UNIVERSITY OF SOUTHAMPTON.

MODELS OF MORPHOGENESIS.

A STUDY OF THE DEVELOPMENT OF THE NEPHRIC SYSTEM IN XENOPUS LAEVIS.

by Daniel Ciantar

M.Phil. Thesis.

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ABSTRACT

Master of Philosophy

MODELS OF MORPHOGENESIS.
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The early development of the pronephros, mesonephros, and nephric duct all show basic similarities. They all start as an accumulation of somatic mesoderm cells, which then arrange themselves into a radial array. Specialised cell junctions, basement membranes, and a lumen all form, and then regional differences become apparent. The basic similarity, however, also extends to the metanephros of higher vertebrates. This indicates that vertebrate kidneys develop from a homologous population of cells. Regional and species-specific differences arise due to differences in environment (including inductive influences and temporal factors). Of special note is the difference in the mechanisms of nephric duct extension between urodeles and anura.

A review shows that cell behaviour shown *in vivo* can also be seen *in vitro*, and quite complex morphogenesis *in vitro* can be produced. The *Xenopus* kidney cell line used here, unfortunately, did not show any organised morphogenesis under any experimental conditions. Some useful information concerning the cytoskeleton was obtained from these cells and a salmon cell line. Thus, it was concluded that the 'microtrabecular lattice' is probably artefactual, and details of the connections between intermediate filaments, microfilaments and microtubules are discussed.

The simple form of morphogenesis shown by the *Xenopus* cell line and normal kidney development were discussed in relation to a number of formal models of development.

ACKNOWLEDGEMENTS.

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ABBREVIATIONS.

ATP - Adenosine Triphosphate.

cAMP - Cyclic Adenosine Monophosphate.

DNA - Deoxyribonucleic Acid

ECM - Extracellular Matrix.

EDTA - Ethylenediaminetetra-acetic acid.

FCS - Foetal Calf Serum.

GMB - Gurdon's Modified Barth's

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

ICC - Intercellular Cavity.

IF - Intermediate Filament.

MAP - Microtubule-Associated Protein.

MDT - Mean Doubling Time.

MEM - Modified Eagle's Medium.

MF - Microfilaments.

mRNA - Messenger Ribonucleic Acid.

MS - Modified Steinberg's.

MT - Microtubule.

MTOC - Microtubule Organizing Centre.

NCS - Newborn Calf Serum.

PEG - Polyethylene Glycol.

PVA - Polyvinyl Alcohol.

RNA - Ribonucleic Acid.

SEM - Scanning Electron Microscopy.

TEM - Transmission Electron Microscopy.

XTC - <u>Xenopus</u> Tissue Culture.

INTRODUCTION

This project is concerned with one particular aspect of development, Morphogenesis. The project itself is concerned with kidney tubule formation in *Xenopus*, and includes an attempt to produce an *in vitro* model system using an Epitheloid Cell Line derived from adult *Xenopus* kidney. The main aspect of interest is the way in which cells produce and maintain their shapes, as well as how they interact to produce higher order structures. This Introduction will progress from an examination of how single cells maintain their shape, to how they interact to produce simple multicellular structures *in vitro* and ending with a review of specific examples of morphogenesis *in vivo*. The emphasis will be on tubule formation, and kidney development in particular.

Obviously care has to be taken when extrapolating from *in vitro* work to normal development. But the behaviour of cells in culture is in many ways similar to their behaviour *in vivo*. By modifying the culture environment, e.g. by using collagen gels, this similarity can become even more striking.

Perhaps surprisingly, all metazoan cells seem to take up one of six or so basic cell morphologies when in culture (Willmer, 1965 and 1970):- Epitheliocytes, Mechanocytes (Fibroblasts), Amoebocytes, Nerve Cells, Neuroglia and Lymphocytes. Of these, the first two are most frequently seen and are described in Table 1. Amoebocytes are derived

TABLE 1.

A Comparison of Epithelial and Fibroblastic Cells.

(Derived from Willmer, 1970.)

Epithelial Cells	Fibroblastic Cells
Grow as a sheet with intimate cell contact.	Grow as a network exhibiting contact inhibition. (Isolated cells survive with difficulty.)
Desmosomes may be present.	Desmosome-like structures may be present.
Pseudopodia, at the edge of growth only, mostly lamelliform but sometimes pointed.	Characteristically pointed pseudopodia (Filopodia).
Cells attached to substrate locally only - tendency for attachments to break.	Cells attach to substrate at extremities.
Colonies fuse.	Colonies fuse.
Gliding movement of the sheet, following extension be the peripheral cells.	Gliding movement of the cells with polarity.
Best growth on a surface.	Limited penetration into a plasma clot.
Some epithelial cells phagocytic *in vivo*. Not normally so *in vitro*.	Not normally phagocytic. Do not segregate vital dyes very actively.
Can pinocytose.	Can pinocytose.
Mucosubstances may collect in the cytoplasm. In other cases, they may be extracellular.	Mucosubstances mostly produced outside the cell.
Tend to produce Keratin.	Tend to produce Collagen.
Acid or Alkaline Phosphatase may be present.	Alkaline Phosphatase.

from Monocytes, Macrophages and Microglia. They show much random movement and the cells remain isolated from each other, even when arranged as a loosely knit sheet. Nerve cells have long elaborate processes, but their cell bodies tend to remain stationary. Neuroglial cells tend to be either spindle-shaped or have large lamelliform membranes. Lymphocytes show a gliding movement with a wave of constriction passing posteriorly. (Obviously, these cell morphologies are not rigidly maintained and the effect of the in vitro system on cell survival may be selecting for such cell types. But these factors will not greatly affect the main conclusions of the following discussion.)

As already stated, the two main types (and most widely studied) are the epithelial and fibroblastic cells. Indeed, most cell culture work has been done on fibroblasts. The usual view is that these morphological cell types are derived from the corresponding tissues in the explant. Thus, epithelial cells are derived from various epithelia and fibroblasts from mesenchyme. (However, see below and the Discussion.) Obviously, these cell types are not rigidly defined or maintained (e.g. Moscatelli et. al., 1980, give an example of change from epithelial to fibroblastic morphology). There is a lot of variation, depending on several factors, e.g. Substrate, Medium, Age of culture, Cell Density, etc. (see Willmer, 1965 and 1970 for fuller discussions).

The point of interest is that the morphological cell types can be at least partially explained or described in terms of differences in their cytoskeletons and cell adhesiveness. Given that these cell types are seen in explants from all the Metazoa yet studied, this indicates that there are basic

similarities in cell properties throughout the Metazoa. (The Protozoa are not considered here due to their having a wide variety of specialised organelles which complicates any description.) These cell properties can be studied and manipulated *in vitro* and compared with the situation *in vivo*.

One more point needs to be made; the difference between Primary and Transformed Cell Lines. Taking the definitions from J. Paul (1975):-

"Primary Culture. - A culture started from cells, tissues or organs taken directly from an organism. A primary culture may be regarded as such until it is sub-cultured for the first time. It then becomes a

Cell Line. - This term implies that cultures from it consist of numerous lineages of the cells originally present in the primary culture.

Diploid Cell Line. - A cell line in which, arbitrarily, at least 75% of the cells have the same karyotype as the normal cells of the species from which the cells were originally obtained.

Established Cell Line. - A cell line may be said to have become 'established' when it demonstrates the potential to be sub-cultured indefinitely *in vitro*.

Heteroploid Cell Line. - A cell line having less than 75% of the cells with diploid chromosome consitution.

Cell Alteration. - A more-or-less sudden change in the character of a cell line, usually associated with the emergence of an established cell line, and with alteration in morphology, contact inhibition, karyotype, viral susceptibility and ability to grow in suspension.

Transformation. - This term has been used synonymously with

'Cell Alteration' in the tissue culture literature. It does not imply the transfer of genetic material and is not necessarily related to the phenomenon of transformation in micro-organisms. Hence, its use in the sense of 'Cell Alteration' is undesirable and it is recommended that it should be discontinued."

Although Paul criticizes the use of the term "transformed" it is in general use and will be used here. Table 2 shows some of the differences that may be found between primary and transformed cell lines. The latter may show one or more of the characters described in Table 2. Figure 1 shows the relationship between normal and transformed cultures.

There are two points for particular notice in Table 2. First, it defines all permanent (=established) cell lines as Transformed. Secondly, some transformed cell lines are oncogenic. That there are similarities between transformed cell lines and tumuour cells has been noted very often and serves as one of the reasons for studying transformed cell lines.

Now to turn to the behaviour of cells in culture; beginning with single cells and then moving to groups of cells.

Cells in suspension tend to be spherical and covered in blebs or microvilli. Microfilament bundles (see below) are absent and Microtubules (MT) are rarely seen (Bragina et. al., 1976; Heaysman et. al., 1982). They flatten out on an appropriate "adhesive" substrate (see below), and their blebs and microvilli disappear (Erickson and Trinkaus, 1976). Surface activity usually becomes confined to one region of the periphery, this becomes the Leading Edge. Ruffles are formed at the active edge, continually moving back over the upper

TABLE 2.

Comparison of Some of the Properties of Primary and Transformed Cell Lines.

(Derived from information summarised in Vasiliev and Gelfand, 1981.)

