using ^{125}I in autoradiographs show a similar lack of resolution (see Darribere et al 1982) and confirms that a major problem lies with the use of ^{125}I as a gamma/weak beta emitter in the autoradiographic technique.

Attempts were made to improve the resolution of the ^{125}I autoradiographs. On several occasions autoradiography of the iodine gels was carried out without the use of intensifying screens. This was found to improve the resolution of the autoradiographs but diminished the sensitivity (by around 8-fold) to the extent that the developing times became excessively long. An alternative involved the use of a high sensitivity, high resolution Xray film designed for use with weak beta-emitting isotopes such as ^{125}I (Hyperfilm- ^3H Amersham). This film was tested both with and without intensifying screens. When used with a screen the film showed little improvement in the overall resolution of the gel. When used without a screen the sensitivity of the film was only slightly better than that obtained with the normal film and again the exposure times were very long. In addition the film was delicate and any adverse handling resulted in disfiguring scratch marks on the surface of the autoradiograph. For these reasons the Hyperfilm was not used in place of the normal autoradiography film.

4.8b Xenopus laevis Embryo Proteins Labelled with ¹²⁵Iodine

The overall pattern of proteins labelled in the different samples was reproducible. In the VM samples five labelled species were detected that were absent in intact and disaggregated samples. On IEF autoradiographs these vitelline membrane specific proteins appear as major spots. This was consistent with the results of the histo-autoradiographic experiment on VM embryos (Figure 27) where the vitelline membrane appeared very heavily labelled. It is suggested that the vitelline membrane is permeable to the labelling mixture, and that iodination of VM embryos also results in labelling of the embryo surface.

Surface labelling of Xenopus laevis embryos with radioactive Iodine has been carried out previously (Richter, 1980). Fertilisation envelopes (the vitelline membranes of the unfertilised egg) were isolated and analysed on SDS-page gels stained with Coomassie Blue. 9-11 bands were detected of which four: 1, (115,000 Mr); 2, (68,000 Mr); 3 (40,000 Mr); and 4, (31,000 Mr) were very strong. On iodination of the isolated envelopes using lactoperoxidase all of the bands, with the exception of band 3, were found to label.

The molecular weights of proteins 1 and 3 on Richter's gel are consistent with the molecular weights of the VM specific proteins A/V2 and I/V5 shown above. However there are discrepancies between the results quoted in Richter (1980) and those discussed in this chapter. This is not thought to be due to differences in the protein composition of fertilisation envelopes and vitelline membranes since these have been shown to be very similar (Richter 1980; Wolf

et al, 1976). A study of the 125 Iodine labelled proteins of isolated vitelline membranes should be carried out to compare the results obtained.

A comparison was made between proteins labelled in intact and disaggregated embryos and labelled proteins specific to disaggregated embryos were detected. Two of these proteins were reproducible in all experiments carried out and could be detected on both 1- and 2-dimensional gels. These proteins, P37 and P47, are thought to be specific to intercellular membranes.

A comparison of the labelled proteins from different embryonic stages showed that the overall pattern of cell surface labelling was very similar. This is in agreement with the conclusions of Chapter 3, where it was shown that the profile of isolated membrane proteins from different stages was very similar. Only one labelled protein was found to show temporal variation. This was protein 5 (77,000 Mr) which on some gels resolves into two spots 5a and 5b. The data concerning these proteins was confusing and more investigation is needed to characterise these proteins further. Apart from proteins 5a and 5b, no temporal variation in labelled proteins was detected in any of the samples using 1- or 2-dimensional gels. This is thought to be due to the problems with resolution of the minor labelled proteins.

These conclusions are very different to those drawn by Darribere et al (1982). They carried out a similar analysis of surface proteins labelled with 125 Iodine in intact and disaggregated *Pleurodeles waltlii* embryos. They detected 10 major labelled proteins in disaggregated embryos

on 2-dimensional gels and showed that 6 of these proteins undergo some form of temporal variation in their expression. Of intact Pleurodeles embryos only seven of the ten disaggregated protein species could be detected, and these showed no temporal variation over the stages studied. The reasons for the different results obtained lies partly in the nature of the two different embryos species used. In addition, since there were similar problems with resolution it is possible that their data was over-interpreted.

4.6c Controls for the ¹²⁵Iodine Surface Labelling Technique

The ¹²⁵Iodine surface labelling technique was assumed to be specific for the cell surface since only a small fraction of the total number of embryo proteins were labelled, and because previous work (Darribere et al 1982) had indicated that this was so. Histo-autoradiographic experiments were carried out to confirm this suggestion on intact, disaggregated and VM embryos. These experiments showed that the labelling was specific to the embryo surface in intact embryos, but could only suggest that cell lysis or penetration of the label was not occurring during the labelling of disaggregated embryos. Since only three or four more protein species are labelled in the disaggregated embryos than are labelled in the intact embryos, it seems very unlikely that the presence of these disaggregated embryo specific proteins on autoradiographs arises through cell lysis or penetration of the label.

It is suggested that the P37 and P47 proteins specific to labelling of the disaggregated embryo are

membrane proteins present on inter-cellular membranes. However other explanations are possible for the labelling pattern seen in the autoradiographs. For example, the P37 and P47 proteins may be inherently present on both internal and external embryo membranes but are washed from the embryo surface in the intact labelling medium. If the proteins were not disturbed by the disaggregating medium the pattern of labelling on gels would be identical to that obtained if the proteins were specific for the inter-cellular membranes. Since disaggregating embryos are first washed in intact labelling medium (Barth X with 5mM glucose) before transferring to the disaggregating medium, this possibility would appear unlikely unless the loss of surface proteins from the embryos is a time dependent process. No controls have yet been carried out to confirm this suggestion. The P37 and P47 proteins may be present on the embryo surface but are masked by a component that is washed off in the disaggregating medium. This component could be another protein or the carbohydrate portion of a glycolipid or glycoprotein. This possibility could be discounted by allowing the embryos to disaggregate and so washing off the masking species, then switching them to the intact labelling medium to reaggregate, and labelling as intact embryos. This experiment was attempted but it was found that reaggregation of the embryos was very poor with the result that previously internal cell surfaces were left accessible to the labelling medium after reaggregation. As these surfaces are thought to contain the proteins P37 and P47 there would be no way of distinguishing between the possibilities of labelling of

unmasked proteins on the outer embryo surface, and the labelling of P37 and P47 on the intercellular membranes. There appears to be no way of controlling for this possibility and it is accepted the masking of protein species on the outer embryo surface is a possible explanation for the labelling pattern of P37 and P47.

4.6d Proteins P37 and P47

It is clear that the disaggregated embryo specific proteins P37 and P47 may be specific to internal cell membranes. However it is not possible to confirm this from the experiments carried out so far. For this reason, and to enable a more general characterisation of the proteins, an attempt was made to raise polyclonal mouse sera against the proteins. The initial work with the antibodies showed that the serum from mouse 37.1 was positive when screened on a Western blot against a Xenopus laevis embryo homogenate. The band detected had a molecular weight of 37,000. This was not sufficient to prove that the serum is recognising P37 since more than one protein species of this molecular weight could have been present on the nitrocellulose which was implanted into the mouse. More conclusive evidence on the nature of the 37,000 Mr antigen would be obtained from screening on Xenopus sections. As yet no signal has been obtained on sections that indicates that the 37.1 serum is recognising an internal membrane antigen.

CHAPTER 5

RESULTS: INVESTIGATION OF CELL CLEAVAGE AND CILIATION

5.1 Introduction

The histo-autoradiographic technique discussed in Chapter 4 provides a useful tool for the study of cell membranes and the cell surface. In this chapter the method is used to follow the redistribution of cell surface proteins during cleavage of the fertilised *Xenopus laevis* egg, and to investigate the origin of cilia from the double layered epidermis.

5.2 The Redistribution of Cell Surface Proteins During *Xenopus laevis* Cell Cleavage

The first cleavage of *Xenopus laevis* eggs occurs approximately 90 minutes post-fertilisation at 23°C, and is vertical, passing through both the animal and vegetal hemispheres and bisecting the grey crescent. On cleavage a furrow is formed in the membrane which is initially asymmetrical, starting in the animal hemisphere and moving progressively ventrally to encircle the embryo. The cleavage furrow is formed by the contraction of a ring of actin filaments which are laid down locally in the direction of the furrow at the cell surface (Selman and Perry, 1970; Perry 1975; Blumink and de Laat, 1977).

In the animal hemisphere the cleavage furrow is clearly visible as a white line lacking cortical granule pigment. Studies have indicated that the cleavage furrow

membrane and the membrane of the furrow surface have very different properties. For example, measurements of ion currents in *Xenopus laevis* embryos during cleavage have shown that current leaves the unpigmented furrow region and enters the pigmented surface. This suggests a heterogeneous ion channel distribution between the two membrane domains (Kline *et al.* 1983). Freeze fracture electron-microscopy has revealed a higher intramembraneous particle density in the pigmented surface membrane compared with the furrow membrane (Bluemink *et al.* 1976, Sanders and DiCaprio, 1976). At the 32-64 cell stage Con-A receptors are preferentially localised in the unpigmented surfaces of amphibian blastomeres (Roberson and Armstrong, 1979) and this surface is more adhesive than the pigmented surface (Holtfreter, 1943; Roberson and Armstrong, 1980).

Histo-autoradiographic studies of membrane proteins in *Xenopus* embryos have indicated significant differences in the composition of the outer pre-existing membrane domain, and the inner newly synthesised membrane domain (Darribere *et al.* 1982; see also Chapter 4). These studies suggest that the membrane of the cleavage furrow is synthesised *de novo*, and is not constructed from the rearrangement of pre-existing membrane (Bluemink and de Laat, 1977). This is supported by the observation that there is no substantial change in surface area of the embryo before cleavage which would be needed to accommodate an increase in furrow surface area (Bluemink and de Laat, 1973; Selman and Waddington, 1955).

In 1986, Byers and Armstrong investigated the contribution of membrane in the embryo surface to the newly

forming membrane furrow (Byers and Armstrong, 1986). They labelled the *Xenopus laevis* embryo surface with 125 Iodine to follow the segregation of membrane proteins during cell cleavage. Near the end of first cleavage, membrane on the outer surface of the embryo and a short band of membrane at the leading edge of the cleavage furrow were found to show a high silver grain intensity. In contrast the remaining membrane in the cleavage furrow was only lightly labelled. The cleavage membrane therefore appeared mosaic in character, originating in part from the pre-existing surface membrane and in part from internal membrane stores or precursors. Furrow membrane adjacent to the outer surface membrane showed very low silver grain density and was underlain by membrane vesicles. It was suggested that new membrane derived from cytoplasmic precursors is primarily inserted at this location, at least during the latter stage of cleavage. The domain of heavily labelled material at the leading edge of the furrow was thought to contain material which is gathered at the animal pole during the initial surface contraction event. This could include membrane anchors for the underlying ring of microfilaments.

Byers and Armstrongs' experiments were carried out on pigmented *Xenopus laevis* embryos. Much of their work involved events at the animal pole where there are substantial amounts of pigment. As mentioned briefly in Chapter 4, the presence of pigment granules in histo-autoradiographic experiments can be very misleading since pigment granules and silver grains are difficult to distinguish under the light microscope. The work described

below involves a partial repetition, confirmation and adaptation of several of the experiments carried out by Byers and Armstrong. Albino Xenopus laevis embryos were used to eliminate the difficulties associated with pigment granules.

5.3 Experimental Investigation of Xenopus laevis Cell Cleavage

Cell surface labelling with ^{125}I Iodine was used to follow the distribution of labelled cell surface membrane proteins during and following first cleavage. A summary of the approach used in these experiments is given in Figure 31. Albino Xenopus laevis embryos were fertilised in vitro and the vitelline membranes removed. The embryos were surface labelled with ^{125}I Iodine as described in Method M.9. Substantial care was needed to ensure that the surface labelling and washing was complete before the onset of first cleavage. Samples of the labelled embryos were fixed immediately following labelling, during first cleavage, second cleavage and at stage 6 of development. Figure 32 shows Xenopus eggs fixed immediately following labelling. The silver grains are mainly associated with the surface of the embryo, although some label is seen on the cytoplasmic regions of the section due to the 'sticky yolk' and leaching effects during fixing discussed in Chapter 4.

Figure 33 shows the autoradiography of albino Xenopus laevis embryos fixed during the latter stages of the first cleavage. There is a decrease in the intensity of the silver granules around the furrow walls compared with the surface of the embryo. The leading edge of the furrow is more

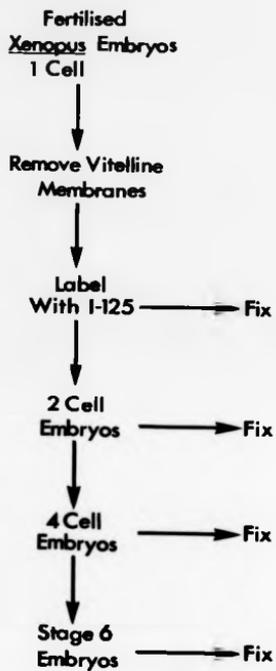


Figure 31 Experimental Procedure to Investigate the Origin
of Cleavage Membrane in the Xenopus laevis Embryo

See Methods M. 9, M. 32 and M. 33.

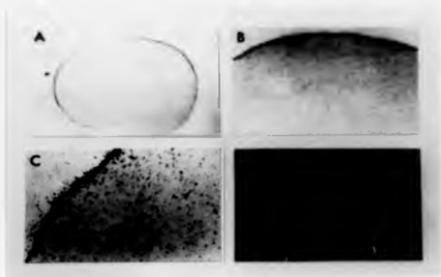


Figure 32 Investigation of Xenopus laevis Cell Cleavage:
Histo-autoradiography of Control Embryos

Xenopus laevis albino embryos were surface labelled with ¹²⁵Iodine before first cleavage. The embryos were fixed immediately after labelling, embedded in wax and sectioned on a microtome. Autoradiography was carried out on the wax sections (Method M.33).

The sections are as follows: A, x10 magnification, bright field illumination; B, x20 magnification, bright field illumination; C, x40 magnification, bright field illumination; D, x40 magnification, dark field illumination.

heavily labelled than the walls of the furrow (see especially 33E and F). Figure 34 shows embryos fixed during the latter stages of the second cleavage. A similar pattern of labelling is seen to that in Figure 33. Three membrane regions within the embryo can be identified on the basis of labelling: the embryo surface which is heavily labelled (see 34A and B), the walls of the furrow which are lightly labelled, and the leading edge of the furrow which appears as a distinct ring of heavily labelled material within the cleavage membrane (arrowed in Figure 34A, C, E, G).

Figure 35 shows sections of *Xenopus laevis* albino embryos labelled at fertilisation, and fixed at stage 8. The surface of these embryos is the most heavily labelled region and very little of the label has penetrated. However, high power photographs of the sections indicate silver grain concentration above background on the internal membranes of the blastomeres. This is shown by a visible demarkation between the cells (e.g. Figure 35F and H).

5.4 Investigation of *Xenopus laevis* Cell Cleavage:

Discussion

The results outlined above are in consistent with those described by Byers and Armstrong (1986). The cleavage furrow membrane in dividing *Xenopus laevis* embryos is mosaic in character. In labelled embryos the walls of the furrow contain a low density of label whilst the leading edge of the furrow shows very high incorporation of label. The surface of the embryo appears heavily labelled with the intensity of label stopping significantly at the edge of the furrow.

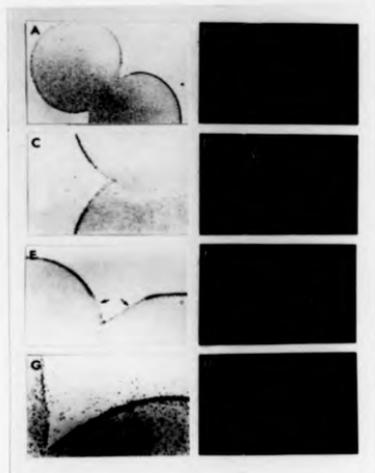


Figure 33 Investigation of Xenopus laevis Cell Cleavage:
Histo-autoradiography of Embryos Surface Labelled at 1-cell
Fixed During the First Cell Division

Xenopus laevis albino embryos were surface labelled with 125 Iodine before first cleavage. The embryos were washed after labelling and left to develop until midway through the first cell cleavage. The embryos were fixed, embedded in wax and sectioned on a microtome. Autoradiography was carried out on the wax sections (Method M.33). Arrows mark the region of the cleavage furrow with reduced silver grains.

The sections are as follows: A, x10 magnification, bright field illumination; B, x10 magnification, dark field illumination; C, x20 magnification, bright field illumination; D, x20 magnification, dark field illumination; E, x20 magnification, bright field illumination; F, x20 magnification, dark field illumination; G, x40 magnification, bright field illumination; H, x40 magnification, dark field illumination.