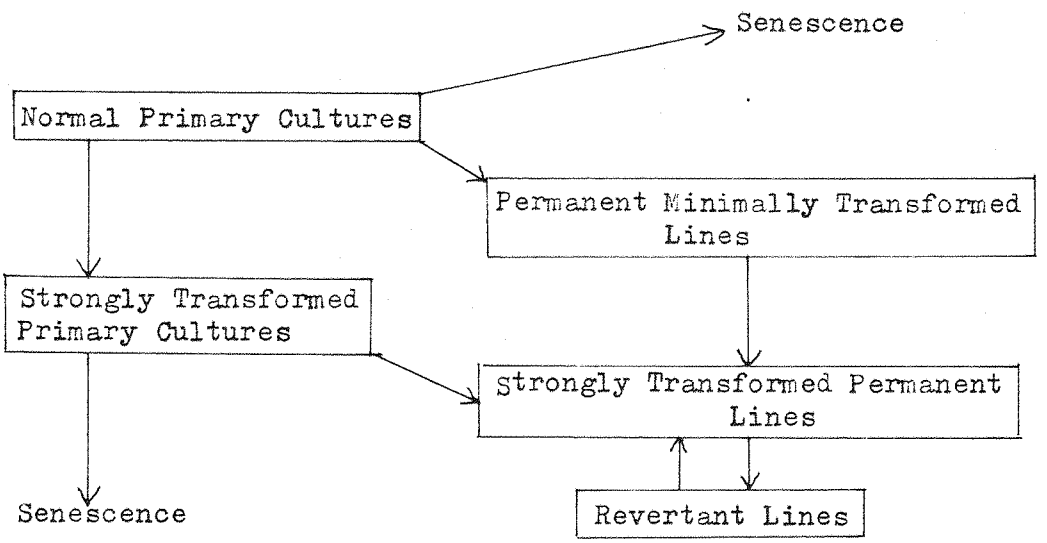
Primary Cell Lines	Transformed Cell Lines
Limited lifespan in culture.	Potentially immortal.
Anchorage dependence of growth.	Can proliferate in suspension.
Require serum for growth.	Require little serum for growth.
Density dependent growth.	Decreased density dependence of growth.
Not oncogenic when transplanted into susceptible animals.	Oncogenic (i.e. produce tumours) when transplanted into susceptible animals.
Cell attachment to substrate taken as Standard.	Decreased cell attachment to substrate.
Karyotype taken as Standard.	Karyotype changes.
Susceptibility to toxic effects of chemical carcinogens taken as Standard.	Increased resistance to chemical carcinogens.
Synthesis of Fibronectin taken as Standard.	Decreased synthesis of Fibronectin.
Agglutination by plant lectins taken as Standard.	Increased agglutination.
Dependence of Proliferation on Calcium content in medium taken as Standard.	Decreased dependence.

From this Table it can be seen that for some characters a cell line can only be described as transformed after comparison with its primary cell line.

FIGURE 1.

The Main Stages of Neoplastic Evolution in Cell Cultures.

(Taken from Vasiliev and Gelfand, 1981.)



Minimally Transformed Lines . . . Cultures with one or a few of the transformed characters noted in Table 2.

Strongly Transformed Lines . . . Cultures with most of the transformed characters noted in Table 2.

Revertant Lines . . . Cultures derived from strongly transformed permanent cell lines, which have lost one or more transformed characters

No reports of Minimally Transformed Primary Cultures have apparently been produced.

All Transformations are genetically stable.

surface of the cell (see below and Heaysman et. al., 1982). (Most of the above information, and much of that given below, is summarised in Vasiliev and Gelfand, 1977 and 1981.)

Transformed fibroblasts flatten out less than normal fibroblasts. Their area of lamellar cytoplasm tends to be less and is more jagged in outline (Fox et. al., 1977; Domnina et. al., 1972). The lower surface of the cells is less well attached to the substrate and may even possess microvilli. Focal contacts (see below) tend to be limited to the cell periphery. (Substances which increase cell adhesion, e.g. cAMP, often induce a more normal phenotype in transformed cells, although locomotion is reduced, e.g. Johnson and Pastan, 1972.) The upper cell surface tends to have more surface features, e.g. blebs and microvilli (Porter, Todaro, and Fonte, 1973; Malick and Langenbach, 1976; Borek and Fenoglio, 1976). There is a decrease in the number and size of microfilament bundles (possibly correlated with the lower adhesion of the cells), and the microfilament meshwork of the cells tends to become disorganised. Intermittent sphering, apparently due to lower adhesiveness to the substrate, may also be seen (Paranjpe and Boone, 1975). Cell movement is also more disorganised, with more turns. Such a morphology and behaviour is also found in tumour cells when compared to their normal counter-parts (Gonda et. al., 1976).

Transformed epithelial cells are less well studied, but share many of the characteristics of transformed fibroblasts (reviewed in Vasiliev and Gelfand, 1981).

Cell surface changes are also produced by a number of other factors (reviewed in Oliver and Berlin, 1982). For example, during division cells tend to round up, produce more surface

features (possibly acting as a membrane reserve for the two daughter cells until they flatten out; Pasternak, 1976; Erickson and Trinkaus, 1976), loose their microfilament bundles and undergo MT rearrangements (Porter, Prescott, and Frye, 1973). Increasing cell density also affects cell morphology, there being an inverse relation between cell density and number of microvilli in Hamster fibroblasts for example (O'Neill and Follett, 1970). It also seems that cell-to-cell contact is necessary for the changes observed during cell division (Rubin and Everhart, 1973). Folkman and Moscona (1978) find a correlation between cell shape and growth in normal cells; rounded cells tend not to show DNA synthesis and do not divide. Finally, trypsin causes rounding up and detachment of cells (it is usually used to detach cells in culture) and also affects the cytoskeleton (e.g. Badley et. al., 1980).

There is now a need for a slight detour. Mention has already been made of various elements of the cytoskeleton and this will continue. Therefore, a brief review of the three elements of cytoskeletons is required.

Microtubules (MT) are composed of the protein Tubulin (110,000 m.w.), which is itself a dimer of α- and β-monomers. The tubulin dimers are arranged in a spiral forming a tube of 22-25nm diameter (c.15nm internal diameter and 5nm wall thickness), the Microtubule itself (Snyder and McIntosh, 1976). Other proteins (Microtubule Associated Proteins; MAP's) are present in MT's (e.g. see Pallas and Solomon, 1982). Some of these (e.g. tau-protein) appear to be essential for MT formation. Intermediate filaments have also been found associated with MT in vivo (Linck et. al., 1982). MT's have an

intrinsic polarity (Heidemann and McIntosh, 1980) and are produced within cells at particular MT Organizing Centres (MTOC's). These are usually "Centrosomes", i.e. the centriole (or its derivative the basal body) plus the electron-dense material surrounding it; although the centriole may be absent in some cases (Brinkley, Cox, and Fistel, 1981; Raff, 1979; Solomon, 1980). ATP is apparently necessary for MT disassembly (Bershadsky and Gelfand, 1981). In fact, the MT seem to be in dynamic equilibrium with the cell's tubulin pool. They assemble and dissassemble at their ends, and may be anchored at least at one end to an MTOC. However, there is still much debate on these points (see the Discussion and Cleveland, 1982; Margolis and Wilson, 1981). There may even be some regulation of tubulin mRNA transcription by the tubulin pool itself (Cleveland et. al., 1981). In many cases, bridges have been seen between MT (e.g. McIntosh, 1974). It seems likely that these stabilise the MT arrays in which they are found.

MT's appear to function in the maintenance and stabilization of cell shape (Roberts, 1974) and possibly as "tracks" for the movement of organelles (e.g. in axons, Lasek, 1981).

Microfilaments (MF; diameter= 4-6nm) are composed of the protein Actin (42,000 m.w.). The actin monomers (G-Actin) polymerise to form helical filaments (F-Actin), the Microfilaments (Clarke and Spudich, 1977; Egelman et. al., 1982). Muscle and non-muscle actins are very similar, both show similar polymerisation reactions and both bind myosin reversibly (Pollard, 1981). Actin filaments possess a polarity which can be visualized under the electron microscope using myosin (Begg et. al., 1978). Actin composes 5-20% of the protein in non-muscle cells (Condeelis, 1981). 40-70% of this

can occur in monomer form (suggesting a dynamic equilibrium as with MT, Cleveland, 1982). A wide variety of factors interact with actin to either keep it as a monomer (e.g. DNase I) or to polymerise and aggregate it (e.g. ∝-Actinin; see the reviews by Groschel-Stewart, 1980; Schliwa, 1981, and Weeds, 1982). Of course, the most well known interaction is with Myosin; especially studied in muscle cells and the molecular basis of their contraction (Stebbings and Hyams, 1979; Monet et. al., 1981). The G-Actin to F-Actin reaction can be produced in vitro. F-Actin solutions are viscous and the actin filaments seem to form a meshwork (Condeelis, 1981; Pollard, 1976), probably due to the cross-linking action of one or more associated proteins (e.g. Actin-Binding Protein; Rosenberg and Stracher, 1982). The meshwork system seems to share many of the characteristics of cytoplasm (see Discussion; Jen et. al., 1982; Zaner and Stassel, 1982). Actin also seems to cross-link with MAP's (Seldon and Pollard, 1982).

MF appear to act in changing cell shape and provide the motile force in locomotion. This they apparently do either by moving relative to one another or by local polymerization and depolymerization (Vasiliev and Gelfand, 1981). MF bundles may also be associated with cell surface glycoproteins important in adhesion (see below).