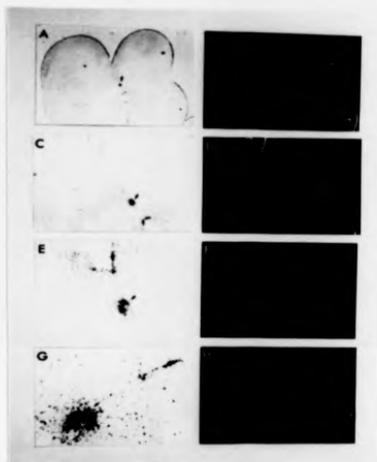


Figure 34 Investigation of Xenopus laevis Cell Cleavage:
Histo-autoradiography of Embryos Surface Labelled at 1-cell,
Fixed During the Second Cell Division

Xenopus laevis albino embryos were surface labelled with ^{125}I iodine before first cleavage. The embryos were washed after labelling and left to develop until midway through the second cell cleavage. The embryos were fixed, embedded in wax and sectioned on a microtome. Autoradiography was carried out on the wax sections (Method M.33). Arrows mark the intensely labelled leading edge of the cleavage furrow.

The sections are as follows: A, x10 magnification, bright field illumination; B, x10 magnification, dark field illumination; C, x20 magnification, bright field illumination; D, x20 magnification, dark field illumination; E, x40 magnification, bright field illumination; F, x40 magnification, dark field illumination; G, x100 magnification, bright field illumination; H, x10 magnification, dark field illumination. H shows a control unlabelled Xenopus laevis albino embryo midway through the second cell cycle.

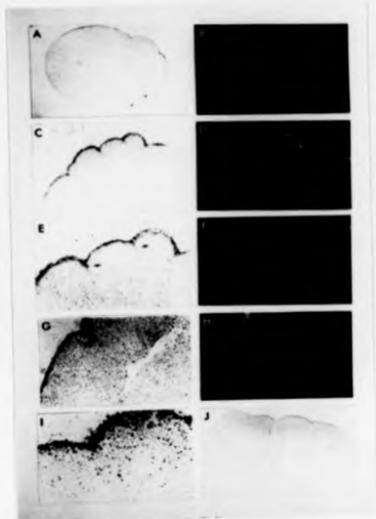


Figure 35 Investigation of *Xenopus laevis* Cell Cleavage:
Histo-autoradiography of Embryos Surface Labelled at 1-cell.
Fixed at Stage 6

Xenopus laevis albino embryos were surface labelled with ¹²⁵Iodine before first cleavage. The embryos were washed after labelling and left to develop to stage 6. The embryos were fixed, embedded in wax and sectioned on a microtome. Autoradiography was carried out on the wax sections (Method M.33). Arrows mark the newly forming cleavage furrows.

Sections are as follows: A, x10 magnification, bright field illumination; B, x10 magnification, dark field illumination; C, x20 magnification, bright field illumination; D, x20 magnification, dark field illumination; E, x40 magnification, bright field illumination; F, x40 magnification, dark field illumination; G, x100 magnification, bright field illumination; H, x100 magnification, bright field illumination; I, x100 magnification, bright field illumination; J, x10 magnification, bright field illumination. J shows a control unlabelled *Xenopus laevis* albino embryo at stage 6.

Byers and Armstrong suggest a dynamic, directed contractile event occurring on the surface of the embryos prior to the penetration of the furrow into the cytoplasm. Membrane lying in the path of the furrow is thought to move towards the future furrow initiation site. The furrow then deepens and carries the cell surface membrane towards its leading edge in the centre of the embryo. At the same time new membrane is inserted into the lateral walls of the furrow to enable the expansion of membrane area required for cleavage. Cell surface membrane lying outside of the path of the furrow is relatively unaffected by the contractile and penetration events.

There is evidence to support the initial contractile event preceding furrow formation. Observations on the movement of cortical pigment granules in cleaving newt embryos indicated the movement of granules in the plane of the future furrow and directed towards the future initiation site (Selman and Waddington, 1955). Hara (1977) described a surface contraction wave which originates from the area of the animal pole and progresses downwards towards the vegetal pole. When the contraction wave reaches the vegetal pole the cleavage furrow appears at the animal pole.

Byers and Armstrong carried out transmission electron-microscopy (TEM) on their samples and illustrated that the heavily labelled region at the leading edge of the furrow was characterised by the presence of microvilli. These microvilli increase the membrane area in the leading edge region and therefore artificially increase the density of label. They suggest that the leading edge of the furrow

includes membrane anchors for the underlying contractile ring of actin microfilaments.

The edges of the cleavage furrow in surface labelled Xenopus embryos show low silver grain intensity suggesting that the cleavage membrane comprises newly synthesised membrane. TEM studies by Byers and Armstrong indicated the presence of vesicles underlying the furrow which supports the suggestion that cytoplasmic precursors are the principal source of new membrane. However, observations of embryos at later stages indicated label present on intercellular membranes (Figure 38). It is therefore suggested that the furrow membrane is essentially mosaic in nature, comprising mainly *de novo* synthesised membrane alongside very low amounts of pre-existing internalised membrane.

5.5 The Origin of Ciliated Cells from the Double Layered Epidermis

In Xenopus embryos the epidermis differentiates into two major cell types at a relatively early stage. The most common cell is mucous secreting (Pflugfelder and Schubert, 1985), but other ciliated cells are also seen (Asheton, 1996). Estimates from light microscopy suggest that about one in every ten epidermal cells in Xenopus embryos eventually becomes ciliated (Billet and Gould, 1971). During late neurula stages of development the beating cilia enable the embryo to rotate within the egg membranes. Cilia are

also thought to play a role in preventing contamination of the embryo surface.

Ciliated cells are found in all regions of the embryo with the exception of those in the neural plate, on the inner sides of the neural fold and on the sucker. Ciliated cells first appear on the epidermal surface of *Xenopus laevis* embryos at stage 13. The number of ciliated cells then increases until hatching (stage 20). From around stage 24 the cells begin to degenerate, the cilia are lost and the cells become dehydrated. In contrast the secretory cells remain intact and become the sole cell type covering the embryo (Kessel *et al.* 1974).

The differentiation of the ciliated and secretory cells coincides with a period of expansion of the epidermis following neurulation. The ectoderm at the end of gastrulation is comprised of several layers of cells. On formation of the neural tube a significant proportion of the ectoderm is removed from the surface of the embryo. The remaining epidermis must then expand during the following elongation of the embryo. This expansion of the ectoderm involves movement of cells from the interior to the surface, as well as a rearrangement of the cells in the innermost layer so that the epidermis becomes a double layered sheet of cells. Cells migrating from the inner layer of the epidermis to the outer layer are thought to become the ciliary cells. Stereoscan studies of the surface of the neural fold stage embryo shows many pit-like depressions, each depression appearing to correspond to a single ciliated cell (Billet and Courtenay, 1973). In stage 15 *Xenopus* embryos

electron-microscope studies show cells containing large numbers of centrioles and dense pre-centriole material lying below the outer epidermal cell layer (Steinman, 1968; Billet and Gould, 1971).

The secretory cells are columnar in shape and contain large numbers of pigment granules (Billet, 1968) and apical mucous droplets (Steinman, 1968). In contrast, the ciliary cells are broader and more rounded and they lack pigment granules. The nucleoli are prominent, and cytoplasmic inclusions such as liposomes and yolk platelets are seen in the bottom two thirds of the cell. The ciliated cells are isolated from each other, and are regularly spaced over the epidermal surface (Billet, 1968). The absence of pigment granules in the ciliated cells means either that the precursors of the ciliated cells lie beneath the surface, or that the cells lose their pigment granules very quickly upon differentiation (Billet, 1968).

Evidence on the origin of the ciliated cells in Xenopus embryos has come from observational studies. The remaining work in this chapter uses the histo-autoradiographic technique as a direct method of investigating the origin of the ciliated cells in Xenopus laevis embryos.

5.6 Investigation of Ciliation: Histo-autoradiography of Labelled Xenopus embryos

Figure 36 summarises the experiment carried out to investigate the origin of ciliated cells from the double layered epidermis in Xenopus laevis embryos. Albino embryos

Xenopus Embryos
Stage 10

Remove Vitelline
Membranes → Fix

Label With
I-125 → Fix

← Ciliation

Stage 28
Embryos → Fix

Stage 28
Embryos

Remove Vitelline
Membranes → Fix

Label With
I-125 → Fix

Figure 38 Experimental Procedure to Investigate the Origin
of Cilia from the Embryonic Mesoderm in *Hydra laevis*
Embryos

See Methods M. 9, M. 32 and M. 33.

were labelled at stage 10 prior to ciliation. They were then fixed immediately as controls, or allowed to develop through ciliation before fixing. Embryos were also labelled and fixed at Stage 28 as controls. Iodination, fixation and the autoradiography were carried out on albino embryos as described in Methods M.9, M.32 and M.33.

Figure 37 shows the control embryos from this experiment. A and B show sections from stage 10 labelled embryos which were fixed immediately following labelling. There is uniform labelling of the embryo surface with fairly low non-specific background. The remaining sections are from *Xenopus laevis* embryos which were labelled and fixed at stage 28. Again there is uniform labelling over the embryo surface.

Figure 38 shows sections from embryos labelled and washed at stage 10, which were fixed following ciliation at stage 28. The sections show that labelling of the embryo surface is not continuous but is broken up by small regularly spaced unlabelled patches. The effect is very clear and is seen over the length of the labelled embryo surface. This pattern was observed in all the embryos labelled before ciliation and fixed following ciliation.

The most likely explanation for the labelling pattern seen is that the composition of the embryo surface has changed between labelling of the embryo surface and fixation. The control embryos which were labelled and fixed at Stage 28 indicated that all regions of the embryo surface at this stage will incorporate the label. The labelling pattern seen is clearly consistent with the migration of cells from the inner unlabelled layer of the epidermis to the

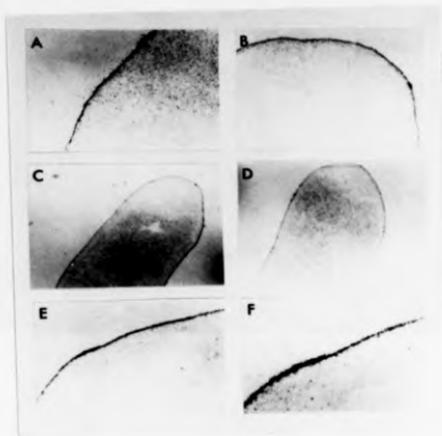


Figure 37 Investigation of Ciliation in Xenopus laevis
embryos: Histo-autoradiography of Controls

Stage 10 and stage 28 Xenopus laevis albino embryos were surface labelled with ^{125}I Iodine. The embryos were washed and fixed after labelling then embedded in wax and sectioned on a microtome. Autoradiography was carried out on the wax sections (Method M.33).

The sections are shown under bright field illumination as follows: A and B, stage 10 embryo, x20 magnification; C and D, stage 28 embryo, x10 magnification; E and F, stage 28 embryo, x20 magnification.

outer, labelled layer. To confirm that the unlabelled areas of the membrane correspond to ciliated cells it is necessary to show the presence of cilia on the unlabelled patches.

Figure 39A and B show cilia on the surface of a stage 28 unlabelled embryo. Figure 39C shows the unlabelled patches on the surface of an 125 Iodine labelled embryo. The cilia are not clearly shown in this section, and indeed proved difficult to visualise under the light microscope due to the high refractive index of the mounting medium used. Cilia could just be seen on the unlabelled patches but were not sufficiently distinct for photography. The appearance of the cells in the unlabelled regions was dissimilar to that of cells in the neighbouring labelled regions. The labelled cells appeared more columnar and had a bright shiny surface thought to correspond to the mucous covering of the secretory cells. The unlabelled cells had a less distinct appearance and lacked the shiny surface covering. These observations again are not clear in photographs of these sections (shown in Figure 39), since the observations were made by changing the focussing plane of the microscope.

5.7 Investigation of Ciliation in *Xenopus laevis* embryos: Discussion

Changes occur in the cell surface membrane between stage 10 and stage 28 *Xenopus laevis* embryos. The observations were consistent with the migration of ciliary cells from the inner layer of the epidermis to the outer layer. Label-free patches of the embryo surface are thought to be caused by ciliated cells and this supports for the idea

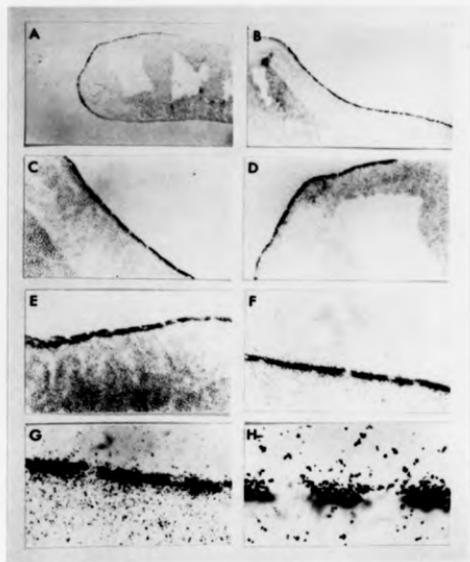


Figure 38 Investigation of Ciliation in *Xenopus laevis*
embryos: Histo-autoradiography of Embryos Surface Labelled at
Stage 10, Fixed at Stage 28

Stage 10 *Xenopus laevis* albino embryos were surface labelled with ^{125}I Iodine. The embryos were washed after labelling and left to develop to stage 28. The embryos were fixed, embedded in wax and sectioned on a microtome. Autoradiography was carried out on the wax sections (Method M.33).

The sections are shown under bright field illumination as follows: A and B, x10 magnification; C and D, x20 magnification; E and F, x40 magnification; G and H, x100 magnification.

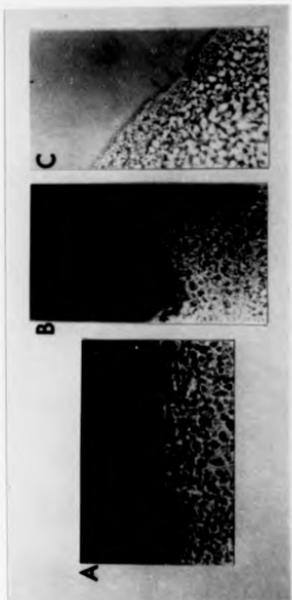


Figure 38 Investigation of Ciliation in *Xenopus laevis* embryos: Appearance of Cilia in the Epidermis of Stage 28 Embryos

Stage 10 *Xenopus laevis* albino embryos were surface labelled with 125 Iodine. The embryos were washed after labelling and left to develop to stage 28. The embryos were fixed, embedded in wax and sectioned on a microtome. Autoradiography was carried out on the radioactive wax sections (Method M.33). Control *Xenopus laevis* embryos at stage 28 which had not been iodinated were also fixed, embedded in wax and sectioned.

The sections are as follows: A and B, epidermis of stage 28 non-iodinated *Xenopus laevis* embryo. The slides were prepared using 1:1 glycerol/PBS mountant, x40 magnification; C, epidermis of a stage 28 surface labelled *Xenopus laevis* embryo. The slide was prepared using DPX toluene based mountant, x40 magnification.

It is possible that turnover of cell surface components by cells present on the external surface of the embryo could cause the patterned effect seen in the labelled embryo sections in Figure 38.

that ciliated cells originate in the inner layer of the epidermis and migrate to the outer layer. However, further evidence is needed to confirm that the label-free patches of membrane on the embryo surface correspond to the presence of ciliated cells.

CHAPTER 6

RESULTS: IDENTIFICATION AND CHARACTERISATION OF THE 2B12 MONOCLONAL ANTIBODY ON SECTIONS

6.1 Introduction

The work in this chapter involves the characterisation of the 2B12 monoclonal antibody on embryo and tissue sections. This work follows indirectly from that carried out in Chapters 3 and 4 where the object of the experiments was to detect membrane proteins showing temporal variation during Xenopus laevis development. Although several membrane proteins showing variation in expression were detected, there was insufficient time to characterise and investigate them further. The work in this chapter and in Chapter 7 involves the characterisation of the 2B12 antigen detected in Xenopus laevis embryos which shows temporal variation in its expression, and which appears to be a membrane component.

6.2 Preparation of the 2B12 Monoclonal Antibody

The preparation of the 2B12 monoclonal antibody, including immunisations, fusion, screening and cloning were carried out by Dr. E.A. Jones. Xenopus laevis adult frog brain was removed and homogenised in PBS. Samples of the brain homogenate were injected into Balb/c mice as outlined in Method M.24. Four days following the final tail-vein boost, the spleens were removed from the mice and fusions were carried out (Method M.26). Supernatant from positive

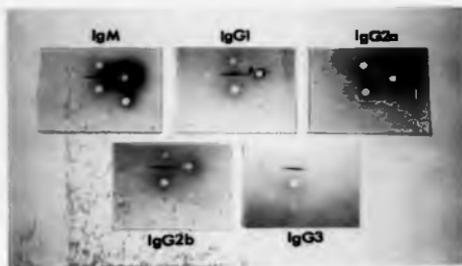


Figure 40 Ouchterlony Immunodiffusion Analysis of the 2B12 Antibody

An Ouchterlony immunodiffusion assay was carried out on the 2B12 antibody as described in Method M.31.

The wells are as follows: Centre well, 2B12 antibody supernatant; Well 1 (top), RAM serum (positive control); Well 2 (clockwise from top), test antibody undiluted; Well 3, test antibody diluted 1/5; Well 4, test antibody diluted 1/25; Well 5, test antibody diluted 1/125; Well 6, test antibody diluted 1/625.

tissue culture wells was screened on *Xenopus laevis* embryo sections for activity using indirect immunofluorescence. The 2B12 antibody was subsequently cloned using the serial dilution technique.

6.3 Ouchterlony Test

An Ouchterlony immunodiffusion test (Method M.31) was carried out to determine the class of the 2B12 monoclonal antibody. The results of this experiment are shown in Figure 40. The RAM serum positive control gave a strong signal against 2B12 on each slide. A faint signal was seen with the 2B12 antibody against the most concentrated solution of anti-Mouse IgG1, shown in Figure 40 with an arrow. No other signal above background was observed. The cross-reactivity of 2B12 with anti-IgG1 was reproducible. It was therefore concluded that the 2B12 monoclonal antibody is a mouse antibody of class Gamma-1.

6.4 Detection of 2B12 Antibody Binding on Xenopus Embryo Sections

Xenopus laevis embryos at varying developmental stages were fixed, embedded in acrylamide and sectioned on the cryostat as described in Method M.27. The embryo sections were screened for 2B12 antibody binding using indirect immunofluorescence. A summary of the entire screening technique is shown in Figure 41.

Xenopus embryo sections were screened with 2B12 to detect the initial appearance of the antigen. An example of the embryo sections obtained are shown in Figure 42. 2B12

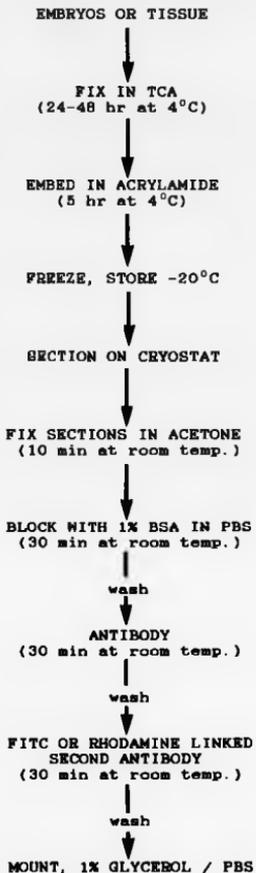


Figure 41 Experimental Protocol for Screening Sections
using Indirect Immunofluorescence

See Method M. 27.

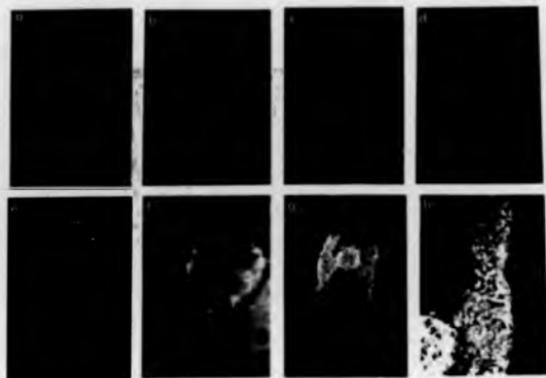


Figure 42 2B12 Antibody Binding on Sections from Late
Neurula Stage Xenopus laevis Embryos

Stage 22-28 Xenopus laevis embryo sections were screened with 2B12 using indirect immunofluorescence. Transverse anterior embryo sections are shown: a, stage 22 embryo, x10 magnification; b, stage 22 embryo, x40 magnification; c, stage 23 embryo, x10 magnification; d, stage 23 embryo, x40 magnification; e, stage 26 embryo, x10 magnification; f, stage 26 embryo, x40 magnification; g, stage 28 embryo, x10 magnification; h, stage 28 embryo, x40 magnification.

Nt, neural tube; No, notochord; Ep, epidermis; En, endoderm.

antibody binding is first detected in the most anterior sections of stage 23 Xenopus laevis embryos (Figure 42c and d). The observed fluorescence is speckled in nature appearing first in the ventral region of the neural tube. In stage 24 Xenopus laevis embryos, fluorescence is detected in more posterior sections, and is observed in both dorsal and ventral neural tube. By stage 25, antibody binding is observed in transverse sections from the posterior most region of the Xenopus laevis embryo. Figure 42e shows fluorescence in the neural tube of a stage 26 anterior section. Under high power, fluorescence is just visible in the somites being mostly in the dorsal tips. By stage 28 (Figure 42g and h), strong fluorescence is seen in the neural tube and in the somites. Associated tissue such as the epidermis, the endoderm and the notochord are negative at this stage for 2B12 binding.

Figures 43, 44 and 45 all show the screening of 2B12 on stage 42 Xenopus laevis sections. In Figure 43, the forebrain optic region of a transversely sectioned embryo is shown after screening with the 2B12 antibody. The diencephalon region of the primitive brain is strongly positive for 2B12 activity. This region has developed directly from the neural tube which was the initial site of 2B12 binding in Stage 23 embryo sections (Figure 42). Both the internal grey matter, and the more external white matter of the brain appear positive, but the signal from the white matter is more intense. In contrast, the diocoel cavity within the diencephalon shows only background fluorescence.

2B12 activity is more intense in the dorsal regions

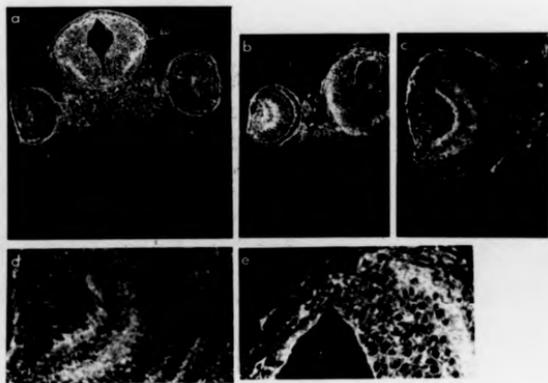


Figure 43 2B12 Antibody Binding on Sections from Stage 42
Xenopus laevis Embryos (1): Forebrain Region of the Embryo

Stage 42 Xenopus laevis embryo sections were screened with 2B12 using indirect immunofluorescence. Transverse sections through the forebrain of the embryo are shown: a and b, forebrain region, x10 magnification; c, eye, x40 magnification; d and e, eye, x100 magnification.

Ey, eye; Ph, pharynx; En, endoderm; Ch, choroid coat; Pl, pigment layer; L, lens; R, retina. The numbers in section d refer to the different cell layers within the eye as follows: 1, nerve fibres; 2, ganglion layer; 3, inner plexiform layer; 4, inner nuclear layer; 5, outer plexiform layer; 6, outer nuclear layer; 7, receptor layer; 8, pigmented epithelium.

of stage 42 Xenopus laevis embryo sections. The pharynx and other endodermal tissue appear negative. The epidermis is negative in the ventral regions of the section, but positive in the most dorsal region around the brain. Significant 2B12 binding is seen in the eye. The retina shows strong activity with a differing intensity of fluorescence observed in bands across the retina (Figure 43d). The negative band of tissue (band 8) around the retina is the pigmented epithelium. This again is surrounded by the choroid which is positive for 2B12 binding. No 2B12 activity is seen in the lens. High magnification of the optic region of these sections (Figure 43d and e) shows the fluorescent signal from individual cells. On the outermost region of the eye, the fluorescence is speckled. On cells in the retina the fluorescence appears to be derived from tissue surrounding the cells (possibly the cell membranes) or from connective tissue. The positive tissue associated with the eye seen in sections 43b and c (arrowed) are cranial ganglia or a section of the optic nerve.

Figure 44 shows more posterior sections from a transversely sectioned stage 42 Xenopus laevis embryo screened with 2B12. Sections 44a and b show the mid brain region of the embryo, around the auditory vesicles. The brain tissue in these sections, present here as the medulla oblongata, shows similar fluorescence to that described previously for the forebrain. The auditory capsules are positive but the internal auditory vesicles are negative for 2B12 binding. The notochord is negative but the surrounding parachordal cartilage (which develops from the somites) is

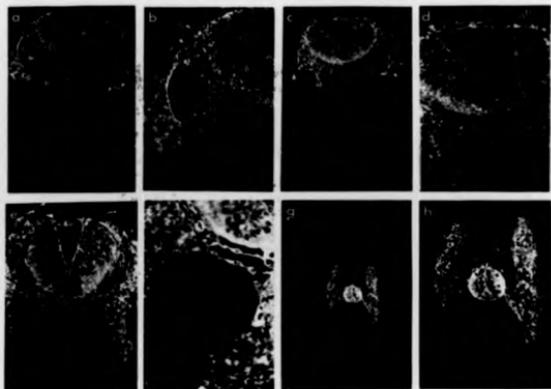


Figure 44 2B12 Antibody Binding on Sections from Stage 42
Xenopus laevis Embryos (2): Midbrain and Posterior Regions of
the Embryo

Stage 42 Xenopus laevis embryo sections were screened with 2B12 using indirect immunofluorescence. Transverse sections from the mid- and hind-brain of the embryo and from the tail region are shown: a, mid-brain, x10 magnification; b, mid-brain, x20 magnification; c, hind-brain, x10 magnification; d and e, hind-brain, x20 magnifications; f, hind-brain, x40 magnification; g, tail region, x10 magnification; h, tail region, x20 magnification.

Av, auditory vesicle; Ac, auditory capsule; Ph, pharynx; Bv, blood vessel; Sc, sclerotome; Ao, aorta; M, myotome; Pc, parachordal cartilage; B, brain; Wh, white matter; Gr, grey matter; Cg, cranial ganglia; Nt, neural tube; No, notochord; Ep, epidermis; D, dermis.



Figure 45 2B12 Antibody Binding on Sections from Stage 42
Xenopus laevis Embryos (3): Longitudinal Sections

Stage 42 Xenopus laevis embryo longitudinal sections were screened with 2B12 using indirect immunofluoresence. a, optic region, x10 magnification; b, eye, x20 magnification; c, section through the notochord, x20 magnification; d, section through the brain and neural tube, x10 magnification.

Av, auditory vesicle; Ac, auditory capsule; M, myotome; Pc, parachordal cartilage; FB, forebrain; MB, midbrain; HB, hindbrain; Wh, white matter; Gr, grey matter; Cg, cranial ganglia; Ey, eye; Sc, scleroid coat; Pl, pigment layer; R, retina; L, lens; Nt, neural tube; Nc, notochord; Ep, epidermis; D, dermis.

<u>TISSUE</u>	<u>2B12 BINDING ACTIVITY</u>
BRAIN (grey and white matter)	+
EYE- retina, choroid	+
- lens, pigmented epithelium	-
AUDITORY CAPSULE	+
AUDITORY VESICLE	-
OLFACTORY ORGAN	+
CRANIAL GANGLIA	+
OPTIC NERVE	+
EPIDERMIS- dorsal surrounding brain	+
- ventral	-
DERMIS	-
NOTOCHORD	-
MYOTOMES	+
PARACHORDAL CARTILAGE	+
SCLEROTOME	+
PHARYNX	-

Table 10 2B12 Activity on Stage 42 Xenopus laevis Embryo
Sections

This table summarises results shown in Figures 43, 44 and 45. + indicates significant 2B12 activity; - indicates no significant 2B12 activity.

positive. The dorsal aorta and the anterior cardinal vein are both negative, but the cranial ganglia are positive.

Sections 44c and d show the transverse mid-brain region of a stage 42 Xenopus laevis embryo screened with 2B12. Section 44c emphasizes the dorsal/ventral distribution of 2B12 activity at this stage in the embryo development. Figure 44e and f show the hindbrain. The tissue ventral to the notochord is the aorta which is negative for antibody binding. The tail sections in Figure 44g and h show 2B12 activity in the neural tube and the myotomes. Note that the dorsal region of the myotome shows stronger 2B12 activity than the ventral region.

Figure 45 shows longitudinally sectioned stage 42 Xenopus laevis embryos screened with 2B12. Section 45a clearly shows 2B12 binding in the eyes, in the auditory capsule and in the olfactory pit. Brain tissue is positive as before. The section clearly shows the myotomes in the posterior of the embryo, and the sclerotome and parachordal cartilage in the more anterior of the embryo which show strong antibody binding. Figure 45c is a more skew section taken through a Xenopus embryo. This indicates 2B12 activity over the length of the brain tissue in the embryo. A summary of the activity of 2B12 on stage 42 Xenopus laevis embryo sections is shown in Table 10.

6.5 Characterisation of 2B12 Antibody Binding on Adult Frog Tissue

Figures 46-50 show the activity of the 2B12 antibody on adult Xenopus laevis tissue sections. Screening

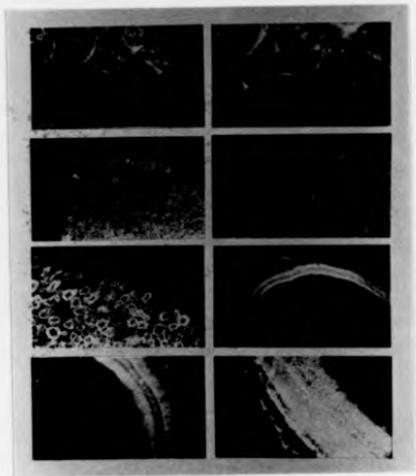


Figure 46 2B12 Antibody Binding on Xenopus laevis Adult Frog Tissue Sections (1): Brain, Nerve and Eye

Xenopus laevis adult frog tissue sections were screened with 2B12 using indirect immunofluoresence. a, brain, x20 magnification; b, brain, x40 magnification; c, nerve, x10 magnification; d, nerve, x20 magnification; e, nerve, x40 magnification; f, eye, x10 magnification; g, eye, x20 magnification; h, eye, x40 magnification.

N, nerve; Nm, nerve membrane; Ma, myelinated axon; Ms, myelin sheath; Sc, scleroid coat; Pl, pigment layer; L lens; R, retina.

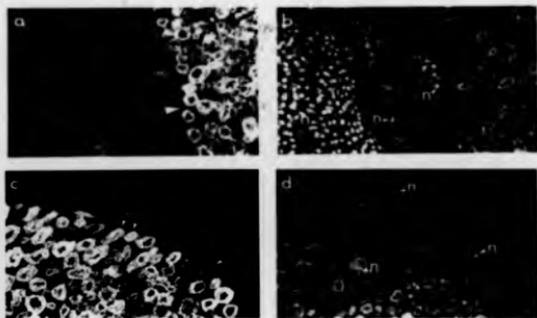


Figure 47 2B12 Antibody Binding on Xenopus laevis Adult
Frog Tissue Sections (2): Nerve and Muscle

Sections of nerve and muscle tissue from Xenopus laevis adult frog were screened for 2B12 binding using immunofluorescence and with quinacrine, to indicate the presence of cell nuclei. Sections were screened as normal using BSA and 2B12. Rhodamine-RAM was used to distinguish between quinacrine fluorescence and that from the antibody. Sections were incubated in 0.02% quinacrine for 8 min following incubation in rhodamine-RAM. Sections are shown at x40 magnification: a, nerve and muscle, 2B12 binding; b, nerve and muscle, cell nuclei; c, nerve, 2B12 binding; d, nerve, cell nuclei.

M, muscle; N, nerve; CT, connective tissue; n, nuclei; Ax axon; Nm, nerve membrane.

of the antibody is by indirect immunofluorescence. Figure 46 shows 2B12 binding on nervous tissue from different sources within a Xenopus laevis frog. Sections 46a and b show brain tissue which is totally positive for antibody binding. The antibody signal appears as a mixture of speckled (white arrows) and fibrous (black arrows) fluorescence. Figure 46c, d and e show 2B12 activity on a frog nerve. The individual myelinated neurons are clearly visible within the nerve fibre. The myelin sheath around each neuron shows strong antibody binding but the axon cylinders within the myelin sheaths show none. The nerve membrane surrounding the cluster of myelinated neurons is also negative for 2B12 activity.