Finally, the Intermediate Filaments (IF; diameter= 7-11nm) which are all proteins. They are divided into four major classes (Lazarides, 1980 and 1981):- 1/Keratin (Tono) filaments. Composed of several keratin polypeptides (42-65,000m.w.). Characteristically found in epithelial cells. 2/Neurofilaments. Composed of three polypeptides (210,000, 160,000 and 68,000m.w.). Characteristically found in neurones. 3/Glial filaments.

Composed of Glial Fibrillary Acidic Protein (50,000m.w.). Characteristically found in cells of glial origin. 4/Filaments containing Vimentin (52,000m.w.), Desmin (50,000m.w.) or Synemin (230,000m.w.) or combinations of all three. Found in most cell types.

More than one class of IF can be found in the same cell. All four classes have basic similarities, based on in vitro work (e.g. in vitro polymerization) and structural studies (e.g. all are fibrous α-helical proteins). The ratio of quantities of IF subunits varies between cell types and during development, but appears to be specifically regulated within each cell type (see Lazarides, 1980 and 1981 for reviews). This variation suggests regulation of function. It seems likely that they act as mechanical integrators of various organelles (Jones et. al., 1982; Lazarides, 1980). However, their function is still a subject of debate. IF also have associated proteins (e.g. Telser, 1982) and appear to have some connection with MT's, possibly via MAP's (Bloom and Vallee, 1982; Blose et. al., 1982).

Now to return to whole cells and continue with cell adhesion. A large number of factors will effect adhesion, e.g. temperature, ion composition of the medium, protein concentration of the medium. One point to note is that divalent cations (e.g. calcium and magnesium) in the medium are normally necessary for cell adhesion and spreading. But many transformed cells have a decreased dependence on calcium for growth (Vasiliev and Gelfand, 1981). Calcium bridging does not appear to be involved in cell adhesion (Hughes et. al., 1980). However, a complete analysis of the effects of divalent cations has not been performed.

Micromanipulation (Harris, 1973), electron microscopy and interference reflection microscopy (Gingell, 1981; Izzard and Lochner, 1976) have all shown that cells are usually closely attached to the substratum at a few sites only. At these sites the cell is only about 25–50nm away from the substratum (Close Contacts), decreasing to 10–20nm occasionally (Focal Contacts; Heath, 1982). The latter are associated with MF bundles (Badley et. al., 1980; Opas and Kalnins, 1981; Rees et. al., 1980) and appear to function in locomotion (see below).

Extracellular substances are often involved with cell adhesion (for a review see Grinell, 1978). Cells normally only attach to wettable surfaces and it is known that proteins adsorb to such surfaces very quickly. Active cell adhesion appears to be dependent on particular serum proteins (see below) or the active secretion of protein by the cells (e.g. Grinnell, 1976; Rajaraman and MacSween, 1980; Rees et. al., 1978).

The most well known serum protein important in cell adhesion is Fibronectin (Olden et. al., 1980; Hynes and Yamada, 1982; Erickson et. al., 1981). This is a glycoprotein, 440,000m.w.. A slightly different form is actually produced by many types of cells (e.g. Atherton and Hynes, 1981). Decreased fibronectin synthesis is one characteristic of transformed cells (Hayman et. al., 1981; Olden et. al., 1980).

There may be a relationship between fibronectin and MF bundles. Vinculin is a protein (130,000m.w.) associated with the ends of MF bundles. A transmembrane relation between Vinculin and Fibronectin has been found by Singer and Paradiso (1981). Whilst Hynes and Yamada (1982) in a review article

publish two electron micrographs showing the actin filaments of an MF bundle leading directly onto Extracellular Material (ECM) fibres associated with fibronectin. However, there is some contradictory work. Most recently, Chen and Singer (1982) using a double immuno-labelling technique at the electron microscope level, concluded that fibronectin is found at close contact sites rather than focal contacts. Whilst vinculin, although present at focal contacts, is absent at close contact sites. The same authors also show that ECM-contacts come to predominate in late (24-36 hr) cultures.

The solution to this inconsistency may be found in Singer's (1981, 1982) work. He showed that at a concentration of 0.3% foetal calf serum (FCS), fibronectin-vinculin associations were very often found at focal contact sites near the centre of the cells (which were arrested at G1 due to the low FCS concentration). Focal contacts at the periphery of such cells lacked the fibronectin-vinculin association. It appears that the latter develop into the former type. At 5% FCS, fibronectin was absent at the vinculin-containing focal contacts.

The situation is still unresolved. However, transformation is known to disrupt the fibronectin-vinculin association and vinculin seems to be a target of at least one transforming virus (Sefton *et. al.*, 1981; Hynes, 1982).

Most of the above work has been done on fibroblasts. But many epithelial cell lines show a similar relation between fibronectin and adhesion (e.g. Bannikov *et. al.*, 1982). However many epithelial cells do not normally produce fibronectin, e.g. bladder epithelium (Trejdosiewicz *et. al.*, 1981).

Fibronectin is also found *in vivo*. For example, it is important in wound repair and during various developmental

processes (e.g. development of the embryonic chick eye, Kurkinen et. al., 1979). Finally, it is also important in cell adhesion to collagen (see below).

However, there are other glycoproteins important in cell adhesion. Of especial interest here is the basement membrane glycoprotein, Laminin (800,000m.w.;Hogan, 1981). This has been identified in an in vitro kidney tubule induction system by Saxen's group (Ekblom, Alitalo, Vaheri, Timpl, and Saxen, 1980; see also Carlsson et. al., 1981).

Now to move on to cell locomotion (reviewed in Dunn, 1980; Abercrombie, 1965 and 1980; Abercrombie et. al., 1977). A wide variety of techniques have been brought together to try to elucidate the mechanism. Interference reflection microscopy enables one to see areas of cell adhesion whilst the cell is moving. The dark streaks seen are interpreted as areas of the cell which are closely apposed to the substrate, i.e. Focal Contacts, as confirmed by transmission electron microscopy (TEM; Heath, 1982). The gap between the cell membrane and the substrate at these points is 10-20nm (Abercrombie, 1980). Focal contacts appear to be sites of strong adhesion (evidence supporting this is reviewed by Abercrombie, 1980; Abercrombie et. al., 1977; Vasiliev and Gelfand, 1977 and 1981). The Close contacts appear as areas of gray. The focal contacts remain stationary in relation to the substratum as the cell moves. New focal contacts appear at the leading edge of the cell. At the level of the nucleus most of them fade out (Izzard and Lochner, 1980).

MF bundles within the fibroblasts are approximately in line with the direction of cell movement (Abercrombie, 1980; Rees et. al., 1980). These bundles may be retractile, the front end

of each bundle terminating on the cytoplasmic side of a focal contact. Contraction of these bundles would tend to pull the nuclear region forward. Meanwhile the front of the cell is extruding, laying down new focal contacts. The leading lamella (100-500nm thick) in fact undergoes local extensions and retractions (Abercrombie et. al., 1970a). It usually possesses "Ruffles" (Abercrombie et. al., 1970b; Ingram, 1969) and is often called a Ruffled Membrane. It contains a fine meshwork of actin filaments and is very vulnerable to cytochalasin (Domnina et. al., 1982). Ruffles travel back along the dorsal side of the leading lamella, as indeed do particles attached to the dorsal surface (Abercrombie et. al., 1970c, 1972; Dembo and Harris, 1981; Heath, 1983; Vasiliev et. al., 1976). This indicates that a backward movement of the dorsal plasma membrane occurs during locomotion. Presumably, cytoplasmic material flows into the leading edge during forward movement, perhaps propelled by sub-surface actomyosin, and associated with the retraction of the trailing edge of the fibroblast (Chen, 1981a,b).

The above is a review of a possible mechanism of fibroblast locomotion in vitro. It is based mainly on studies of avian and mammalian fibroblasts (especially by Abercrombie and his co-workers) and some conflicting evidence exists. For example, Lewis et. al. (1980) found that cells with many large MF bundles (visible under the light microscope and called Stress Fibres) tended to be stationary. They also contradict Chen's (1980a,b) work on fibroblast "tail" retraction, showing that "tailless" cells travel at the same rate as normal cells. Dunn (1980) notes work performed on primary chick fibroblasts which possess neither MF bundles nor focal contacts, yet are

able to locomote perfectly well (see also Couchman and Rees, 1979). Kolega *et. al.* (1982) found that cells with many focal contacts tended to be flat and moved very little, whilst cells with close contacts were relatively fast moving. Ruffled membranes themselves may be artifacts produced by the flat plastic substrate itself (Tomasek and Hay, 1981; Lofberg and Ebendal, 1980). Other locomotory specialisations such as filopodia may be important in the 3-dimensional *in vivo* situation (Robinson and Karnovsky, 1980). Finally, although actin appears to be essential for cell locomotion in most cases (Spooner *et. al.*, 1971), special cases are found which apparently do not use actin (e.g. amoeboid locomotion of *Caenorhabditis elegans* spermatozoa; Nelson *et. al.*, 1982; Roberts and Ward, 1982).

Few studies have been made on epithelial cell locomotion. Epithelial cells tend to form sheets, where only the cells at the periphery show locomotory specialisations, e.g. ruffled membranes (Dipasquale, 1975a,b; Vasiliev and Gelfand, 1977 and 1981; Heath, 1982). These specialisations seem to be produced by the actin meshwork of the cell, as they are sensitive to cytochalasin B (Dipasquale, 1975b). This suggests a similar mechanism to that suggested for fibroblasts. But again there is contradictory evidence. Heath (1982) notes work which shows that epidermal cells showing focal contacts moved more slowly than cells without, and that adhesion plaques were absent in these cells *in vivo* during wound healing. (On the latter point, however, Donaldson and Dunlop, 1981, did find focal adhesions during wound healing.)