Figure 46f-h show 2B12 on Xenopus laevis eye sections. The retinal tissue, which comprises large numbers of rod and cone cells, shows strong antibody binding whereas the pigment layer surrounding the retina is negative. The outermost sclerotic layer is positive, whereas the lens is negative. Figure 47 shows sections of nerve fibres alongside muscle and connective tissue. The sections were screened with 2B12 and a rhodamine linked second antibody, and also with quinacrine which binds to cell nuclei. Figure 47a and c show the sections viewed with red fluorescence for rhodamine visualisation. The myelinated nerve axons are positive for 2B12 binding whereas the muscle tissue and associated connective tissue (in Figure 47a) are negative. When the sections are viewed under blue light for quinacrine visualisation, the nuclei of the muscle and connective tissue are clearly evident. At high power (Figure 47d) the quinacrine staining shows the cell nuclei of the Schwann

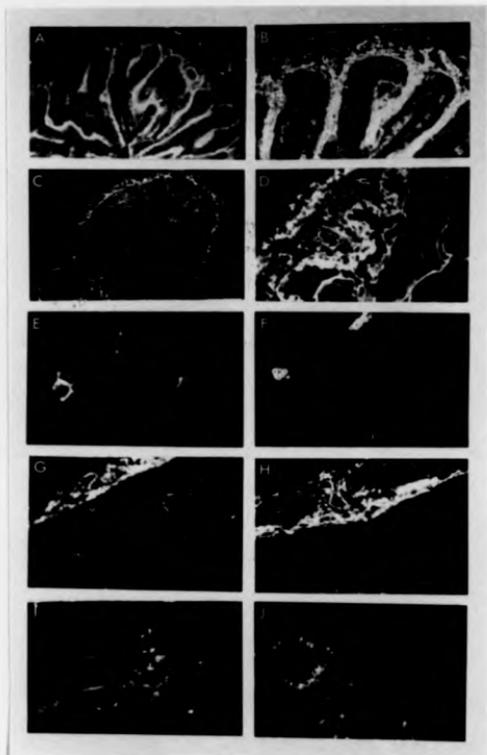


Figure 4B 2B12 Antibody Binding on Xenopus laevis Adult Frog Tissue Sections (3): Gut, Kidney, Muscle, Skin and Liver

Xenopus laevis adult frog tissue sections were screened with 2B12 using indirect immunofluoresence. A, gut, x10 magnification; B, gut, x40 magnification; C, kidney, x10 magnification; D, kidney, x40 magnification; E, muscle, x40 magnification; F, muscle, x40 magnification; G, skin, x10 magnification; H, skin, x20 magnification; I, skin, x40 magnification; J, liver, x40 magnification.

CM, circular muscle; LM, longitudinal muscle; Pl, plexes; Sm, submucosa; Lu, lumen; Tm, tunica mucosa; Ur, ureter; Fc, fibrous coat; Cx, cortex; Me, medulla; N, nerve; M, muscle; MG, mucous gland; Ep, epidermis; GD, gland duct; Ch, chromatophores; Vn, vein.

cells, as well as nuclei in the nerve membrane which surrounds the myelinated axons.

Figure 48 shows the screening of the 2B12 antibody on various other adult Xenopus laevis tissues. Figure 48A and B show 2B12 activity on the Xenopus frog gut. The outermost layer of the gut comprises two major layers of muscle, a layer of circular muscle and a layer of longitudinally oriented smooth muscle fibres. Both of these layers show no 2B12 activity. Between the layers is a vascular plexus and a nerve plexus containing numerous small ganglia. This intermediate layer is positive for 2B12. The next innermost layer of the gut is the submucosa which contains coarse connective tissue with some elastic fibres. Within it are plexes of blood vessels and nerves, and ganglion cells. The nerves present in the submucosa layer form part of both the sympathetic and parasympathetic nervous systems. The submucosa shows positive 2B12 binding. Within the submucosa is the tunica mucosa which is essentially a wet surface membrane of epithelial cells lubricated by mucous. The tunica mucosa contains numerous mucous secreting glands with connective tissue, blood and lymph capillaries. The tunica mucosa does not contain nerve cells. Both the tunica mucosa and the innermost lumen of the gut are negative for 2B12 binding.

Figure 48C and D show kidney sections from adult Xenopus laevis frogs screened with 2B12. The outer fibrous coat of the kidney shows no 2B12 binding. Within the fibrous coat is a positive layer of tissue which corresponds to a region of circulating blood vessels. Within the cortex and

medulla regions of the kidney there is a mixture of 2B12 positive and negative tissue, both intermingled, and with the positive tissue running alongside negative tissue. The specific location of nerves in the kidney has not been clearly identified. It is however known that nerves are found alongside the larger blood vessels in the kidney and this would explain the ring of antibody positive tissue around the kidney.

Figure 48E and F show adult *Xenopus laevis* thigh muscle. The muscle blocks show no 2B12 binding but there is positive tissue associated with the muscle blocks. In many cases this has the characteristic appearance of myelinated nerve fibres in cross section (Figure 48F). Under x100 white light illumination, the striations within the muscle blocks are visible. In contrast the 2B12 positive tissue is not striated suggesting that this is not muscle tissue.

Figure 48G, H and I show *Xenopus laevis* frog skin. Frog skin is characterised by the large mucous glands within the dermis. Gland ducts can be seen passing from the glands through the epidermis to the surface of the skin (Figure 48 G and I). The epidermal layer of the skin shows no 2B12 activity, whereas areas within the dermis are positive. Regions of 2B12 activity within the dermis have the characteristic appearance of myelinated axons (Figure 48I). A layer of 2B12 positive tissue runs over the mucous granules within the dermis layer of the skin which is characteristic of the known pattern of nerves. By far the highest concentration of 2B12 binding on the skin sections is seen in the muscle tissue underlying the dermis. Not all of this

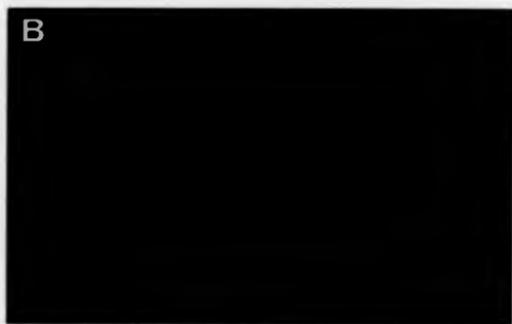
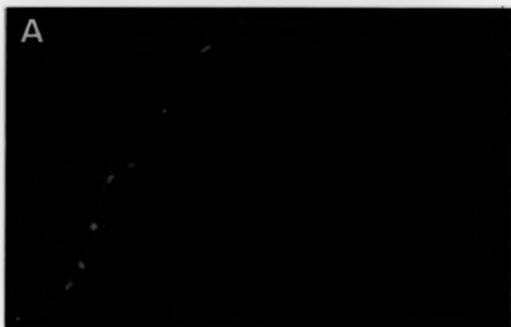


Figure 4B 2B12 Antibody Binding on Xenopus laevis Adult Frog
Tissue Sections (4): Skin and Nerve Colour Sections

Xenopus laevis adult frog skin and nerve sections were screened with 2B12 using indirect immunofluorescence. Colour photographs are shown to emphasise antibody binding. A, skin; B, nerve.

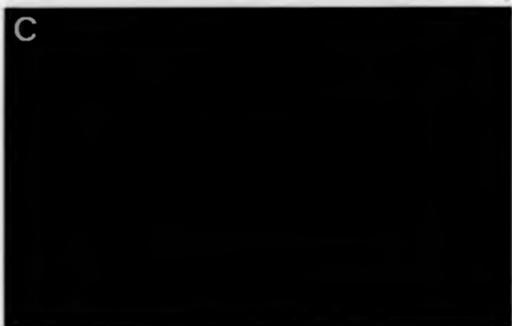


Figure 50 2B12 Antibody Binding on Xenopus laevis Adult Frog
Tissue Sections (5): Gut and Kidney Colour Sections

Xenopus laevis adult gut skin and kidney sections were screened with 2B12 using indirect immunofluoresence. Colour photographs are shown to emphasise antibody binding. C, gut; D, kidney.

muscle tissue shows antibody binding but there is a continuous stretch of tissue running beneath the skin which is positive for 2B12.

Figure 48K shows a section of Xenopus laevis liver. The liver tissue is generally uniform in nature, being composed of epithelial cells that are interconnected to form a continuous three-dimensional lattice. The section shows a central vein within the liver tissue. The walls of this vein show 2B12 activity. The majority of the liver tissue is negative.

Colour photographs of the screening of 2B12 on tissue sections from adult Xenopus laevis are shown in Figures 49 and 50. These are included to illustrate the appearance of 2B12 fluorescence on sections.

6.6 Characterization of 2B12 on Adult Mouse Tissue

Figures 51 and 52 show the activity of the 2B12 antibody on tissue sections from adult Balb/c mice. Figure 51a and b show spinal cord sections screened with 2B12. The appearance of the antibody binding on the sections is different to that seen on Xenopus spinal cord sections (data not shown). On the mouse sections the majority of the tissue is negative with a few positive strands of fluorescence. In comparison, all tissue present on Xenopus laevis adult spinal cord sections screened with 2B12 is positive. In cross-section the Xenopus spinal cord white matter shows the fluorescence patterns characteristic of myelinated axons.

Figure 51c-f show mouse thigh muscle sections screened with 2B12. Each of the muscle blocks is negative but

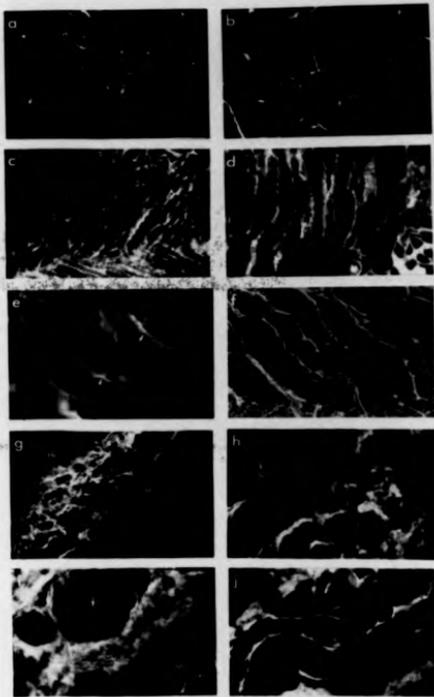


Figure 51 2B12 Antibody Binding on Tissue Sections from
Adult Mouse (1): Spinal Cord, Muscle and Skin

Adult mouse tissue sections were screened with 2B12 using indirect immunofluoresence. a, spinal oord, x10 magnification; b, spinal cord, x40 magnification; c, muscle, x10 magnification; d, muscle, x40 magnification; e, muscle, x100 magnification; f, muscle, x100, viewed under white light; g, skin, x10 magnification; h, skin, x20 magnification; i, skin, x40 magnification; j, skin, x100 magnification.

Hr, hair; D, dermis; M, muscle. Arrows indicate 2B12 binding in sections a, b and e.

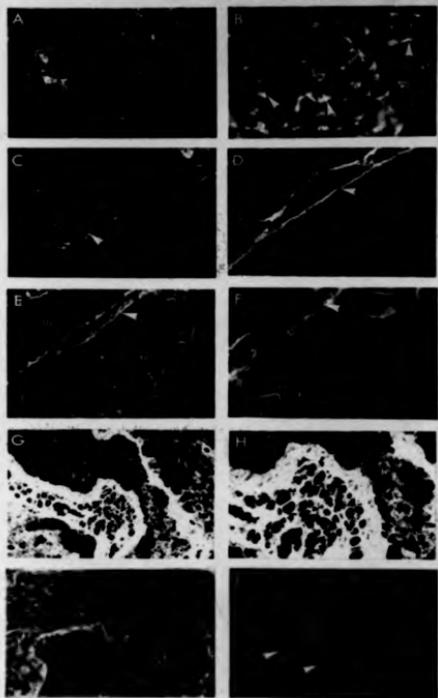


Figure 52 2B12 Antibody Binding on Tissue Sections from
Adult Mouse (2): Liver, Kidney, Lung and Heart

Adult mouse tissue sections were screened with 2B12 using indirect immunofluoresence. A, liver, x10 magnification; B, liver, x100 magnification; C, kidney, x10 magnification; D, kidney, x20 magnification; E, kidney, x40 magnification; F, kidney, x100 magnification; G, lung, x10 magnification; H, lung, x40 magnification; I and J, heart, x10 magnification. Arrows indicate 2B12 binding.

Vn, vein; Me, medulla; Co, cortex; CM, cardiac muscle.

positive tissue is present between the blocks. Figure 51f shows the muscle section viewed under white light with the striations clearly visible. Comparison with the same section viewed for fluorescence (Figure 51e) indicates that muscle tissue is negative for 2B12 but that tissue present on the surface of the muscle blocks shows 2B12 activity. The appearance of 2B12 binding on mouse muscle is, therefore, similar to that seen on the adult *Xenopus laevis* muscle sections (Figure 48e and f).

Figure 51g-j show the screening of 2B12 on mouse skin sections. The histology of mammalian skin differs from that of amphibians. The large mucous glands are absent and the epidermis represents a smaller percentage of the cross-sectional area. In Figure 51g the epidermis and large amounts of the dermis show positive 2B12 binding. This differs from that seen in frog skin where the epidermis was negative for the antibody. The underlying tissue comprises fat cells which are negative, and muscle tissue which shows similar fluorescence to that seen in the muscle sections from *Xenopus* (Figure 51e-h). The large circular object in Figure 51i is a gland (possibly a sweat gland) and shows no 2B12 activity. There is no significant layer of strongly positive material lining the innermost layer of the skin as was seen in the *Xenopus* skin sections.

Figure 52A and B show mouse liver sections screened with 2B12. A similar lattice of epithelial cells is seen to that observed on the frog liver section in Figure 48J. In the mouse section, positive tissue is present surrounding the liver cells. This tissue is particularly evident surrounding

the veins which is consistent with the labelling observed in the Xenopus liver section.

Figure 52C-F show mouse kidney sections screened with 2B12. There is a clear distinction in the morphology of the kidney tissue in the mouse and the frog. Most of the tissue present in the mouse kidney shows no 2B12 activity. However, strands of positive tissue are seen which are mainly present between the cortex and medulla layers. This is most likely to correspond to the layer of major blood vessels which encircle the kidney between these two layers. Some minor fluorescence is seen surrounding the individual cells particularly in the cortex layer.

Figure 52G and H show mouse lung tissue screened with 2B12. Mammalian lungs consist of numerous alveoli to which air passes by means of bronchioles. These sections indicate that some of the mouse lung tissue is positive for 2B12 whereas neighbouring tissue is negative. Small nerve fibres are found in the lung, particularly in the region of major bronchi and blood vessels.

Figure 52I and J show mouse heart screened with 2B12. Figure 52I shows a heart in cross section and Figure 52J a longitudinal section through the cardiac muscle. The majority of the heart tissue shows no 2B12 binding. Small regions of fluorescent tissue are seen (arrowed) particularly running through the muscle of the heart. Numerous small nerves are present in mammalian heart and the distribution of the antibody binding is consistent with a possible distribution of nerves.

6.7 Discussion

The work in this chapter has involved the characterisation of the 2B12 antibody on embryo and tissue sections in Xenopus laevis and mouse. The aim was not to perform a total and thorough histological investigation of the antibody, but rather to carry out a preliminary study of its activity on tissue and embryo sections. In this way information has been produced on the extent of activity of 2B12 binding and suggestions can now be made concerning the nature and tissue specificity of the antigen. More data would be obtained from an in depth histological study of 2B12 activity on embryo and tissue sections, and there is case for this to be carried in the future. Information on the cell and tissue types present in the sections was made by reference to the following textbooks (Rowett, 1966; Bloom and Fawcett, 1975; Romer and Parsons, 1986; Leeson and Leeson, 1987).

The first experiment involved the detection of the initial appearance of 2B12 binding on Xenopus laevis sections. This occurs in the most ventral region of the anterior neural tube in Stage 23 Xenopus laevis embryos. Antibody binding progressed into the dorsal and posterior regions of the neural tube as the embryo developed. By stage 26 activity was just visible in the somites. This is consistent with the outgrowth of neurons from the neural tube entering the somites at this stage (Nieuwkoop and Faber, 1967). This pattern of expression is similar to that of another monoclonal antibody, 2G9, which is totally nervous system specific (Jones and Woodland, in preparation). In stage 42 Xenopus laevis embryo sections, 2B12 activity was

seen in most tissues. The exceptions were the ventral epidermis, blood vessels such as the aorta, and the endodermal tissues such as the pharynx. In adult frog and mouse sections 2B12 activity was present to a lesser or greater extent in all the tissues types studied.

2B12 appears to be a monoclonal antibody specific for tissue of the nervous system. Detection of 2B12 activity on sections of non-nervous tissues is thought to indicate the presence of nerves within these tissues. The antibody was originally raised against Xenopus laevis brain tissue, and on nervous tissue sections 2B12 shows high activity. The appearance of 2B12 activity in somites also coincides ^{in part} with the known outgrowth of nerves from the neural tube. The distribution of 2B12 activity in non-nervous tissue on Xenopus sections may coincide with the distribution of nerves within these tissues. However there is no proof that 2B12 stains only nerves, and in some tissues (e.g. somites and the adult frog lung) more fluorescence is seen than would be expected from the known distribution of nerves. Double staining with nerve markers such as anti-neurofilament antibodies could be used to confirm the distribution of nerves in these tissues.

Assuming that the antigen is specific to nervous system, then the appearance of antibody binding on the myelinated nerve axons indicates that the antibody may be specific for a component of the myelin sheath. This is supported by the distribution of 2B12 binding on sections of the brain where a stronger signal is obtained from the

myelinated white matter than from the largely unmyelinated grey matter. However activity is also observed on non-myelinated nervous tissue such as that seen in the retina of the eye. It is therefore suggested that the 2B12 antigen is a component of myelin and of other nervous tissue membranes.

CHAPTER 7

RESULTS: BIOCHEMICAL CHARACTERISATION OF THE 2B12 ANTIGEN

7.1 Introduction

Chapter 6 established the temporal and tissue distribution of the 2B12 antigen on embryo and tissue sections. 2B12 activity appeared to be specific to nervous tissues and it was suggested that the antigen was a constituent of myelin and of other nervous system membranes. Antibody activity was first detected on Xenopus laevis embryo sections at stage 23. Significant differences were observed in the specificity of the antibody on adult frog and mouse sections, particularly in brain and spinal cord. This is likely to indicate differences in the composition of the nervous tissue in these two animals, at least at the level of expression of the epitope identified by 2B12.

The work in this chapter further characterises the 2B12 antigen using biochemical techniques.

7.2 Analysis of the Activity of 2B12 on a Western Blot

The characterisation of the 2B12 antibody on adult Xenopus laevis sections in Chapter 6 showed that antibody binding to adult frog brain and spinal cord sections was very strong. These tissues were chosen to investigate whether the 2B12 antibody gave a positive signal on a Western blot.

An adult Xenopus laevis frog was killed and the brain and spinal cord removed. These were homogenised in SDS-page sample buffer and samples were run on an acrylamide

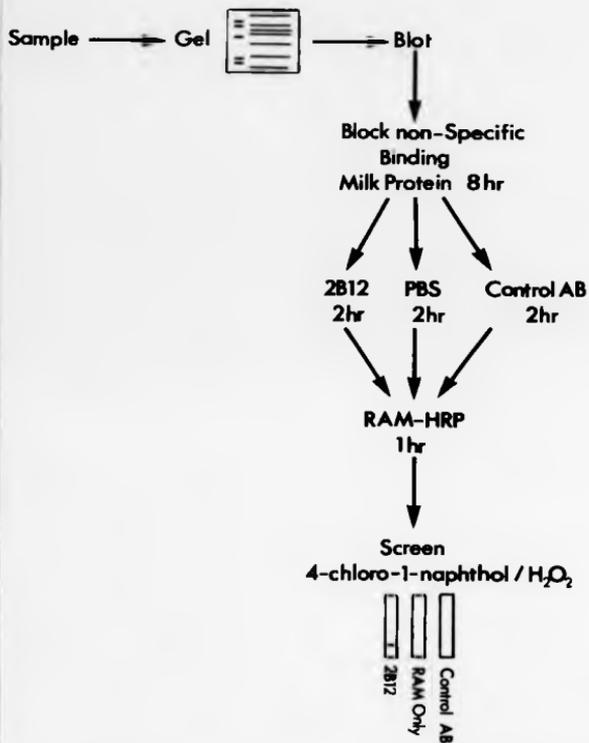


Figure 53 Experimental Protocol for Western Blotting
SDS-page Gels and Screening with 2B12 Antibody

See Method M. 15.

gel. After electrophoresis the gel was electroblotted onto nitrocellulose (Method M.15). The blot was then screened with the 2B12 antibody (Method M.28). A summary of the method used is shown in Figure 53.

The results of this experiment (Figure 54) show that 2B12 gives a positive signal on the Western blot against both the *Xenopus* brain and spinal cord homogenates. No band is seen in either of the control samples which were screened with EpA (an epidermis specific antibody. Jones, 1985) and in the absence of first antibody. This indicates that the antibody reaction obtained is specific. The signal from the brain homogenate is faint compared with that from the spinal cord. This is due to the difference in protein loading of the two tracks shown on the silver-stained tracks.

The band in the spinal cord track is 'ballooned' upwards corresponding to a maximum Mr of around 23,000. A similar ballooned area is seen on the stained gel in the spinal cord track. One explanation for the distortion of the bands is the presence of large amounts of lipid in the samples which run near the dye-front of the gel and disrupt the passage of low molecular weight proteins. This effect is not seen in the brain track possibly due to the lower sample loading. Large amounts of lipid would be expected in these tissues due to the presence of myelin in the nervous tissue.

The bands in both tracks on the Western blot correspond to areas on the gel which have not silver-stained. This effect was reproducible, the bands on the nitrocellulose always appearing at a lower Mr than the lowest stained band on the gel. Two possible explanations for this effect can be

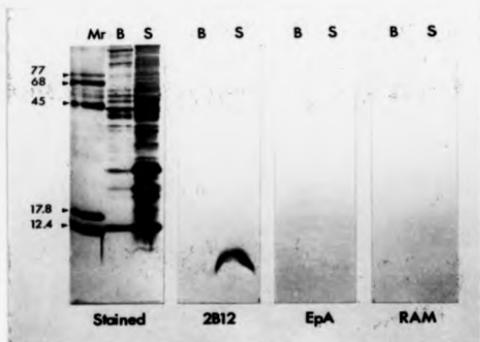


Figure 54 Western Blot of Xenopus laevis Brain and Spinal Cord Tissue Screened with 2B12

The brain and spinal cord from an adult Xenopus laevis frog were homogenised in 500 μ l of SDS-page sample buffer. The samples were centrifuged for 2 mins and insoluble material was discarded. 50 μ l volumes of the samples were run in tracks on a 12% high bis SDS-page gel. A section of the gel was fixed and silver-stained after running. The remaining portion of the gel was Western blotted. The nitrocellulose was cut into strips each containing 1 track of brain homogenate and 1 track of spinal cord homogenate. These strips were screened separately as follows: 2B12, strip incubated in 2B12 antibody and RAM-HRP; EpA, (Control antibody) strip incubated in EpA antibody and RAM-HRP; RAM, strip incubated in RAM-HRP antibody only. The silver-stained gel and nitrocellulose strips are shown.

Mr, Molecular weight markers; B, Brain; S, Spinal Cord.

suggested. Firstly, the antigen may be present on the gel at too low a concentration for detection by silver-stain. Alternatively it is possible that the antigen is present on the gel but does not stain with silver-stain. These alternatives will be discussed in more detail later in the chapter.

Figure 54 shows a positive signal obtained with the 2B12 antibody on a Western blot against brain and spinal cord tissue at approximately the same molecular weight. This technique was extended to analyse the activity of the antibody against a variety of Xenopus tissues. Figure 55 shows the results of an experiment where 2B12 antibody was screened on a blot containing adult Xenopus laevis brain, spinal cord, nerve, eye and muscle tissue, and against a stage 42 Xenopus laevis embryo homogenate.

In Figure 55 a strong band is seen in the brain, spinal cord and nerve tracks, and a weaker band in the eye track. These are all nervous tissues on which significant 2B12 binding was seen on tissue sections in Chapter 6. No band is seen in the control tracks which were screened only with the RAM-HRP antibody. The muscle track is negative which agrees with the conclusions from screening on tissue sections. Muscle sections appeared essentially negative for 2B12 binding with the exception of very low levels of fluorescence due to the presence of nerves. The level of the 2B12 antigen in muscle is almost certainly too low to be detected using the Western blot technique. Both the stage 42 total embryo homogenate and the dissected embryo homogenate are negative in this experiment. On Xenopus laevis embryo

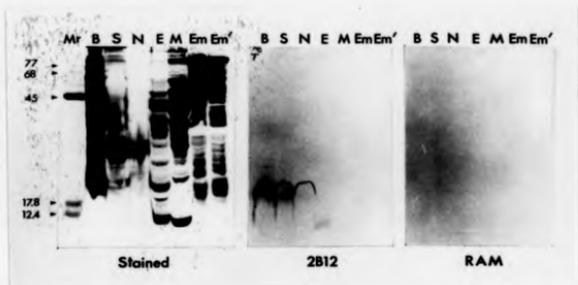


Figure 55 Western Blot of Xenopus laevis Embryo and Tissue Samples Screened with 2B12

Samples of Xenopus laevis adult frog tissue and stage 42 embryos were homogenised in SDS-page sample buffer. 5µl samples containing approximately 50µg of protein were run on 3 identical 12% high bis SDS-page gels. One gel was fixed and silver-stained after running. The other 2 gels were Western blotted onto nitrocellulose. Screening of the nitrocellulose was carried out separately as follows: 2B12, nitrocellulose incubated in 2B12 antibody and RAM-HRP; RAM (negative control) nitrocellulose incubated in RAM-HRP antibody only. The silver-stained gel and nitrocellulose strips are shown.

Mr, Molecular weight markers; B, Brain; S, Spinal Cord; N, nerve; E, eye; M, muscle; Em, stage 42 Xenopus laevis embryo; Em', dorsal dissected region of stage 42 Xenopus laevis embryo containing brain and neural tube.

sections a positive antibody reaction is seen from stage 23 onwards. The absence of a signal in the embryo tracks on the blot must therefore be ascribed either to an insufficient amount of the antigen present in the embryo for detection, or to the possibility that the antibody recognises a different species in the embryo to that in the adult nervous tissue, and the embryological species does not give a positive reaction on a Western blot. It is not immediately possible to distinguish between these alternatives. However, another nervous system specific monoclonal antibody, 2G9, which does not blot on whole embryo homogenates has since been shown to blot on dissected brains from tadpole stages of *Xenopus* embryos (Jones, 1988. Personal Communication). Given further time, this could have been attempted for 2B12, and might thus distinguish between these possibilities.

The 'ballooning' of the bands seen in Figure 55 is observed again on this blot in the brain, spinal cord and nerve tracks. These bands on the nitrocellulose all correspond to similar distortion of bands on the stained gel, and correspond to maximum molecular weights of around 23,000 Daltons. However the signal obtained against the eye homogenate on the Western blot is a straight band of around 8,000 Mr. The major protein seen in the stained eye track appears at around 9,000 Mr. If this band is the antigen recognised by 2B12 then it is present as a major species in the eye, yet the signal on the blot is very faint. A molecule of this molecular weight is absent in the silver-stained tracks of the brain, spinal cord and nerve, and yet the signal in the blots of those tissues is very strong.

Figure 56 Experimental Protocol for Immunoprecipitation

See Method M.29. Det. Buffer is detergent mix
buffer.

Therefore, it seems unlikely that this 9,000 Mr band represents the 2B12 antigen.

From these initial experiments it appears that the 2B12 antigen has a molecular weight of around 8,000 on SDS-page gels. The antigen often runs on gels as a disrupted band possibly due to the large amounts of lipid present in tissue of neural origin. It is also not possible to identify the antigenic species as a particular band on a silver-stained gel.

7.3 Immunoprecipitation of the 2B12 Antigen

An alternative technique for the biochemical detection of the antigen is immunoprecipitation. A 2B12 immunoprecipitation was carried out on a ^{125}I Iodine labelled Xenopus spinal cord homogenate (Method M.29). A summary of the protocol used for this experiment is shown in Figure 56 and the results of the experiment are shown in Figure 57.

Two labelled spinal cord homogenates were used. In the first the spinal cord was homogenised in PBS, then centrifuged to remove insoluble material before labelling with ^{125}I Iodine (H2, see Figure 56). In the second sample the spinal cord was cut into small pieces before labelling with ^{125}I Iodine, then washed and homogenised in detergent mix buffer (H1). This was then centrifuged for 5 min before immunoprecipitating. The aim of this protocol (in H2) was to maximise the solubilisation of the membrane in the detergent buffer and so avoid potential loss of a membrane antigen when centrifuging to remove insoluble material. Immunoprecipitation with H1 was carried out as a control.

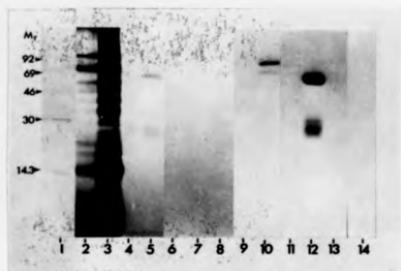


Figure 57 Immunoprecipitation of Xenopus laevis Spinal Cord Homogenate with 2B12

Xenopus laevis spinal cord was labelled with ¹²⁵Iodine as illustrated in Figure 56. Immunoprecipitations were carried out on the spinal cord samples (H1 and H2) using 2B12 antibodies at normal concentration, or concentrated x10 in an Amicon concentrator. Control precipitations were carried out in the absence of labelled sample, 2B12 antibody, RAM antibody or Stabh A envelopes. The samples were analysed on a 15% low bis SDS-page gel. The autoradiograph of this gel is shown.

The tracks are as follows: 1, Molecular weight markers; 2, H1 labelled homogenate (1 μ l); 3, H2 labelled homogenate (1 μ l). Tracks 4-14 are immunoprecipitates as follows: 4, 10x concentrate 2B12, H1, RAM, Stabh A; 5, 1x concentrate 2B12, H1, RAM, Stabh A; 6, 10x concentrate 2B12, H2, RAM, Stabh A; 7, 1x concentrate 2B12, H2, RAM, Stabh A; 8, 10x concentrate 2B12, H1, Stabh A; 9, 10x concentrate 2B12, H2, Stabh A; 10, 10x concentrate 2B12, H1, RAM; 11, 10x concentrate 2B12, H2, RAM; 12, H1, RAM, Stabh A; 13, H2, RAM, Stabh A; 14, 10x concentrate 2B12, RAM, Stabh A.

Tracks 1 and 2 in Figure 57 show samples of the labelled homogenate for both H1 and H2. There is a greater incorporation of the 125 Iodine label in the unspun homogenate (H2) than in the spun (H1) homogenate. This would be expected from the increased solubility of the spinal cord tissue in the detergent containing buffer. Tracks 4, 5, 6 and 7 represent the immunoprecipitation of H1 and H2 with 2B12 antibody, RAM and *Staphylococcus A.* membranes present. Tracks 4 and 5 which show the immunoprecipitation of the H1 homogenate indicate two bands at 85,000 Mr and 27,000 Mr, these are also seen in track 12 which shows the immunoprecipitation of H1 in the absence of 2B12. These are therefore background bands. No signal above background is seen for the 2B12 immunoprecipitation of either the H1 or H2 homogenates. The experiment was therefore unsuccessful in its attempt to immunoprecipitate the 2B12 antigen from *Xenopus laevis* spinal cord homogenate.

Further attempts (data not shown) to obtain an immunoprecipitate of the 2B12 antigen from iodinated tissue, and from tissue labelled with 35 S-methionine also proved unsuccessful. This is not totally surprising since monoclonal antibodies are often of relatively low affinity and difficult to use in immunoprecipitation analysis.

7.4 Neuraminidase Treatment of Sections and Western Blots

The neuraminidase enzymes catalyse the removal of sialic acids (N-acetyl neuraminic acid and N-glycoonyl neuraminic acid) from chains of sugar residues. Treatment of *Xenopus laevis* nervous tissue samples with neuraminidase can

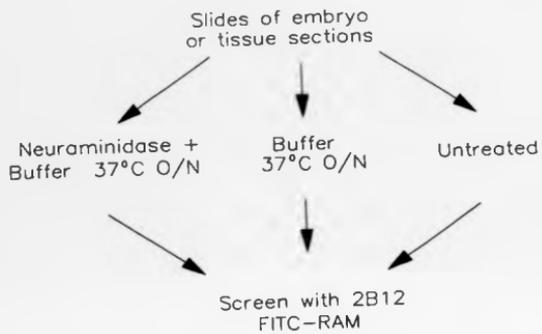


Figure 5B Experimental Protocol For Treating Sections with
Neuraminidase

See Method M. 30.

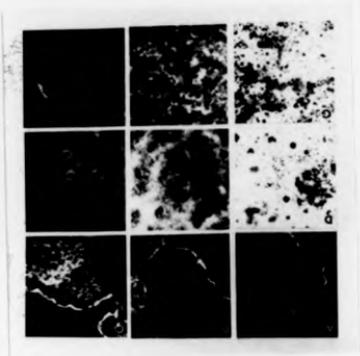


Figure 59 2B12 Activity on Xenopus laevis Nerve Sections
Treated with Neuraminidase

Sections of Xenopus laevis adult nerve and stage 42 Xenopus laevis embryo were treated with neuraminidase or with buffer as outlined in Figure 58 (see also Method M.30). Nerve sections were screened with 2B12, embryo sections were screened as controls with EpA, an antibody specific for Xenopus laevis epidermis.

The sections are as follows: A, Embryo section, untreated, screened with EpA antibody; B, Embryo section, treated with neuraminidase buffer, screened with EpA antibody; C, Embryo section, treated with neuraminidase, screened with EpA antibody; D, nerve section, untreated, screened with 2B12 antibody, x40 magnification; E, nerve section, treated with neuraminidase buffer, screened with 2B12 antibody, x40 magnification; F, nerve section, treated with neuraminidase, screened with 2B12 antibody, x40 magnification; G, nerve section, untreated, screened with 2B12 antibody, x10 magnification; H, nerve section, treated with neuraminidase buffer, screened with 2B12 antibody, x10 magnification; I, nerve section, treated with neuraminidase, screened with 2B12, x10 magnification.

be used to investigate whether 2B12 binding is inhibited by the removal of sialic acid. Three separate protocols were used to investigate the effect of the enzyme on antibody/antigen binding. The first of these is summarized in Figure 58. In this experiment Xenopus laevis nerve or embryo sections were treated with neuraminidase before screening using immunofluorescence with 2B12.