Cell shape and locomotion are also affected by the type of substrate. "Contact Guidance" is a term coined by P. Weiss

(e.g. 1961) to describe the orientation of cells produced by the shape of the substratum (reviewed in Dunn, 1982). For example, glass fibres or aligned collagen fibres can be used (Dunn and Ebendal, 1978b; Yoshizato et. al., 1981). The cells align along the fibres rather than across them. This suggests that physical factors have a role in contact guidance (Dunn and Ebendal, 1978a; Lofberg and Ebendal, 1980). It may be that the shape of the substratum imposes mechanical restraints on the formation of linear MF bundles (Dunn and Heath, 1976). Transformed cells often show decreased alignment in contact guidance experiments. This often seems to be correlated with their more disorganized cytoskeleton, especially the loss or reduction of MF bundles (Fisher and Tickle, 1981). Contact guidance may have a role in vivo, e.g. in axon growth cone guidance (Bray, 1982; Ebendal, 1976; see also Trinkaus, 1982) or myoblast alignment in tadpole tails (Warren, 1981). Note, however, that contact guidance will only align cells, not give them direction. The latter may come via chemical (Zigmond, 1982; Bray, 1982) or electrical means (Trinkaus, 1982), or possibly Contact Inhibition.

Early contacts between normal fibroblasts result in "Contact Inhibition" (first described and named by Abercrombie and Heaysman, 1954). Heaysman (1978) distinguishes two types of Contact Inhibition. In type 1, two fibroblasts stop immediately on contact. The ruffling of their leading lamellae ceases. (Ruffling is associated with cell movement but is not essential.) Retraction finally occurs and the leading edges seperate. The cells may collide again, but eventually seperate. If one fibroblast collides with the side of another, the latter is unaffected, but the former

shows contact inhibition (though it may pass below the second cell to a certain extent). Given this behaviour, continued cell divisions should result in a monolayer culture.

Dead cells, inanimate material and contact with non-adhesive substrates do not usually elicit this reaction (see Heaysman, 1978, for a few exceptions). Forward locomotion ceases, but ruffling continues and no retraction occurs. In fact, ruffling may become very active. Heaysman (1978) names this reaction Contact Inhibition Type 2. Examples are known of it occurring between living cells, e.g. hypoblast cells (see Heaysman, 1978, for the original reference). This type of contact inhibition may be due to a failure to adhere to the substrate (or dead cell, etc).

Heaysman and Pegrum (1973 and 1982) have studied early cell contacts using the TEM. Within twenty seconds of impact special areas of cytoplasm are seen, resembling focal contacts. Within one minute, MF bundles appear associated with these areas. After two minutes these bundles are well defined and the cells begin to retract. The specialised areas appear to be reciprocal.

To summarise, it appears that colliding cells actually stop locomotion although focal contacts persist in the contact inhibition region (Abercrombie and Dunn, 1975). Specialised intercellular adhesions (resembling focal contacts) are produced, but breakdown when the cells seperate. It is not known why the cells stop locomotion, nor why they finally seperate. It has been suggested that seperation occurs because the intercellular adhesion is too weak to support locomotory behaviour (Heaysman, 1978; Heaysman and Pegrum, 1982).

Some cells do not show contact inhibition type 1. For

example, many transformed cells show reduced contact inhibition behaviour (even when enucleated, Pollack et. al., 1974). Much of this work has been done by looking at Nuclear Overlap Indices. But cell overlapping may be brought about by other mechanisms. For example, weak adhesion to substrates may allow underlapping (Heaysman, 1978) or "retraction clumping" of part of a cell sheet (Harris, 1973). However, transformed cells' altered contact inhibition behaviour has been linked to malignancy by Abercrombie (1979), possibly helping to account for metastasis. To try to test this much work has been done on colliding outgrowths from explants (e.g. Stephenson, 1982a,b; Marcel, 1980). Cells from malignant tissue are often able to infiltrate the outgrowth from normal tissues. However, there is the problem that such experiments are carried out on 2-dimensional substrates in a very abnormal environment. Work with 3-dimensional substrates (e.g. collagen gels, see below) should go some way to overcoming this.

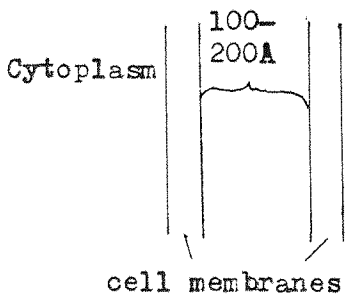
Much less work has been done on cell-cell contacts in epithelial cells (reviewed in Middleton, 1982). Part of the problem appears to be that many epithelial cells do not move about very much, and tend to form stable specialised inter-cellular contacts (e.g. gap junctions or Desmosomes, Fig. 2; Dipasquale, 1975a; Sanders and Prasad, 1981). Thus, these cells tend to form sheets in culture, in which only cells at the periphery and at scattered intervals within the sheet are closely attached to the substrate (Dipasquale, 1975a). Only cells at the periphery have leading lamellae. When sheets collide, the leading lamellae are inhibited, movement normally stops and one sheet is formed. Thus, contact inhibition type 1 does appear to occur between and within epithelial cell

FIGURE 2.

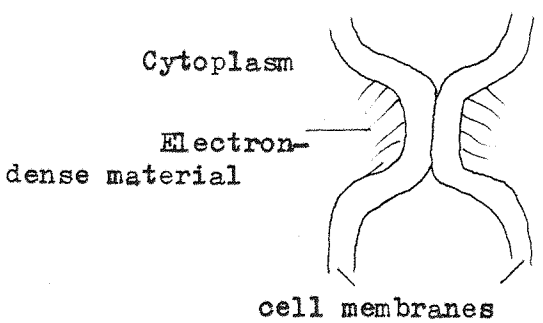
Specialised Cell Junctions.

(See the review by Gilula, 1973, for original references.)

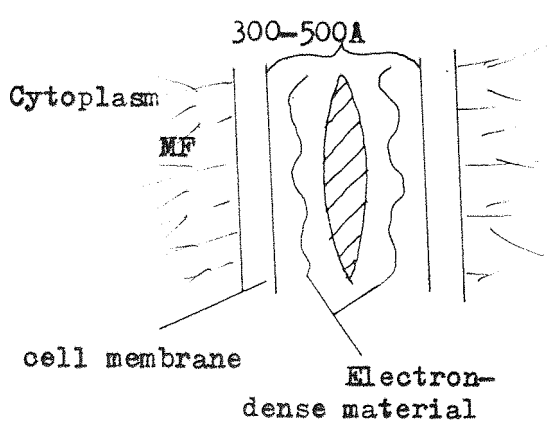
Loose Junction, or Intermediate Junction, or Zonula adherens.



Tight Junction, or Zonula occludens.



Desmosome, or Macula adherens.



Septate Desmosome (Found in invertebrates).

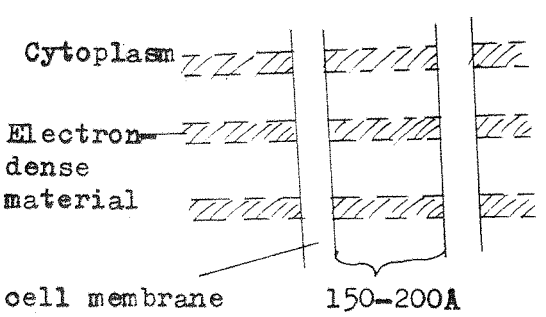
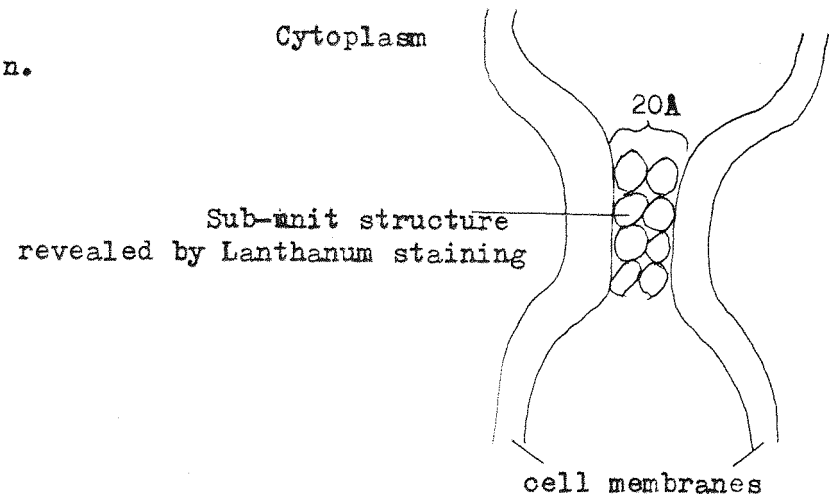


FIGURE 2. (Contd.)

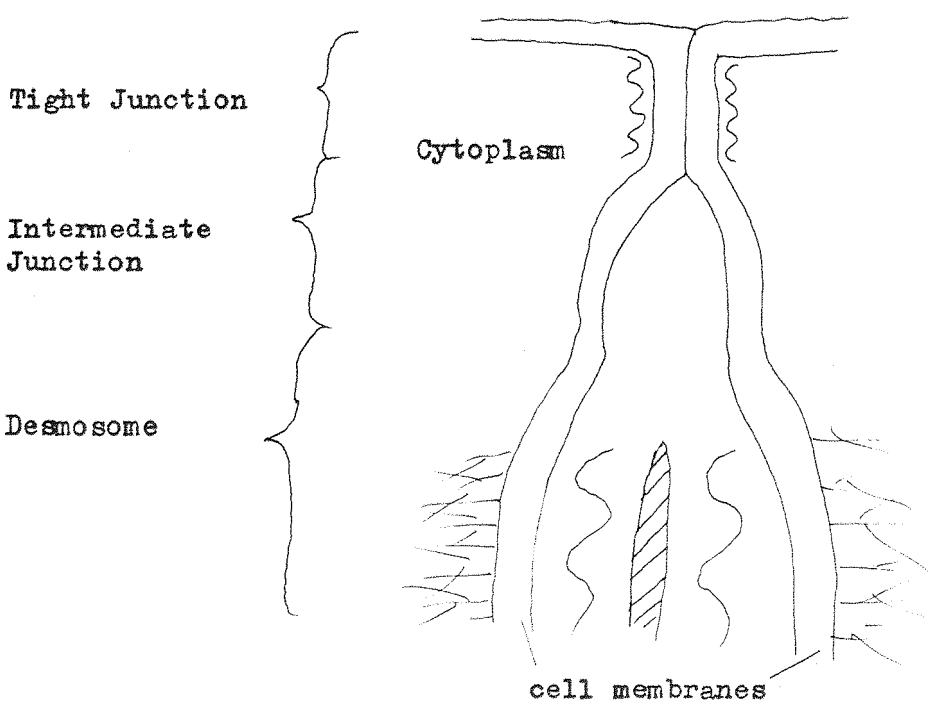
Specialised Cell Junctions.

(See the review by Gilula, 1973, for original references.)

Gap Junction.



The Junctional Complex.



sheets. Evidence from wound closure experiments tends to confirm that contact inhibition type 1 occurs (e.g. Udoh and Derby, 1982).

Cell interactions also have an effect on growth (reviewed in Loewenstein, 1979; Stoker, 1967; Vasiliev and Gelfand, 1981). First of all, normal cells usually only grow attached to a solid substrate. Transformed cells show reduced anchorage dependence. (Mukherjee et. al., 1982, have shown that retinoic acid can induce anchorage-independent growth and density-dependent growth without affecting the cytoskeleton or fibronectin formation in transformed cells. Whilst Peehl and Stanbridge, 1981, were able to induce anchorage-independent growth in normal fibroblasts using extra FCS and hydrocortisone.) Normal cells usually stop dividing at a certain density. This may be due to contact inhibition or to local depletion of the medium. There is evidence supporting both ideas. For example, shaking cultures or continuous flow of medium can elicit cell division in confluent "resting" cultures (e.g. Froehlich and Anastassiades, 1975; Kruse Jr. and Miedema, 1965). The contact inhibition idea is supported by the fact that cell division reoccurs at wound sites. It may be due to close packing of the cells resulting in a cell shape that does not allow DNA synthesis (Folkman and Moscona, 1978; Folkman and Greenspan, 1975; Pasternak, 1976). Transformed cells show a decreased response to cell shape (Wittelsberger et. al., 1981). Martz and Steinberg (1972) note, however, that cell-cell contacts or high local density do not inhibit division directly, since there is often a one generation lag. They support four possibilities to account for this:- 1/. The cells have a sufficient stock-pile of materials to divide

once. 2/. The cell-cell contacts have to "mature". 3/. Contacts with inhibited cells may work better than contacts with uninhibited cells. 4/. Interleaving cell contact may be required. Lastly, one should note that some cells may even produce proliferation inhibitors (e.g. Strobel-Stevens and Lacey Jr., 1981).

Other factors are known to influence growth. For example, most cells have an optimum pH (Froehlich and Anastassiades, 1974; Zetterberg and Engström, 1981) - though Rubin (1971) shows that low density cultures are less sensitive - an optimum temperature and ion concentration (reviewed in Vasiliev and Gelfand, 1981).

Specific proteins (Growth Factors) are also required (reviews in Vasiliev and Gelfand, 1981; Gospodarowicz, 1979; Shuth, 1981). These are usually provided in the medium as a constituent of the serum. Therefore, serum-free media usually have to include them, (e.g. Walthall and Ham, 1981; Yamane *et. al.*, 1981). Growth factors can produce cytoskeletal changes (amongst a wide variety of other effects; Keski-Oja *et. al.*, 1980 and 1981; Schlessinger and Geiger, 1981). They are also found associated with MT and MF in triton-extracted cytoskeletons (Schechter and Bothwell, 1981; Nasi *et. al.*, 1982).

Thus far this Introduction has reviewed concepts in cell culturing and information on cell morphology, locomotion and growth. All this is necessary background information for the Discussion. Now, work on morphogenesis *in vitro* will be briefly reviewed before moving to examples of morphogenesis *in vivo*. For the latter, Saxen's work on the metanephric kidney will be discussed in detail as it was one of the initial

stimuli for this project. Finally, a brief review of kidney formation (especially in X. laevis) is presented, which then allows a fuller description of the aims and work involved in this project.

Simple multicellular shapes and patterns can be produced by cells in vitro. Elsdale and Foley (1969) and Elsdale and Bard (1972a) obtained complex ridge patterns in embryonic human lung fibroblasts. These ridges seemed to be produced by cells moving over the ECM produced by the cells themselves. Although all the cells seem to be capable of forming ECM, Elsdale and Foley (1969) suggest that only non-motile cells actually do so. Fibroblasts are spindle-shaped, in confluent culture they tend to form up in arrays (Photo. 14c). Cells are parallel within the arrays, but arrays are arranged randomly within a culture. Only the cells at frontiers between arrays are non-motile (Elsdale, 1968). Thus ridges are produced at these sites, rather than general multilayering. The ridges finally disappear as the different cell layers merge.

Transformed cells often show multilayering in which the cells cross-over each other. This was first interpreted as due to decreased contact inhibition type 1. However, work by Bell Jr. (1972 and 1977) and Erickson (1978) shows that contact inhibition type 1 can still be found in transformed cells. Multilayering apparently results by underlapping of cells due to a low adhesion to the substrate.

Dipasquale and Bell Jr.(1974) find that neither epithelial nor fibroblastic cells will move over each others' dorsal surfaces. However, Elsdale and Bard (1974) conclude that whereas the dorsal surface of epithelial sheets will not support cell locomotion, epithelial sheets are able to grow

over fibroblast monolayers. They consider this behaviour to be due to an intrinsic polarity within epithelial cells and associate this with gland formation *in vivo*. Prop (1975) challenges both these two sets of workers. He suggests that all one can say is that cells vary in their adhesiveness, both to one another and to the substrate. He quotes work in which fibroblasts apparently spread and grew over epithelial sheets. However, this work has in turn been critized by Dipasquale and Bell (1975), who consider that the resolution used was not good enough to tell whether the fibroblasts were actually attached to the epithelial sheet or to the substrate (exposed in gaps between the epithelial cells). They also critize Elsdale and Bard's (1974) work for similar reasons. In their turn, Elsdale and Bard (1975) note that apparent fibroblast growth on epithelial sheets (both derived from mammary glands) only occurs in the absence of hormone. In its presence, fibroblast cell cords pass *over* the epithelial sheets to settle on the substrate.

Finally, Bard (1979) confirms the view that most epithelia will not support cell locomotion on their dorsal surfaces. He examined outgrowths from human embryonic kidney explants. Epithelial sheets appeared first, then came cords of fibroblasts. These cords did not settle on the dorsal surface of the epithelia. Instead, they grew out above the epithelia and only settled on the plastic substrate beyond the edge of the epithelia. However, the epithelial cells could grow over the top of the settled fibroblasts. Bard also notes that only epithelia which maintain a free surface *in vivo* should be expected to maintain one *in vitro*. Epithelia which stratify *in vivo* are known to multilayer *in vitro* (e.g. epidermis; Van

Scott and Flaxman, 1968). Epithelia which occur as clusters or strands *in vivo* possess no free surface *in vitro*, being enveloped by fibroblasts (e.g. monkey peridontal ligament cells; Brunette *et. al.*, 1977).

Many primary cell cultures can show quite complex organised morphogenesis. For example, tubes are produced by the outgrowth from mammalian epidermis (Van Scott and Flaxman, 1968). This is associated with the development of elongated cells in the outgrowth. Other cells accumulate around these elongated cells. Central cells then die to form a lumen (see also Matoltsy, 1960).

Cell behaviour seems to be nearer the *in vivo* state if grown on an ECM rather than plastic. This has been well studied using collagen as the ECM. Collagen is a polymer of rigid, rod-shaped molecules, Tropocollagen (Miller, 1977; Eyre, 1980). The tropocollagen is formed from three polypeptide chains (α-chains) wound round each other, forming a triple helix. There is actually a family of collagen molecules differing in a number of criteria (see Table 3; Bornstein and Sage, 1980). Collagen forms at a variety of sites during development (e.g. Green *et. al.*, 1968; Hay, 1973) and is of major importance to many aspects of morphogenesis (see Bornstein and Sage, 1980, and Hay, 1981, for brief reviews). The collagen normally used for *in vitro* studies is Type I, extracted from rat tail tendons by dilute acid (see Methods). This can be used to make a variety of substrates (Fig. 3).