Figure 59 shows the results from neuraminidase treatment of sections (Method M.30). The first three sections (A, B, C) are controls and show the treatment of stage 40 Xenopus embryo sections left untreated, treated with the neuraminidase buffer, and treated with the buffer and neuraminidase respectively, before screening with the EpA antibody which is specific to Xenopus epidermis. No variation in the intensity of the signal is seen for any of the sections. In this case the action of neuraminidase does not affect the binding of the antibody. The remaining sections in Figure 59 show similar treatment of Xenopus adult nerve sections screened with 2B12. The level of signal in the untreated sections (D and G) is very high and all tissue present is positive. In the sections treated with the neuraminidase buffer the level of binding is lower and a streaky appearance of the fluorescence is seen. This suggests that the antigen is sensitive to the incubation treatment. The neuraminidase treated sections are totally negative, and no fluorescence is seen whatsoever. This indicates that the binding of the 2B12 antibody, unlike that of the EpA antibody, is sensitive to the action of neuraminidase.

The results suggest that the 2B12 antigen contains

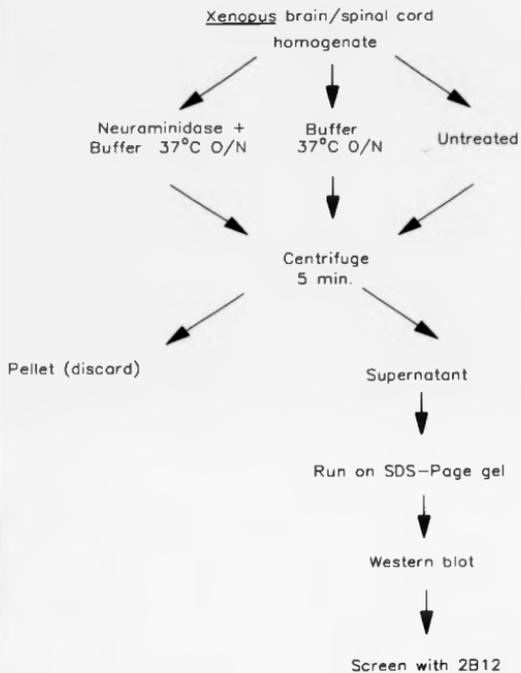


Figure 60 Experimental Protocol For the Treatment of Brain
and Spinal Cord Homogenates with Neuraminidase

Neuraminidase buffer in this experiment is
described in Method M. 30.

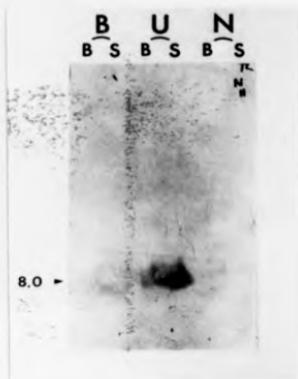


Figure 61 Western Blot of *Xenopus laevis* Neuraminidase
treated Brain and Spinal Cord homogenates Screened with 2B12

See Figure 60. The brain and spinal cord from an adult *Xenopus laevis* frog were homogenised in 600µl of neuraminidase buffer (Method M.30). The homogenates were aliquotted into 3 fractions each. To the first was added 125mU of neuraminidase (Sigma) in 125µl of neuraminidase buffer (N); to the second was added 125µl of neuraminidase buffer (B). These samples were incubated at 37°C overnight. The third samples were mixed with 125µl Neuraminidase buffer and were frozen at -20°C (U) until needed. 25µl of the 6 samples were mixed with 25µl of 2x concentrated SDS-page sample buffer and were run on a 12% high bis acrylamide gel. The gel was blotted onto nitrocellulose and was screened with 2B12.

B, brain; S, spinal cord. 8.0 indicates a molecular weight in kiloDaltons.

sialic acid. However, sialic acid may not form part of the antigen but may be an associated molecular species such that removal of the sugar prevents 2B12 antibody/antigen binding through masking effects or through alteration of the antigenic tertiary structure. To investigate this suggestion, the neuraminidase experiment was repeated using Western blots. The protocol is outlined in Figure 60. In this experiment brain and spinal cord homogenates were treated with neuraminidase before running on an SDS-page gel, electroblotting, and screening with 2B12. The results of this experiment are seen in Figure 61.

A strong signal is seen in the untreated homogenate tracks at around 8,000 Mr. This is consistent with the results obtained previously (Figures 54 and 55). The band in the tracks where the homogenates had been treated with the buffer was fainter which agrees with the observations from the neuraminidase treatment of sections where the antigen was shown to be sensitive to the incubation conditions. Little or no signal is seen in the tracks where the homogenates had been treated with neuraminidase enzyme indicating again that the 2B12 antibody/antigen binding is sensitive to the action of the neuraminidase enzyme.

This experiment can also be carried out by running the samples on the gel and blotting as normal, then treating the blot with the enzyme and buffer as shown in Figure 62. The results of this experiment are shown in Figure 63. Again a decreased amount of antibody binding is seen in the tracks treated with the buffer, but the signal in the neuraminidase treated tracks is much less.

Xenopus brain/spinal cord
homogenate



Run on SDS-Page gel



Western blot



Neuaminidase +
Buffer 37°C O/N



Buffer
37°C O/N



Untreated



Screen with 2B12

Figure 62 Experimental Protocol For the Treatment of
Western Blots with Neuraminidase

Neuraminidase buffer in this experiment is described in Method M.30.



Figure 63 Western Blot of Xenopus laevis Neuraminidase Treated Brain and Spinal Cord Tissue Screened with 2B12

See Figure 62. The brain and spinal cord from an adult *Xenopus laevis* frog were homogenised in 1ml each of SDS-page sample buffer. 50ul samples were run on an 12% SDS-page gel which was Western blotted onto nitrocellulose. Strips from the blot containing 1 track each of brain and spinal cord homogenate were screened separately as follows: N, nitrocellulose incubated in 1mU/ml neuraminidase in neuraminidase buffer, 37°C overnight; B, neuraminidase buffer only, 37°C overnight; U, PBS, 4°C overnight. The three strips of nitrocellulose were then screened as normal for 2B12 binding.

B, brain; S, spinal cord. 8.0 indicates a molecular weight in kiloDaltons.

The overall conclusions from these neuraminidase experiments is that 2B12 antibody/antigen binding is inhibited by the action of neuraminidase. This suggests that the antigen contains sialic acid either as part of the antigenic site, or associated with this site such that removal of sialic acid disrupts the tertiary structure of the active site.

7.5 Activity of 2B12 on Membrane Samples

The neuraminidase experiments showed that the 2B12 antigen contains sialic acid. These acylated neuraminic acids are found predominantly in plasma membrane glycoproteins and glycolipids. To confirm that the 2B12 antigen is present in the membrane, a Western blot was carried out against purified membrane samples.

Figure 64 shows a Western blot of spinal cord tissue which had been fractionated into membrane and cytosol using the crude membrane method generally used for embryos (Method M.3). The blot was then screened with 2B12 and with a RAM-HRP only control. Strong signals are seen in the total homogenate and membrane tracks, and a weak signal is seen in the cytosol track. From this crude experiment it appears that the antigen is present in the membrane fraction.

From the screening of nerve sections with 2B12 in Chapter 6, the antibody was seen to bind to the myelin sheath of myelinated neurons. A preparation of myelin was made from adult Xenopus laevis brain and spinal cord tissue (Method M.8). A summary of the preparation is shown in Figure 65. Samples of Xenopus laevis myelin were run on a gel alongside

Figure 64 Western Blot of Xenopus laevis Spinal Cord Crude Membrane and Cytosol Screened with 2B12

A crude membrane preparation was carried out on an adult *Xenopus laevis* spinal cord (see Method M.3). The crude membrane and cytosol fractions obtained were solubilised in 1ml of SDS-page sample buffer and 50µl samples were run in three tracks each on a 12% high bis acrylamide gel beside unfractionated spinal cord samples (1/20 total spinal cord in each track). After running, a portion of the gel was silver-stained. The remaining gel was Western blotted and the nitrocellulose was screened for 2B12 binding. The stained gel and nitrocellulose strips are shown.

2B12, screened with 2B12 antibody and RAM-HRP; RAM, screened with RAM-HRP only. Mr, molecular weight markers; T, total spinal cord homogenate; M, spinal cord membrane; C, spinal cord cytosol.

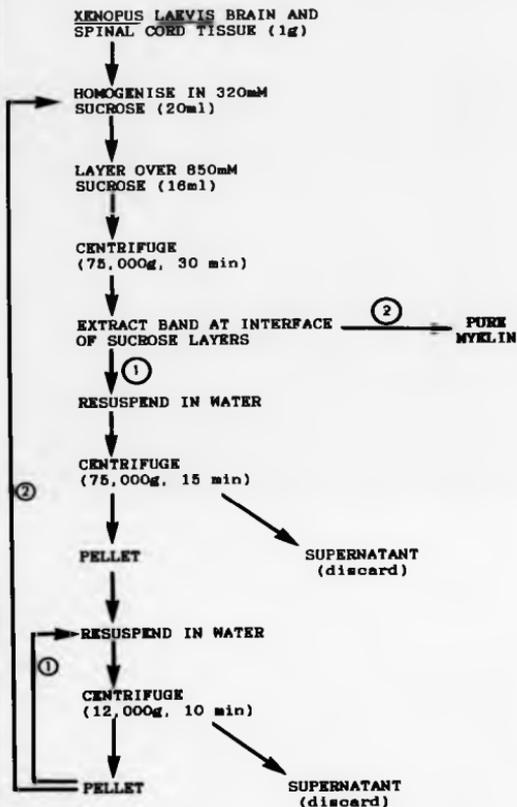


Figure 65 Experimental Protocol for the Purification of
Mvelin

See Method M.6.

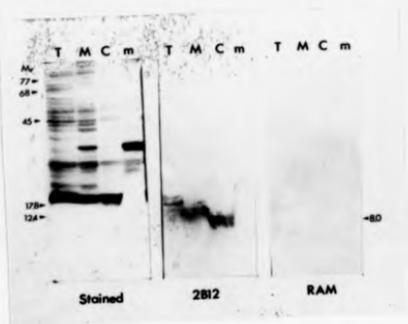


Figure 66 Western Blot of Xenopus laevis Spinal Cord Myelin, Crude Membrane and Cytosol Fractions Screened with 2B12

Samples of *Xenopus laevis* spinal cord crude membrane and cytosol were prepared as described in the legend to Figure 64. Myelin was purified from *Xenopus laevis* spinal cord as outlined in Figure 65 (see also Method M.6). Samples of total spinal cord homogenate, crude membrane, cytosol and myelin comprising 1/20 total spinal cord equivalent were run in three tracks each on a 12% high bis SDS-page gel. A portion of the gel was silver-stained. The remaining gel was Western blotted and the nitrocellulose was screened for 2B12 binding. The stained gel and nitrocellulose strips are shown.

2B12, screened with 2B12 antibody and RAM-HRP; RAM, screened with RAM-HRP only. Mr, molecular weight markers; T, total spinal cord homogenate; M, spinal cord membrane; C, spinal cord cytosol; m, spinal cord myelin. 8.0 indicates a molecular weight in kiloDaltons.

The weak band in the myelin track (m) may be due to a component of myelin which has been degraded during purification of the membrane. Alternatively it may indicate that the 2B12 antigen is not a myelin component and is seen as a weak band in this sample through contamination.

samples of spinal cord fractionated using the crude membrane fractionation as before. The gel was electroblotted and screened with the 2B12 antibody and with a RAM-HRP only control. The results of this experiment are shown in Figure 66.

Only a weak signal is obtained in the myelin track screened with 2B12, despite a substantial protein loading as indicated by the silver-stained track. A possible explanation is that the 2B12 antigen has degraded during the preparation of the myelin membrane. Alternatively the antigen may be only a minor component of myelin. Figure 66 also shows a repeat of the fractionation of spinal cord using the crude membrane technique. A good degree of fractionation was achieved as indicated by the silver-stained tracks. However, both the membrane and cytosol are positive for 2B12 binding. This is inconsistent with the results shown in Figure 64 and indicates that the method is not adequate for the fractionation of membrane from brain and spinal cord.

7.6 Trypsin Treatment of the 2B12 Antigen

The 2B12 antigen appears on SDS-page gels at very low molecular weight in the region where lipid is thought to be present in the gel. A highly polar glycolipid might be expected to run on an SDS-page gel in a similar way to a low molecular weight protein, and would also electroblot and bind to nitrocellulose. No definite silver-stained band can be ascribed to the antigen, and no success was achieved from immunoprecipitation experiments which could be explained by a glycolipid antigen not being labelled by the iodination and

³⁵S-methionine techniques used. If the 2B12 antigen is a glycolipid, then it could be a ganglioside. Gangliosides are complex sphingolipids containing sialic acid which are found in very high concentration in the nervous system. This would be consistent with the distribution of the antigen as revealed by the immunofluorescence experiments in the last chapter.

To investigate whether the 2B12 antigen is a glycoprotein, trypsin treatment of nerve sections and of Western blots followed by 2B12 screening was carried out. The treatment of the sections was unsuccessful since increasing the concentration of trypsin used on the section, or the length of the incubation time had the effect of 'chewing' the sections from the slides. Several repeats of this experiment produced similar results and it remains impossible to determine the extent to which trypsin treatment of sections inhibits 2B12 antibody binding.

Figure 67 and 68 show the effect of trypsin treatment on antibody binding to Western blots. Figure 67 is a control and shows the effect of treating ovalbumin protein with increasing concentrations of trypsin before running the protein samples on a gel, electroblotting, and screening with an anti-ovalbumin polyclonal antibody. The stained gel in Figure 67 shows an increasing degradation of the 45,000 Mr ovalbumin molecule as the concentration of trypsin is increased. In track 5 little antibody binding is evident and in track 6 no binding is present. The antibody binding is clearly sensitive to the action of trypsin, and since little reaction can be seen in the last two tracks it is clear that

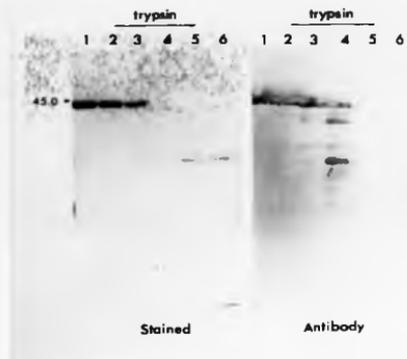
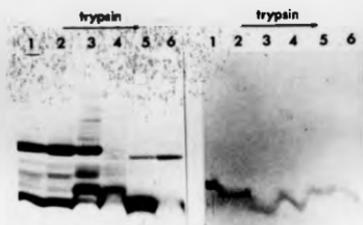


Figure 67 Western Blot of Trypsin Treated Ovalbumin Protein Screened with Anti-ovalbumin Antibody

A solution of 100 μ g of Ovalbumin protein (Sigma) in 600 μ l of T buffer (50mM NaCl, 10mM Mg-acetate 20mM Tris, pH 7.8) was prepared and divided into 6 samples. 10 μ l of Trypsin solution (Difco, 1:200) in T buffer was added to each sample which was then incubated at room temperature for 30 min. 10 μ l of Trypsin inhibitor at twice the original concentration of trypsin (Sigma, ovomucoid) was then added and the samples incubated for a further 30 min at room temp. 120 μ l of 2x concentrated SDS-page sample buffer was added and 50 μ l of the samples were run on 2 tracks each of a 12% acrylamide gel. After running half of the gel was silver-stained. The remaining half was Western blotted and the nitrocellulose screened with rabbit polyclonal anti-ovalbumin antibody (Dr. E. A. Jones), and with GAR-HRP as described in Method M.27. The stained gel and nitrocellulose are shown.

The tracks are as follows: 1, T buffer only added (positive control); 2, 0.01mg/ml trypsin; 0.02mg/ml trypsin inhibitor; 3, 0.1mg/ml trypsin; 0.2mg/ml trypsin inhibitor; 4, 1mg/ml trypsin; 2mg/ml trypsin inhibitor; 5, 10mg/ml trypsin; 20mg/ml trypsin inhibitor; 6, 20mg/ml trypsin; 40mg/ml trypsin inhibitor. 45.0 indicates a molecular weight in kiloDaltons.



Stained

Antibody

Figure 68 Western Blot of Xenopus laevis Trypsin Treated
Spinal Cord Homogenate Screened with 2B12

A Xenopus laevis spinal cord was homogenised in 1ml of T buffer (see the legend to Figure 67). Six 100 μ l samples of the homogenate were treated with trypsin and with trypsin inhibitor and analysed on a gel and Western blot as described in the legend to Figure 67. The nitrocellulose blot was screened with 2B12 and RAM-HRP. The stained gel and nitrocellulose are shown.

The tracks are as outlined in the legend to Figure 67.

trypsin has destroyed the antigenic site of the ovalbumin.