It is known that fibronectin plays a key role in cell adhesion to collagen (Fig. 4; reviewed in Kleinman *et. al.*, 1979). The fibronectin first binds to the collagen, the cells then attach to this complex and flatten out. However, much of

TABLE 3.

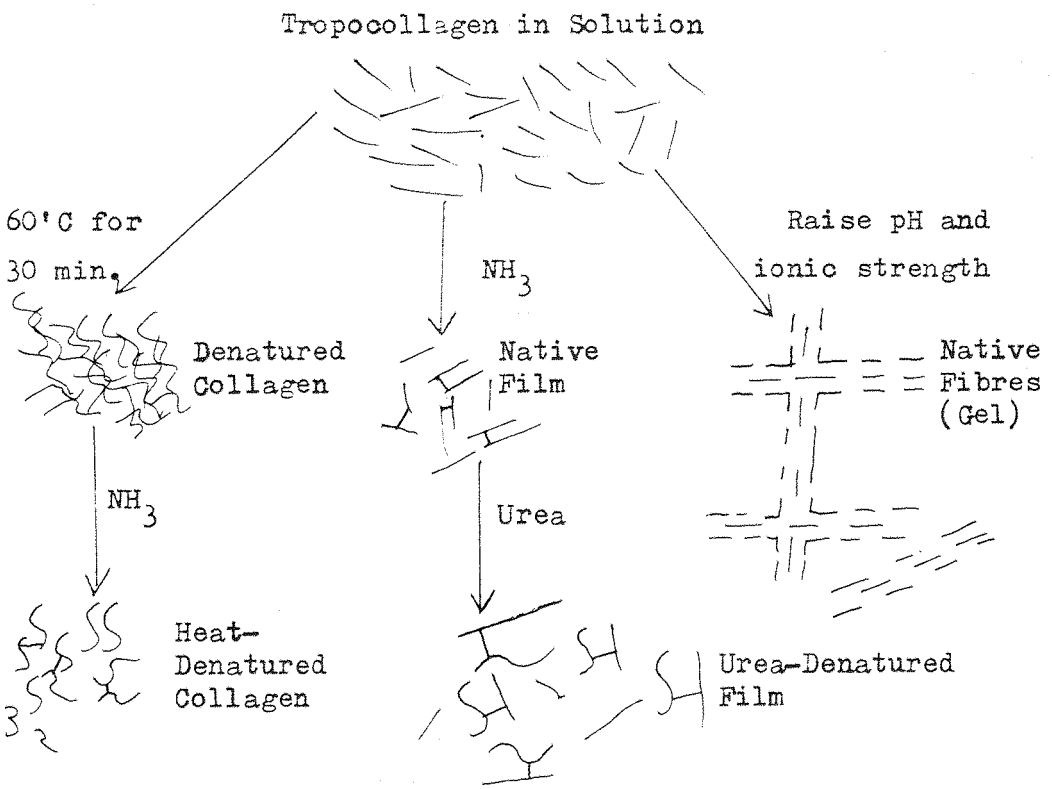
The Collagen Isotypes.

(Taken from Kleinmen et. al., 1981.)

Collagen Type	Composition	Tissue Location	Cell Types that synthesize various Collagen Types
I	$[\alpha 1(I)]_2 \alpha 2(I)$	Skin, bone, tendon, cornea.	Fibroblasts, Osteoblasts, Smooth muscle cells, Epithelium.
II	$[\alpha 1(II)]_3$	Cartilage, cornea, vitreous body.	Chondrocytes, Neural retinal cells, Notochord cells.
III	$[\alpha 1(III)]_3$	Fetal skin, blood vessels, Organs.	Fibroblasts, Myoblasts.
IV	$[\alpha 1(IV)]_3$ $[\alpha 2(IV)]_3$	Basement Membrane.	Endothelial and Epithelial cells.
V	$[\alpha 1(V)]_2 \alpha 2(V)$ (formerly αA- and $\alpha\beta$- chains)	Blood vessels, Smooth muscle.	Smooth muscle cells, Chondrocytes under certain conditions.

FIGURE 3.

Preparation of the Different Collagen Substrata.
(Taken from Schor and Court, 1979)

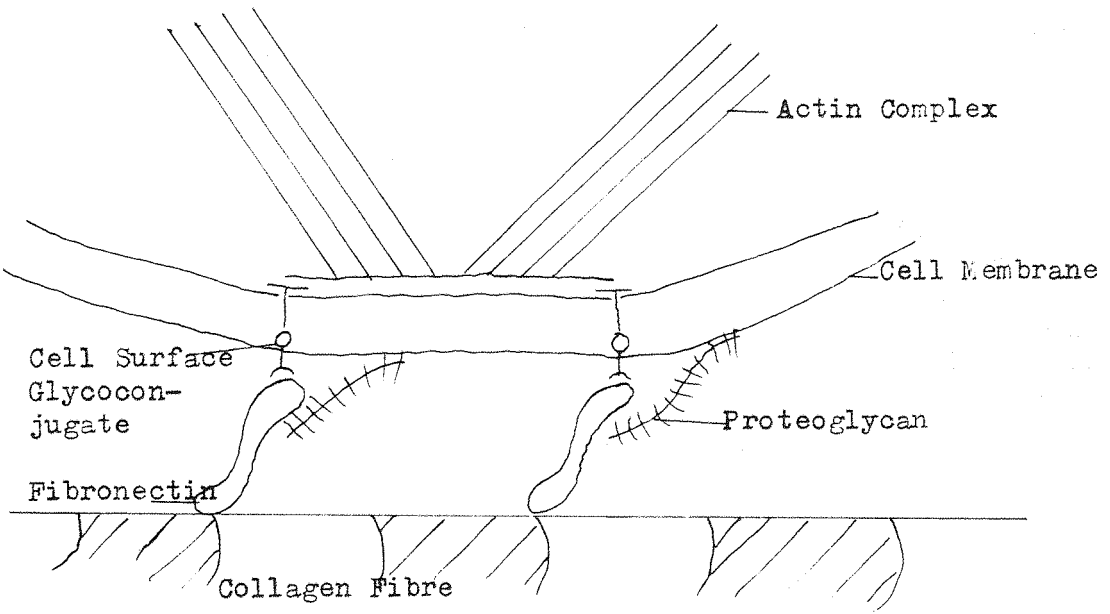


Type I Collagen is extracted from rat tail tendons in dilute acetic acid (see Methods). The resulting solution of Tropocollagen is used to prepare the different collagen substrates.

The diagrams presented here are clearly not intended to be an accurate representation of the molecular detail, but are shown to emphasize the salient differences between the organisation of collagen within the substrata.

FIGURE 4.

Schematic Model of a Collagen Adhesion Site.
(Taken from Kleinmen et. al., 1981.)



Collagen fibres contain specific sites to which the fibronectin molecule binds. The fibronectin molecule contains a region that recognises collagen and another region that recognises the cell surface. The cell surface receptor is thought to be a Glycoconjugate, possibly a Glycolipid that interacts with the cytoskeleton in some as yet unknown manner. Proteoglycans are also present and stabilize the interaction. Components not to scale.

this work has been criticized as dried collagen gels were used. Grinnell and Bennett (1981) found that fibronectin was not essential for cell attachment to hydrated collagen gels and they suggest that this is closer to the *in vivo* situation. This conclusion seems to be supported by Schor and Court (1979) and Briles and Haskew (1982). However, Rubin, Hook, Obrink and Timpl (1981) and Rubin, Johansson, Hook and Obrink(1981) found that rat hepatocytes appear to bind directly to both denatured collagen fibres and native gels, without requiring fibronectin.

Epithelial cells tend to attach preferentially to type IV, basement membrane, collagen (Murray *et. al.*, 1979) and this is not enhanced by fibronectin but by laminin (Terranova *et. al.*, 1980). Whilst Grotendorst *et. al.* (1981) found that smooth muscle cells bind to type V via an intrinsic glycoconjugate, but use fibronectin to attach to types I and III.

Collagen matrices also have a variety of other effects. For example, the life-span of some primary cultures is increased. Growth enhancement also occurs with some cells. Finally, differentiation is maintained or elicited in various cell types (e.g. myoblasts form myotubes on collagen).

One should also not forget that most cell lines produce collagen in varying amounts.

Hydrated collagen gels seem to present the most favourable *in vitro* environment. For example, parenchymal liver cells maintain many of their *in vivo* characteristics on collagen gels (Michalopoulos and Pitot, 1975), so do mammary epithelial cells (Emerman and Pitelka, 1977). The latter even respond appropriately (e.g. produce more caesin - milk proteins) to treatment with lactogenic hormones. Bone marrow cells can

undergo haemopoiesis within collagen gels (Lanotte et. al., 1981). Finally, skin fibroblasts grown within collagen gels seeded with epidermal cells have even been successfully used as skin grafts (Bell et. al., 1981).

Cell behaviour is different to that seen on plastic substrates. Cells tend to be bipolar and lack ruffled membranes, both on a collagen gel (Elsdale and Bard, 1972b; Bolender and Seliger, 1981) or within it (Davis, 1978 and 1980; Davis and Trinkaus, 1981; Greenburg and Hay, 1982a,b). This change is also associated with the loss of any stress fibres, at least in some cell lines (Tomasek and Hay, 1982; Tomasek et. al., 1982). Also, Schor (1980) found that fibroblasts tend to infiltrate collagen gels, whereas epithelial cells do not. No degradation of the collagen is involved in this infiltration (Schor et. al., 1980). Fibronectin can inhibit infiltration in some cell types (Schor et. al., 1981) but is required by others (e.g. cardiac mesenchyme, Markwald and Kitten, 1982). Note, however, that Bennett (1980) was able to elicit ingrowth and morphogenesis of Mammary Epithelial Cells into floating collagen gels. But the cell line she used was derived from a probable Stem Cell Line (Bennett et. al., 1978) and this may have been a factor (see below).

Normal fibroblasts can contract a floating collagen gel. This ability is reduced in transformed fibroblasts (Harris et. al., 1981; Steinberg et. al., 1980) which may be associated with the reduction in the number of their stress fibres and their reduced adhesion. Harris (1982) has studied the way cells can distort their substratum (using plastic sheets as well as collagen gels), and suggests ways in which this may be of importance in vivo. For example, in the organisation of

ligaments, tendons and muscles (Stopak and Harris, 1982; see also Bellows *et. al.*, 1981).

Yang *et. al.* (1979) were able to get organised morphogenesis from primary mammary tumour epithelial cells embedded within collagen gels. Cord-like cell outgrowths were produced which contained intercellular cavities resembling typical epithelial lumina. *In vivo* transplantation of such outgrowths resulted in a mammary adenocarcinoma histologically similar to the donor tumour.

Perhaps of even more interest is the cell line used by Bennett's group (e.g. Bennett, 1980) especially as this work was one of the initial stimuli for my project (with Saxen's work; see below). Rama 25 was derived from a rat mammary tumour (Bennett *et. al.*, 1978). During rapid proliferation the cells have an epithelial morphology. However, at high cell densities two other cell types appear. These resemble secretory and myoepithelial cells of the mammary gland, as indicated by TEM and immunofluorescent staining for caesin. The proportion of these two cell types can be increased using dimethylsulphoxide in different conditions. Thus, Rama 25 appears to be a mammary stem cell line (Fig. 5).

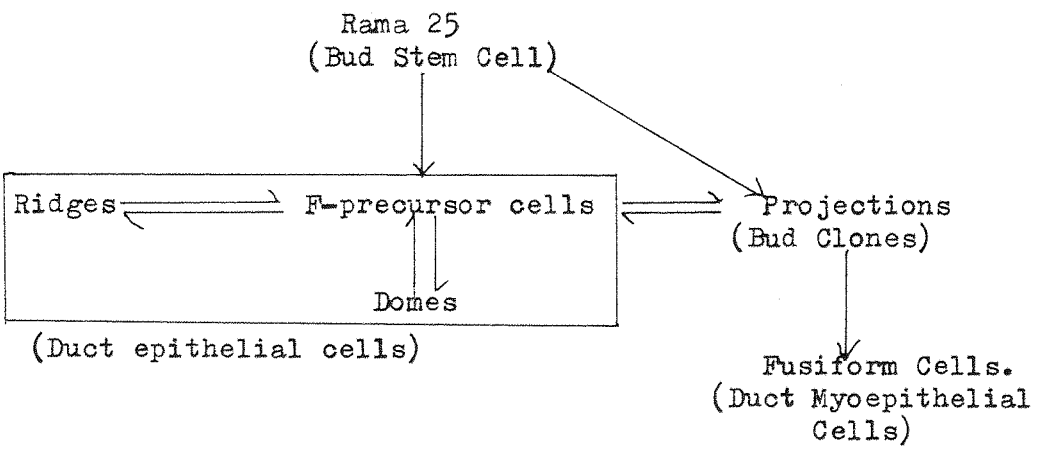
The development of the myoepithelial cells has been studied by Dulbecco's group (Dulbecco *et. al.*, 1979a,b and 1981; Lennon *et. al.*, 1978). They found that multicellular "projections" produced by Rama 25 produce these fusiform cells. They isolated sub-clones from such projections and found that these spontaneously produce domes, projections and ridges. (Domes are fluid-filled blisters formed by local detachment of the epithelial layer from the dish.) A proportion of the cells in projections irreversibly produce fusiform cells (Fig. 5). The

FIGURE 5.

Possible Scheme of Differentiation in the Rama 25 Line and Sub-lines.

(Taken from Dulbecco et. al., 1979a; see also Dulbecco et. al., 1982.)

In parentheses are the possible corresponding cells in the mammary gland.



probability of forming ridges, domes and projections varies with the culture conditions and between sub-clones. For example, dome formation in the sub-clone LA7 was studied in detail by Dulbecco and Okada (1980). They found a number of inducers, some produced by the cells themselves. Cell concentration was also important and hydrocortisone was required. They suggest that the dome-forming cells are analogous to duct luminal cells *in vivo* (see also McGrath, 1975).

Bennett (1980) found that when Rama 25 cells were grown on floating collagen gels, branching tubules were produced by ingrowth into the gels. These resemble the ducts of the glands *in vivo*. Bennett *et. al.* (1981) found that the tubules were produced only if both epithelial and fibroblastic cells were present. What appears to happen is that the fibroblastic cells grow into the gel first, and then the epithelial cells invaginate at these points to produce tubules. Other epithelial cell lines did not produce tubules or ingrowth when mixed with the fibroblastic cells. This seems to negate any simple mechanical explanation. However, some of these conclusions have been challenged by Ormerod and Rudland (1982). They found that incomplete tubules were formed at collagen concentrations of 0.1-0.3% (w/v); Bennett *et. al.* (1981) used 0.3%. At 0.6% long tubules (upto 0.5mm) with large lumina (upto 26 cells in circumference) were found. No fibroblastic cells were associated with the tubules (also noted by Bennett *et. al.*, 1981). Thus, they suggest that the fibroblastic cells may only be necessary as a source of growth-promoting factors required for tubule formation. They also show that tubule formation occurs by central necrosis and/or seperation of cells

in cell cords, rather than by invagination. Finally, they show that sandwiching the cells between two collagen gels only results in the production of fibroblastic cells, not tubules.

The latter point is of interest since MDCK (Madin-Darby canine kidney) cells in the same situation produce large cavities, similar in size to a tubule. Whilst NMuMG (normal murine mammary gland) cells produce clusters of small lumina, resembling the acini of glandular tissue (Hall et. al., 1982). These workers suggest that the epithelial cells are polarized, with their apical surfaces free. Overlaying with collagen produces a "conflict" which is resolved by the production of lumina. The type of lumen is in part determined by the tissue of origin. However, this polarization of epithelial cells may not be present in all cell lines, since they note a mammary cell line which does not produce lumina on collagen overlay. Certainly, it is known that MDCK cells have an asymmetrical morphological polarity (resembling renal tubular epithelium) and will form domes (Lever, 1979; Cereijido et. al., 1978 and 1980; Valentich et. al., 1979; Slaughter et. al., 1982). They even possess a cell surface antigen found on kidney distal tubules (Herzlinger et. al., 1982). Finally, collagen may even produce a polarity in certain cell types. For example, Sugrue and Hay (1981) have shown that solubilized collagen will produce a basal mat of MF in corneal epithelium, similar to that found in vivo. Hay (1982) shows that a collagen gel has the same effect.

This review is enough to show that cell culture systems can show quite complex morphogenesis and that such systems can be used to elucidate the mechanism of same. Collagen and collagen

gels appear to be the most widely used ECM in such studies. However, others have been used or suggested; e.g. ECM produced by the cells themselves, plasma clot and cellulose sponge (Leighton et. al., 1962) and Hydra mesoglea (Day and Lenhoff, 1981).

But there are still some aspects of development that cannot yet be readily studied by cell culturing. In many cases organ cultures are needed. ("Organ Culture. - The maintenance or growth of tissues, organ primordia, or whole or parts of an organ in vitro in a way that may allow differentiation and preservation of the architecture and/or function." Paul, 1975.) A detailed review of organ culture work is unnecessary; the way such systems have been used to study inductive interactions is of most interest here. A large number of inductive tissue interactions have been studied by such means. Of especial interest are the Epithelial-Mesenchymal Interactions, some of which are tabulated in Table 4. (Further examples are found in Holtfreter, 1968, and Saxen, Karkinen-Jääskeläinen, Lehtonen, Nordling, and Wartiovaara, 1976.)

As can be seen, most of these studies have been performed on mouse tissues, and chick to a lesser extent. However, the studies that have been made on other organisms do not seem to alter any of the general principles. This is at least partly due to the fact that the various mammalian and avian systems seem to cover most, if not all, of the physically possible ways of getting a "message" from one tissue to another. These general principles will now be outlined, then a specific example (the work of Saxen's group on the Metanephric kidney) will be examined in greater detail to try and clarify these points.

TABLE 4.

Some of the Epithelial-Mesenchymal Interactions Studied by Organ Culture (normally involving Transfilter Experiments).

Developmental System	Inductor	"Target"	Reference & Notes
Mouse Metanephric Kidney	Ureteric bud (Spinal cord Submandibular Embryonic Epithelium; etc.)	9-Day Metanephric Mesenchyme	e.g. Ekblom et. al., 1982. Discussed in detail in the text.
Mouse Lung	(Older) Bronchial Mesoderm. (Gut; Older Salivary Mesenchyme)	20-26 Somite Gut Endoderm.	Spooner and Wessells (1970). Only Bronchial Mesoderm induces branching of the lung bud produced by the "targets".
	(Older) Bronchial Mesoderm. (Submandibular Mesoderm; Stomach)	Tracheal Epithelium.	Wessells (1970).
Mouse Mammary Gland	12-Day Mammary Mesenchyme	12-16-Day Mammary Epithelium	Kratochwil (1969)
	13-Day Submandibular Gland Mesenchyme	"	" Morphology of epithelium similar to that of salivary gland.