Figure 68 shows the similar experiment carried out for 2B12. Xenopus laevis spinal cord homogenate was treated with the same concentrations of trypsin as for the ovalbumin shown in Figure 67. The stained gel in Figure 68 shows that degradation of protein species in the spinal cord homogenate occurs with increasing concentrations of trypsin. In track 6 the protein species that remain are possibly trypsin insensitive lacking the necessary amino-acid sequences for cleavage by the enzyme. Alternatively, the concentration of trypsin used or the incubation time may have been insufficient for total digestion of the protein present. The Western blot screened with 2B12 shows a signal in all the tracks, but with a slight decrease in the intensity of the signal with increasing trypsin concentration. The molecular weight of the reacting species appears to stay approximately constant over all concentrations of trypsin.

The experiment with 2B12 has not proved as conclusive as for the ovalbumin antibody. A decrease in intensity of the signal is seen with increasing trypsin concentration, but this may be due to the sensitivity of the antigen under harsh conditions. The signal seen at the highest concentrations of trypsin may also be explained by a protein antigen containing no trypsin sensitive cleavage sites. A third alternative is that the persistent 2B12 antibody band indicates a non-protein antigenic species as suggested above. These experiments remain inconclusive and should be repeated using other protease and lipase enzymes to clarify the nature of the 2B12 antigen.

7.7 Immuno-absorption of the 2B12 Antigen

Characterisation of the 2B12 antibody has so far used the techniques of immunofluorescence on sections, and antibody screening of Western blots together with specific enzymes chosen to try and identify the nature of the 2B12 antigen. Another method, useful for its simplicity and speed is immuno-absorption. This method can provide qualitative information about the degree of antibody binding by using biochemically distinct samples to absorb the antibody. An outline of the technique is shown in Figure 69. Essentially, a concentrated solution of the antibody is mixed with a sample containing the antigen. The mixture is incubated at room temperature for 30 min, centrifuged and the supernatant screened on sections using immunofluorescence. Substantial antibody/antigen binding in the mixture produces low levels of fluorescence on sections. A crude quantification of the technique is possible by taking light readings from the fluorescent sections. Results from an experiment using the immunoabsorption technique are shown in Table 11.

Aqueous preparations or organic soluble components of nervous tissues were tested with both 2B12 and the control antibody EpA which is specific for epidermis. None of the samples caused a reduction in the intensity of binding of the EpA antibody. 2B12 gave strong positive fluorescence when mixed with water (1) or with PBS (2). An unspun spinal cord homogenate caused total absorption of the antibody (3) and no activity was detected on sections. When the spinal cord was homogenised in PBS and spun to remove insoluble material it

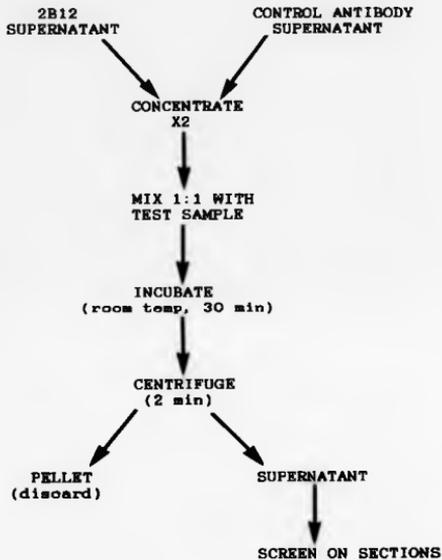


Figure 69 Experimental Protocol for Immunoabsorption
Experiments

<u>SAMPLE</u>	<u>EPA</u>	<u>2B12</u>
1. WATER	++	++
2. PBS	++	++
3. SPINAL CORD (total)	++	--
4. SPINAL CORD (PBS soluble)	++	-+
5. SPINAL CORD (PBS insoluble)	++	--
6. MYELIN	++	-+
7. CHLOROFORM / METHANOL (1:1)	++	++
8. SPINAL CORD (soluble in chloroform / methanol)	++	--
9. SIALIC ACID	++	++

Table 11 Immunoabsorption of *Xenopus laevis* Spinal Cord Fractions By 2B12

Samples of 2B12 and EpA antibodies were concentrated x2 in an Amicon concentrator. EpA is a monoclonal antibody specific for *Xenopus laevis* epidermis. Immunoabsorption using the concentrated antibodies was carried out as described in Figure 69 on various *Xenopus laevis* spinal cord fraction. ++ indicates positive antibody binding; +- indicates low level antibody binding; -- indicates no antibody binding.

The samples were as follows: 1, water; 2, PBS; 3, spinal cord homogenised in 1ml of PBS; 4, spinal cord homogenised in 1ml of PBS, supernatant after centrifugation for 2 mins; 5, spinal cord homogenised in 1ml of PBS, pellet after centrifugation for 2 mins resuspended in 1ml of PBS; 6, myelin prepared from one spinal cord suspended in 1ml of PBS; 7, 1:1 chloroform/methanol mixture; 8, spinal cord homogenised in 1ml of 1:1 chloroform/methanol, supernatant after centrifugation for 2 mins; 9, 2mg/ml solution of sialic acid (Sigma) in PBS.

was found that both the soluble (4) and insoluble fractions (5) were antigenic, but the insoluble material had a greater ability to absorb out the antibody. This could be explained by the poor solubility of membrane in aqueous environment. Myelin also proved antigenic (6), thus confirming the weak Western blot signal achieved on myelin.

Homogenisation of spinal cord in a 1:1 chloroform/methanol buffer was carried out. This was centrifuged and the supernatant tested in an attempt to identify any lipid component to the antigen. The buffer itself was shown to have no effect on the activity of the antigen (7), but the homogenised supernatant (8) was very immunogenic and absorbed out all apparent antibody binding. This suggests that the antigen is soluble in the organic buffer and is consistent with the antigen being a membrane glycolipid.

A solution of sialic acid was found to have no immunogenic effect (9) suggesting that although removal of sialic acid from the antigen prevents antibody binding, sialic acid itself is not sufficient to elicit an antibody response.

7.8 Conclusions

The work in this chapter involved the biochemical characterisation of the 2B12 antigen. On Western blots 2B12 gave a positive signal against samples of *Xenopus laevis* adult brain, spinal cord, nerve and eye tissue. No signal could be detected from stage 42 embryos despite the antibody being positive on embryo sections. It was suggested that the

antigen was present at a concentration too low for detection. The signal detected on nervous tissues appeared as a ballooned band towards the bottom of the Western blot with an apparent protein molecular weight of 8,000. No signal using 2B12 could be detected on 2-dimensional IEF Western blots of the nervous system tissues (data not shown).

All attempts to immunoprecipitate the antigen were unsuccessful despite labelling of tissue with both 125 Iodine and 35 S-methionine, and including RAM to amplify the response. Attempts were made to maximise solubilisation of membrane in these experiments. Neuraminidase treatment of sections and Western blots were conclusive in showing that removal of sialic acid from the antigen prevented antibody binding. An attempt to absorb out the antibody with a concentrated solution of sialic acid was unsuccessful. This suggests that sialic acid alone is non-immunogenic, and it is the presence of sialic acid in, or near, the antigenic site which is necessary for antibody binding. The results from the neuraminidase experiments indicated that the antigen was most likely a membrane component. Myelin purified from Xenopus laevis brain and spinal cords was tested both on blots and by absorption. These experiments confirmed that the 2B12 antigen is a component of myelin although a signal of reduced intensity in both cases suggests possibly some destruction of the 2B12 antigen during the purification of the myelin. This would be consistent with the results from the neuraminidase experiment where even mild incubation conditions appeared to have a degrading effect on the antigen. Other crude membrane fractions used on Western blots proved inconclusive at

indicating whether the antigen was specific to membrane. However, the reactivity of 2B12 against myelin and the results from the neuraminidase experiments would suggest that this is the case.

The precise nature of the 2B12 antigen as a glycolipid or a glycoprotein remains unproven. However, there is significant evidence to suggest that the molecule is a ganglioside and the evidence for this suggestion is summarised briefly below. The band from 2B12 on Western blots against Xenopus laevis nervous tissue does not correspond to a silver-stained band on the polyacrylamide gel. This may indicate a protein present at very low concentration, or one that does not silver-stain. In addition the bands on the blots are 'ballooned' upwards. This could be explained by the presence of lipid in the gel which would be consistent with the nature of the 2B12 antigen as a glycolipid.

The neuraminidase experiments indicated that the antigen contains sialic acid. The relatively strong carboxyl group of sialic acid (pKa 2.6 for N-acetyl neuraminic acid) would mean that a glycolipid containing sialic acid would have a net negative charge and this could explain the separation of the molecule on the SDS-page gels and its trans-blotting onto nitrocellulose. If the antigen were a glycolipid then the presence of sialic acid would suggest a ganglioside, a class of glycolipids found predominantly in nervous tissue membrane. This is consistent with the apparent distribution of antibody binding on embryo sections.

The trypsin experiments showed that total digestion of ovalbumin using the protease prevented binding of an

anti-ovalbumin antibody. The similar experiment with 2B12 and a spinal cord homogenate indicated that, although digestion of the spinal cord homogenate did occur, antibody binding was still observed at the highest concentration of trypsin used. Some decrease in signal intensity was seen with increasing trypsin concentration, and hence the results of the experiment are somewhat inconclusive. It is possible that the antigen is a glycoprotein and that the antigenic site is insensitive to the action of trypsin. Alternatively, the antigen as a glycolipid would not be digested by the protease, and may have undergone some degradation due to the incubation conditions.

Immunoabsorption experiments indicated that the antigen was soluble in a 1:1 mixture of chloroform/methanol. This is a good solvent for glycolipids (Slack, 1984). These points discussed above support the suggestion that the 2B12 antigen is a ganglioside. Further work using lipase and protease enzymes should be carried out to indicate clearly the nature of the 2B12 antigen.

CHAPTER 8

GENERAL DISCUSSION

The aim of this discussion is to review the main conclusions from the experimental work and to outline areas for future investigation. The significance of the findings within Xenopus development will be discussed and the results compared with those obtainable using alternative approaches to the study of the Xenopus embryo cell surface.

8.1 Analysis of Variation in Membrane Proteins During Xenopus Development

The experiments described in Chapters 3 and 4 investigated the extent of membrane protein variation during the early stages of Xenopus development. In the first experiments embryo membranes were purified using a variety of techniques and were analysed on 1- and 2-dimensional gels. Proteins were found which showed reproducible temporal variation (see Tables 4 and 5). More significant results were obtained from the analysis of membrane protein synthesis at different stages of Xenopus development using ^{35}S -methionine incorporation (see Figures 15-17). Proteins showing temporal variation are summarised in Tables 6 and 7.

The overall composition of detectable membrane proteins in the embryo remained reasonably constant over the developmental stages studied. This observation held true for each of the membrane extraction methods used. In contrast the range of de novo synthesised membrane proteins varied

considerably from the blastula stage onwards, possibly indicating increased gene activation following the mid-blastula transition (Newport and Kirschner, 1982a; 1982b). The composition of *de novo* synthesised membrane proteins was also found to differ markedly from the range of membrane proteins present within the embryo at the same stage (see Figure 16). These conclusions are consistent with present ideas concerning the nature of embryo membranes during development. The absence of substantial detectable variation in total membrane proteins during early Xenopus development is understandable considering the large amounts of membrane present in the embryo at any one time. Proteins essential to the structure and function of all embryonic cells would not be expected to undergo temporal variation and are likely to be the more major proteins within the membrane. Membrane proteins observed to undergo temporal variation may be present in the membrane at lower concentrations which would explain the apparent consistency of the overall composition of membrane proteins detected compared with the dramatic variation in *de novo* membrane protein synthesis.

The development of the 2-dimensional IEF system for protein analysis by O'Farrell (1975) has significantly increased the capabilities of electrophoretic analysis of complex protein preparations. A purified plasma membrane preparation from a mutant of Neurospora crassa analysed using this technique by Stotish and Somberg (1981) was shown to contain over 180 distinct polypeptides whereas SDS-page of the preparation revealed only 15 to 30 bands. The IEF technique has been used on several occasions to analyse

changes in total protein composition or in protein synthesis during development, and in some cases inconsistent results have been obtained. One such example are the apparently conflicting results obtained from the studies of de novo protein synthesis in Xenopus embryos carried out by Ballantine et al (1979), Brock and Reeves (1978) and Bravo and Knowland (1979) which were discussed in Chapter 3. 2-dimensional gel analyses of total protein synthesis during development have also been carried out on Drosophila embryos, and similar discrepancies have been noted in the published results (eg compare Savoini et al, 1981 and Trusby and Jarry, 1983). These discrepancies have lead to the suggestion that 2-dimensional IEF gels may be unsuitable for the analysis of changing developmental protein patterns in embryos. This suggestion was investigated using Drosophila embryos (Summers et al. 1986), when it was shown that changing patterns of protein synthesis during early development could be analysed reliably and reproducibly using 2-dimensional gel analysis, but that serious consideration must be given to all steps of the sample labelling and gel electrophoresis protocol before comparisons can be made. Experiments involving 2-dimensional gel analysis must therefore be interpreted with care, and repetition used to confirm the significance of observed variation.

One further disadvantage of the IEF gel technique in the analysis of membrane proteins is the limited pH range which causes the loss of highly basic integral membrane proteins present in the samples. For this reason the Nephge gel technique was attempted but proved unsatisfactory. It is

clear, therefore, that that only part of the total number of membrane proteins have been analysed using the IEF gels. However the general pattern of results obtained using the IEF gels has been confirmed using 1-dimensional SDS-page and it is therefore unlikely that the overall conclusions drawn from the IEF analysis in this thesis have been affected by this limitation.

The work in Chapter 3 represented a preliminary study of variation in membrane proteins during early Xenopus development. More work is needed to confirm the results obtained and to develop and expand the techniques used to analyse proteins of interest showing temporal variation. The analysis of membrane protein synthesis shown in Figures 15-17 was carried out using the crude membrane purification. More relevant information concerning variation in the cell surface would be obtained using a plasma membrane protein purification following ³⁵S-methionine microinjection, and this should be carried out. Membrane protein synthesis should also be studied in Xenopus oocytes and eggs. This would enable determination of those embryonic membrane proteins which are synthesised for the first time as a result of new embryonic gene activation at the mid blastula transition (see Ballantine et al, 1979 for a comparable study of total protein synthesis). The analysis of protein synthesis in Xenopus eggs was attempted but proved unsuccessful due to activation of the eggs following microinjection (data not shown).

Characterisation of membrane proteins showing reproducible temporal variation could be carried out using

antibodies raised against protein spots cut directly from the acrylamide gel (Smith *et al.* 1986). This was the technique used in the attempted production of polyclonal sera against the P37 and P47 proteins in Chapter 4. This approach would enable investigation of the nature of the individual proteins, and could be used to confirm the observed patterns of expression of the proteins in the different regions of the membrane.

The techniques developed in Chapter 3 to analyse membrane proteins in Xenopus embryos could be extended to look at animal/vegetal differences in membrane protein synthesis in Xenopus embryos. This analysis has previously been carried out on total protein synthesis in Xenopus embryos by Ballantine *et al* (1979). They dissected Xenopus embryos at several stages of development into animal and vegetal halves roughly containing ectoderm and endoderm respectively although both halves contained mesoderm. Very few differences were found between proteins synthesised in the animal and vegetal halves of the embryo before the blastula stage of development. Later stages were found to show several 'ectoderm' or 'endoderm' specific spots. An alternative study would use the membrane purification/IEF techniques to look for membrane proteins specific to either the dorsal or ventral halves of the embryo. A previous study of total protein synthesis in dorsal and ventral regions of Xenopus embryos has shown that proteins specific to either half of the embryo can be detected as early as gastrula stage embryos, and such proteins can serve as reliable markers of dorsal and ventral differentiation (Smith and Knowland,

1984). The techniques developed could also be used to detect species specific membrane proteins in Xenopus by comparison of protein profiles from Xenopus embryo membranes with those from other animal embryos.

In Chapter 4 the analysis of 125 Iodine labelled surface proteins in intact and disaggregated embryos at various stages of Xenopus development was carried out using 1- and 2-dimensional gels. This technique lacked the sensitivity of the methods described previously and no significant stage differences in the proteins labelled were detected. This was due partly to the limited resolution of the autoradiographs and is understandable in view of the results from Chapter 3 which showed that very few changes in the overall protein composition of the membrane occur during early development.

Two proteins, P37 and P47, specific to the labelling of disaggregated embryos were detected on 1- and 2-dimensional gels. It was suggested that these proteins are specific to intercellular membranes in the embryo. This is significant in view of the known membrane polarity and presence of membrane domains in the early Xenopus embryo (discussed in the introduction). If P37 and P47 can be proven to be restricted to intercellular membranes then it would appear that differences between the proteins of internal and external membranes exist in Xenopus embryos from the early cleavage stage. This is consistent with results from lipid diffusion studies of pre-existing membrane and cleavage furrow membrane (Tetteroo *et al.*, 1984).

The molecular nature of the P37 and P47 proteins

remains uncertain. They do not immediately identify themselves in terms of their molecular weight as previously characterised intercellular membrane proteins such as junctional molecules or cell adhesion molecules. In order to characterise these proteins and to confirm their distribution in the cell an attempt was made to raise polyclonal antibody sera against them. The serum from one mouse immunised with P37 gave a positive signal on a Western blot against an embryo protein of 37,000 Mr. However no activity of the antibody serum could be detected on embryo sections, and it remains unclear whether the serum is specific for the P37 protein, or for another embryo protein of the same molecular weight.

The conclusions drawn from the cell surface labelling experiments contrast with previous work carried out using similar techniques. Darribere *et al* (1982) carried out a similar analysis of surface labelling using ¹²⁵Iodine on Pleurodeles waltli embryos. They detected 7 proteins present in disaggregated embryo samples that were absent in intact embryos, and observed significant stage specific variation. As mentioned previously, it is felt that the results from these experiments have been over-interpreted considering the poor quality of their autoradiographs. Woods *et al* (1987) used the iodogen surface labelling technique to study alterations in cell surface proteins of Drosophila embryos during morphogenesis. Although the level of resolution is not excellent their autoradiographs appear more highly resolved than those shown in Chapter 4. This improved resolution cannot be ascribed to the lack of an intensifying screen

(which was discussed as a possibility for the poor resolution of the autoradiographs in Chapter 4), since screens were used during autoradiography. The iodogen technique was attempted on several occasions as part of the work in Chapter 4, but the incorporation of ^{125}I and the resolution of the gels was found to be poor in contrast to that obtained with the lactoperoxidase/glucose oxidase method eventually adopted. Further work should be carried out either to improve the resolution of the ^{125}I autoradiographs, or to develop an different technique for cell surface labelling. Examples of such alternative labelling methods, and the results obtained from them when used to label the cell surfaces of amphibian embryo are discussed briefly below.

The labelling of membrane proteins in *Xenopus* eggs using the ^{131}I isotope has been used to investigate proteins exposed on the outer face of dissected egg membranes in *Xenopus* (Richter, 1980). In this study only 4 proteins were detected in the *Xenopus* egg surface membrane. No 2-dimensional gel analysis of labelled proteins was carried out, and the 1-dimensional gels were analysed by scintillation counting of gel slices. Since the resolution of this technique would be very low it is not surprising that so few labelled membrane proteins were detected in these experiments. It would be straightforward to use the ^{131}I isotope in place of ^{125}I in the lactoperoxidase/glucose oxidase labelling technique. However, this would be unlikely to dramatically improve the resolution of the autoradiographs.

Dejellied *Xenopus* oocytes were surface labelled

with the irreversibly binding non penetrating isothiocyanate derivative ^3H -DIDS (Richter and Tintschl, 1983). The technique was used to investigate membrane proteins exposed on the outer surface of the membrane. Analysis of labelled proteins using 2-dimensional gels was not attempted and no illustration of the SDS-page gel was included to enable a determination of the effectiveness of this labelling technique on *Xenopus* membranes. ^{35}S -sulphanilic acid can be used as a cell surface labelling reagent (Berg and Hirsh, 1975; Reinwald and Risse, 1978). Bretzel *et al* (1986) used this reagent to investigate the purity of their plasma membrane preparations from *Xenopus* embryos. However no gel analysis was carried out on their labelled membrane fractions.

An alternative approach uses tritiated sodium borohydride to label cell surface glycoproteins. This has been carried out on *Pleurodeles waltlii* embryos (Darriberre *et al.* 1987) with analysis using 2-dimensional gels. No glycoproteins were detected on the external surface of intact embryos, but in excess of 23 glycoproteins were detected on the surface of disaggregated embryonic cells. They also observed qualitative changes in cell surface glycoproteins during *Pleurodeles* gastrulation. This suggests that the cell surface glycoproteins are largely restricted to intercellular membranes within the embryo, and signifies again the presence of membrane domains in the embryo at very early stages of development.

More work is needed to complete the investigation of cell surface labelling in *Xenopus* embryos. The resolution

of the autoradiographs should be improved, possibly by using a different labelling method as described above. This would enable a more thorough comparison to be made of the cell surface at different stages of development. The P37 and P47 proteins should be characterised to confirm their membrane specificity in the cell, and further experiments should be carried out to investigate the polyclonal serum raised against P37. If this antibody serum does not prove specific to P37 then there is a case for repeated mouse immunisations to obtain antibodies required for the characterisation of these proteins.

8.2 The Analysis of the *Xenopus* Embryo Cell Surface using Histo-autoradiography

The 125 Iodine cell surface labelling technique has been successfully used to study changes occurring in the membrane as a whole during *Xenopus* development. Using histo-autoradiography two specific aspects of membrane development in *Xenopus* embryos were considered in Chapter 5. The analysis of the origin of new membrane during cleavage confirmed the results of previous experiments carried out by Byers and Armstrong (1986) on *Xenopus laevis* embryos. Membrane within the cleavage furrow of a dividing *Xenopus* embryo was shown to originate mainly from *de novo* synthesised membrane and from internal membrane stores. A very small proportion of the furrow membrane is comprised of pre-existing membrane that was accessible to the surface labelling mixture. The leading edge of the cleavage furrow was seen in the embryos studied to be heavily labelled with

¹²⁵Iodine. This is thought to be due to the presence of large amounts of membrane in the form of microvilli which provide attachment sites for the underlying ring of actin microfilaments.

Although the analysis of cell cleavage described in Chapter 5 represented an adaptation and partial repetition of work previously carried out elsewhere, the use of albino embryos confirmed that the conclusions drawn from the earlier work were valid and had not arisen from mis-interpretation. The results from the study are significant in supporting the concept of membrane domains within Xenopus embryos (Bluemink and de Laat, 1973). In particular the results support the concept of membrane domains between the pre-existing membrane and membrane in the cleavage furrow. Such spatial variation in cell surface membrane organisation may prove to be vital in Xenopus embryos. Experiments should be developed to characterise differences between membrane domains, looking specifically at variation in lipid and protein composition. In this way the significance of these membrane domains in Xenopus development could be determined.

The histo-autoradiographic technique was also used to study the origin of ciliated cells in Xenopus embryos. The results obtained were consistent with the view that ciliary cells migrate from the inner layer of the epidermis to the outer layer during ciliogenesis. This was seen in the presence of label free patches of the embryo surface appearing in embryos labelled prior to ciliogenesis, but fixed later following the appearance of cilia on the cell surface. It was suggested that these label free patches

correspond to ciliated cells present on the embryo surface having migrated from the inner, unlabelled epidermal layer. Although it was not possible to show categorically that these label free patches of the cell surface corresponded to ciliated cells, the presence of cilia on these patches had been observed but was not photographed. Further repetition and careful photography should be carried out to present these results.

The analysis of ciliogenesis in Xenopus embryos involved using surface labelling and histo-autoradiography techniques to investigate changes in the embryo that had previously only been studied using microscopic techniques. Surface labelling can thus prove a valuable tool in studying the cell surface in development, not only for analysing differences in protein composition as in Chapter 4, but also as a marker for the cell surface. In this way the technique can be used to label cells present on the embryo surface at a particular stage in development, and to follow the distribution of those cells as development proceeds, using histo-autoradiography or IEF and autoradiography. The technique could prove useful in experiments involving fate mapping, and in embryological and grafting experiments involving changes to the cell surface.

8.3 Characterisation of 2B12 Anti-membrane Antibody

The final two chapters of experimental work in this thesis involved the characterisation of 2B12, an anti-membrane antibody specific for nervous system. The 2B12 antigen is first detected in the anterior neural tube in

Xenopus embryos at stage 23. The expression of the antigen is therefore dependent on the stage of development of the embryo, and as such the characterisation of 2B12 reflects the experimental approach that could be used to characterise and investigate antibodies raised against the membrane proteins showing temporal variation in Chapter 3.

2B12 antibody binding was analysed on embryo and tissue sections from Xenopus, and on adult mouse tissue sections using indirect immunofluorescence. The antibody appeared specific to tissue of the central and peripheral nervous system in both Xenopus and mice, and the distribution of 2B12 binding on nerve fibres suggested that the antigen could be a component of the myelin membrane. The analysis of 2B12 binding carried out on sections of Xenopus and mouse tissue was a preliminary study, and more information on the binding specificity of 2B12 would be obtained from a detailed and thorough histological investigation. In this way the distribution of the 2B12 antigen in specific cell types of the nervous system could be determined. This would enable 2B12 to be used as a marker in embryological experiments. Precise knowledge of the binding specificity of 2B12 could also aid the molecular characterisation of the antigen. The immunofluorescence technique used could be extended to identify the distribution of 2B12 binding on tissue and embryo sections from other animals. Work so far carried out on axolotl embryos and on Xenopus borealis tissue has confirmed that the pattern of 2B12 binding is remarkably similar to that seen in Xenopus laevis (data not shown).

In Chapter 7, a biochemical characterisation of the

2B12 antigen was carried out using Western blotting, enzymatic treatment of the antigen and immunoabsorption. 2B12 gave a signal on Western blots against brain and spinal cord tissue, but no signal was detected against blots of Xenopus embryo homogenate suggesting that the antigen may be present in the Xenopus embryo at very low concentrations. Treatment of the antigen with neuraminidase inhibited antibody binding indicating that sialic acid is likely to be present in the antigen. In addition, the appearance of the 2B12 band on Western blots and the results from experiments using trypsin have lead to the suggestion that the 2B12 antigen is a ganglioside. This suggestion is consistent with the distribution of antibody binding detected on Xenopus and mouse sections in Chapter 8. However, more work is needed to confirm the biochemical nature of the 2B12 antigen. Experiments using lipases or proteases on Western blots should be used to investigate whether the molecule is glycolipid or glycoprotein. Experiments carried out so far using glycolipid preparations from Xenopus brain and spinal cord which were separated using thin layer chromatography have proved inconclusive (data not shown).

There are various experimental procedures which could be used to investigate whether the 2B12 antigen is an significant molecule in the development of the Xenopus embryo. One approach would involve the microinjection of RNA purified from 2B12

hybridoma tissue culture cells into *Xenopus* embryos at an early stage. In this way an attempt could be made to interfere with the normal expression of the antigen in the developing embryo, and to investigate whether disruption of the normal developmental process occurs. The experiment involves the micro-injection of polyadenylated purified hybridoma RNA into *Xenopus* embryos at an early stage (1 or 2 cell), with the aim that the RNA is translated within the cytoplasm of the embryo cell and the resulting antibody protein inserted through the membrane into the lumen of the endoplasmic reticulum as a secretory protein. In this way the 2B12 antibody made within the embryo could be targeted to the 2B12 antigen which is believed to be a membrane antigen.

Experiments of this type have previously been carried out. There is now substantial evidence to suggest that that antibodies injected into living cells are relatively stable (Yamaizumi *et al.* 1979) and that they preserve their antigenic specificity, bind to their respective antigens and can interfere with intercellular processes (eg. Antman and livinstone, 1980; Lin and Feramisco, 1981; Bennett *et al.* 1983; McGarry *et al.* 1983; Stacey and Allfrey, 1984; Mercer *et al.* 1984; Blöse *et al.* 1984; Schlegel *et al.* 1985; Scheer, 1986). Microinjected hybridoma RNA is successfully translated and can interfere with cellular processes (Burke and Warren, 1984). Microinjection of hybridoma RNA, its expression within the cell and the interaction of antibodies with their antigens have also been confirmed in *Xenopus laevis* (Valle *et al.* 1982).

2B12 Hybridoma cells

Control monoclonal antibody
hybridoma cells

Extract RNA

Poly A+ purify

Microinject RNA into 2 cell
Xenopus laevis embryos

Leave embryos to develop

Fix at stage 10

Fix at stage 25

Fix at stage 35

Screen: BSA/PBS

RAM-Fitc

GAR-Fitc

2B12/Control Antibody

Rhodamine-GAM

Figure 70 Experimental Protocol for the Microinjection of
2B12 RNA into Xenopus laevis embryos

Figure 70 indicates the design of such an experiment to investigate the developmental significance of the 2B12 antigen in Xenopus laevis development. This experiment was attempted as part of the work in Chapter 7 but the results proved inconclusive and difficult to interpret, and consequently the data has not been presented in this thesis.

8.4 The Analysis of Cell Surface Membranes in Xenopus Embryos

There are many varying approaches to the study of cell surface membranes in Xenopus development. The experiments described in this thesis have concentrated on detecting and characterising membrane components that may prove important in Xenopus development with the exception of the work described in Chapter 5 where overall changes within cell surface membranes during development were studied. The membrane purification and cell surface labelling techniques proved effective for looking at the number of membrane proteins that vary temporally or spatially during development. However alternative techniques could have been chosen to study changes in the cell surface and to look for components of the membrane that are potentially significant to Xenopus embryo development. One such approach is the analysis of cell surface glycoproteins and glycolipids using radioactive sugar incorporation, or using monoclonal antibodies or lectins (see Slack *et al.*, 1985). In the past lectins have been used to reveal surface differences during the cell cycle and between normal and malignant cells

maintained in culture (Sharon and Lis, 1972; Burger, 1973; Lis and Sharon, 1973). Concanavalin A, the Jack Bean lectin has been used to detect cell surface changes during development (Kleinschuster and Moscona, 1972), and incubation of embryos in Con A can affect the developmental process (Lallier, 1972; O'Dell *et al.* 1974; Boucaut *et al.* 1979). Lectins can be used to detect glycoproteins separated on Western blots using peroxidase stains (Clegg, 1982) and the distribution of glycoconjugates on sections can be studied using fluorescent lectins.

The analysis of glycoconjugates, and the membrane protein and cell surface labelling techniques can be used to detect membrane components showing regional or temporal variation in the Xenopus embryo. These techniques have the disadvantage that further characterisation must be carried out to determine the molecular nature and function of the membrane component. An alternative approach would be to investigate and characterise a particular group of cell surface components showing a common function known to be important in embryonic development. Examples would include the study of cell adhesion molecules, cell junctional molecules or cell surface receptors. There are obvious advantages in adopting such an approach and much of this work has previously been considered in the introduction.

The results obtained in Chapter 4 from the cell surface labelling of intact and disaggregated Xenopus embryos were consistent with results obtained previously from studies on lipid diffusion within Xenopus embryo plasma membranes. These studies revealed the presence of lipid domains within

the membranes of Xenopus eggs and embryos (Tetteroo et al. 1984; Dictus et al. 1984). These experiments were important in illustrating the highly specialised nature of the embryo membrane and in showing that the Xenopus embryo membrane does not fulfill the concept of a fluid mosaic membrane (Singer and Nicholson, 1972). Further information on the specialised nature of the Xenopus embryo plasma membrane has come from microscopic studies which enable the study of changes in the appearance of the membrane to be followed during development. Examples so far discussed include the use of light and electron microscopy to characterise ciliated cells in Chapter 5 (Steinman, 1988; Billett and Gould, 1971; Billett and Courtenay, 1973), and the use of freeze fracture electron microscopy to study the distribution of IMP's between animal and vegetal membranes in the Xenopus egg (Bluemink and Tertoolen, 1978).

One of the most powerful experimental techniques used in the study of the cell surface are monoclonal antibodies. Membrane antigens can be characterised with monoclonals using a variety of techniques including immunofluorescence on sections, Western blotting and immunoprecipitation which were discussed and illustrated in Chapters 6 and 7. Membrane components can also be purified using affinity absorption techniques. In these ways, antibodies can be used to detect and isolate cell surface antigens, even when these antigens are expressed within the membrane at relatively low levels.

Using hybridoma technology (Kohler and Milstein, 1975) it is possible to generate monoclonal antibodies

against purified membrane fractions without the prior isolation of individual antigenic species. Screening can then indicate potential antibodies of interest and these can be studied and characterised. Using *Xenopus* nervous tissue as immunogen, this approach was used to obtain the 2B12 monoclonal antibody.

There has been much interest in the use of monoclonal antibodies as region specific markers in development (see Slack, 1984a). Monoclonal antibodies against tissue specific antigens can be used as markers in studies of embryonic induction and in grafting and sandwich experiments. One such example is the monoclonal antibody 2F7.C7 specific for epidermis in amphibian embryos (Jones, 1985) which has been used to study epidermal development in *Xenopus* embryos using grafting and sandwich techniques.

The use of monoclonal antibodies to investigate the role of the antigen in development has already been discussed. This is clearly one of the more powerful applications of monoclonal antibody technology. A direct illustration of the use of monoclonals to investigate the role of a membrane antigen in development was outlined in the introduction. An antibody to a *Xenopus laevis* gap junction molecule was found to selectively disrupt dye transfer and electrical coupling between cells, when injected into an 8 cell *Xenopus* embryo. This resulted in developmental defects suggesting that blocking intercellular communication can have a profound effect on embryonic development (Warner *et al.* 1984).

8.5 Conclusions

There is substantial evidence to suggest that the Xenopus embryo plasma membrane plays a important role in development and morphogenesis. The experiments described in this thesis have supported this suggestion, and have extended the concept of the embryonic cell membrane as a highly specialised responsive system. More work is needed using a variety of experimental approaches before the complete picture of the role of the cell surface in development is determined.

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