TABLE 4. (Contd.)

Some of the Epithelial-Mesenchymal Interactions Studied by Organ Culture (normally involving Transfilter Experiments).

Developmental System	Inductor	"Target"	Reference & Notes
Chick Cornea	ECM (e.g. Collagen).	Corneal Epithelium	Meier and Hay (1974, 1975); Hay (1977)
Mouse Submandibular Salivary Gland	Submandibular Capsular Mesenchyme	Submandibular Epithelium	Grobstein (1953). Addition of spinal cord or cartilage caused formation of new capsular tissue (possibly from remains of the old one) and formation of the gland (Borghese, 1950a,b). ECM derived from both tissues is necessary (Bernfield et. al., 1973).
Quail Salivary Gland.	Quail anterior lingual or mouse submaxillary mesenchyme	Quail anterior submaxillary epithelium	Nogawa and Mezceno (1981). Branched Morphology results.
	Quail anterior submaxillary mesenchyme	Quail anterior lingual or mouse submaxillary epithelium	Elongate morphology results. Thus, the morphology of quail salivary glands is controlled by the mesenchyme.

TABLE 4. (Contd.)

Some of the Epithelial-Mesenchymal Interactions Studied by Organ Culture (normally involving Transfilter Experiments).

Developmental System	Inductor	"Target"	Reference & Notes
Mouse Tooth Development.	17-Day Dental Mesenchyme	17-Day Dental Epithelium	Thesleff (1977). See Burgess and Katchburian (1982) for TEM of normal development.
Mouse Pancreas	Various Mesenchymes (Mesodermal Factor; a cell-free extract of whole chick embryos or mesenchymal tissue.)	15-Somite Midgut	Reviewed in Wessells (1977); see also Grobstein (1962)

There are basically four types of cell-cell contacts (Saxen, 1977a; but see below):-

Long-Range (50,000nm)

a). Diffusion.

b). Matrix Interaction.

Short-Range (5nm)

c). Exchange of Small Molecules.

d). Surface Associated Compounds.

There appear to be examples of all four types (see reviews by Saxen, 1972, and 1977a,b; Saxen, Karkinen-Jääskeläinen, Lehtonen, Nordling, and Wartiovaara, 1976; Saxen et. al., 1980). In each specific example the two tissues are only Competent (Waddington, 1962) for a given period during development. Before or after this time, one or both of the tissues will not show the same reaction, if any reaction occurs at all. This can lead to a sequential process of development, with tissues interacting in various ways during the course of development (Saxen, 1977a). The interaction involved can be of two types: Directive or Permissive (Saxen, 1977b). In the Directive Interaction, extracellular factors exert a true directive action on differentiation. In the Permissive Interaction, the tissue is already committed to a certain pathway of differentiation, but requires a stimulus from the extracellular factors to express this phenotype. Obviously, this division is not meant to be a strict one, but simply an aid to study.

In Directive Interactions, it is usually the mesenchyme which guides the epithelium (e.g. in tooth development, Slavkin et. al., 1977; see also Table 4). Thus the mesenchyme is specific but the epithelium need not be (e.g. embryonic rabbit mammary gland mesenchyme can induce avian flank epithelium to

differentiate into mammary gland-like epithelium; see Slavkin et. al., 1977, for the original reference).

In Permissive Interactions the epithelium will show a specific reaction (e.g. tubule formation) when combined with a wide variety of inductors (e.g. Saxen's work on mouse metanephric tubule formation).

So some of the points to note in such studies are:-
The period of Competence of the tissues involved.
The type of cell-cell contact required,
The type of interaction involved.
The "signal" substance, if any.
The reaction of one or both tissues.

One last point to note is that with in vitro studies the inductor may simply be making the environment suitable for the "test" tissue. So it may not be an accurate representation of what occurs in vivo. Now to move on to Saxen's work to illustrate some of the above points.

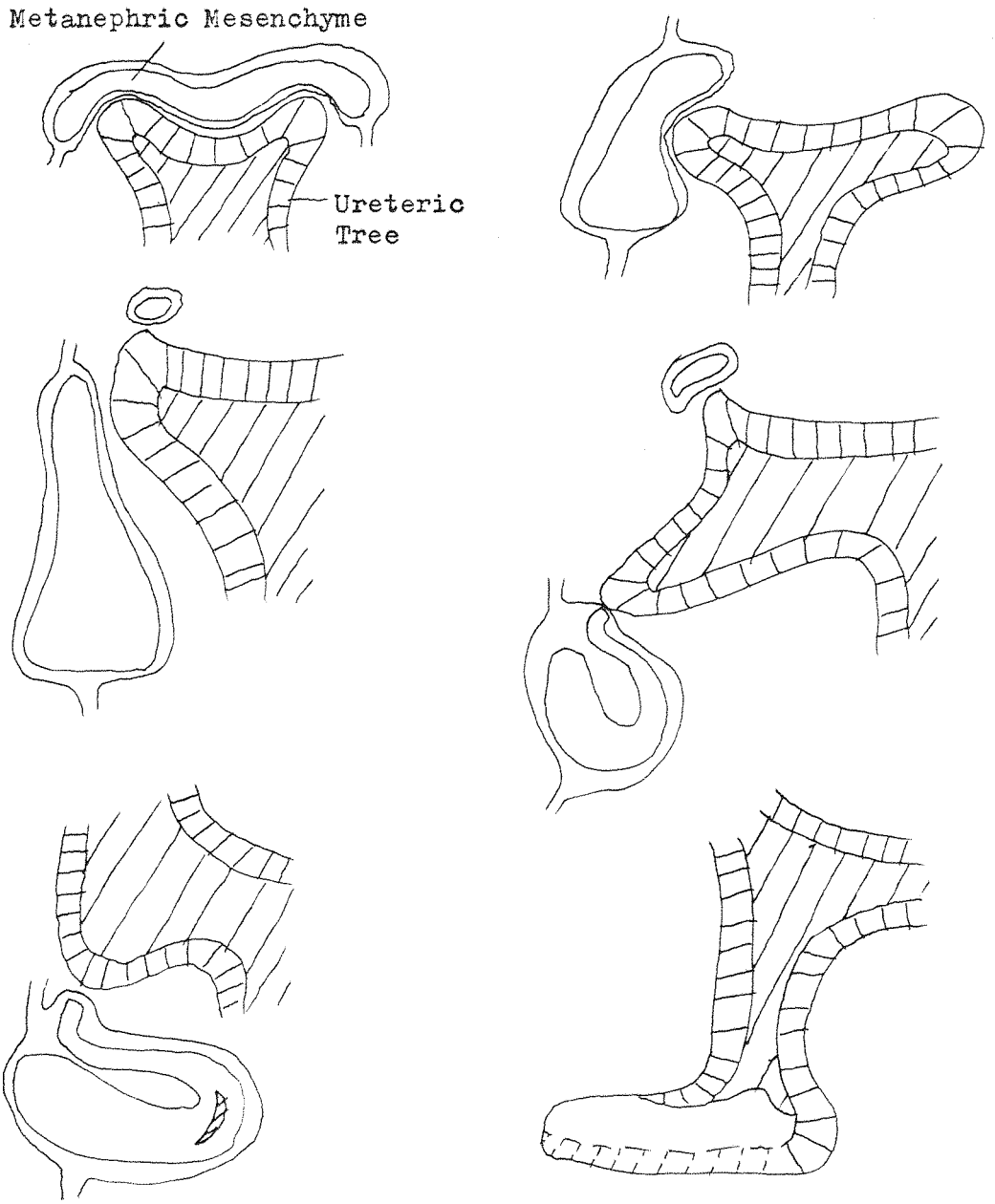
First, a brief outline of the normal development of the metanephric kidney (Fig. 6), taken from Saxen (1971). The ureter bud grows into the loose mesenchyme, forming branches. The mesenchyme becomes condensed around these branches. Dense areas of mesenchymal tissue form around each tip of the ureteric tree. The renal vesicle is formed by differential cell growth within this mass. Then an S-shape is formed which attaches to a branch of the ureteric tree, forming the future tubule collecting system. Time-lapse and TEM techniques show the greater mutual adhesiveness of the cells as they aggregate. Once aggregates have formed, the cells develop a certain asymmetry, a lumen forms and then a basement membrane.

Saxen's group carried on Grobstein's work (e.g. Grobstein,

FIGURE 6.

Early Development and Remodelling of the Renal Vesicles of the Metanephric Kidneys.

(Taken from Saxen, 1971.)



The loose metanephric mesenchyme is seen to become condensed or packed around the tips of the branching ureteric tree, and this dense mass subsequently gives rise to the primitive renal vesicle. This is then converted into the typical S-shaped body preceding the segregation into different portions of the secretary tubule.

1955 and 1961; Unsworth and Grobstein, 1970). They confirmed that heterologous inductors (e.g. spinal cord) would elicit tubule formation in 11-12 day-old mouse metanephric mesenchyme. However, the same inductors had no effect on heterologous mesenchymes, indicating a Permissive Interaction (Saxen, 1970). They also found that the inductor can be removed from transfilter experiments quite early, before the mesenchymal cells have aggregated (i.e. aprox. 24hr) and morphogenesis still occurs (Saxen et. al., 1968). There are a number of associated changes that also occur, summarised in Fig. 7.

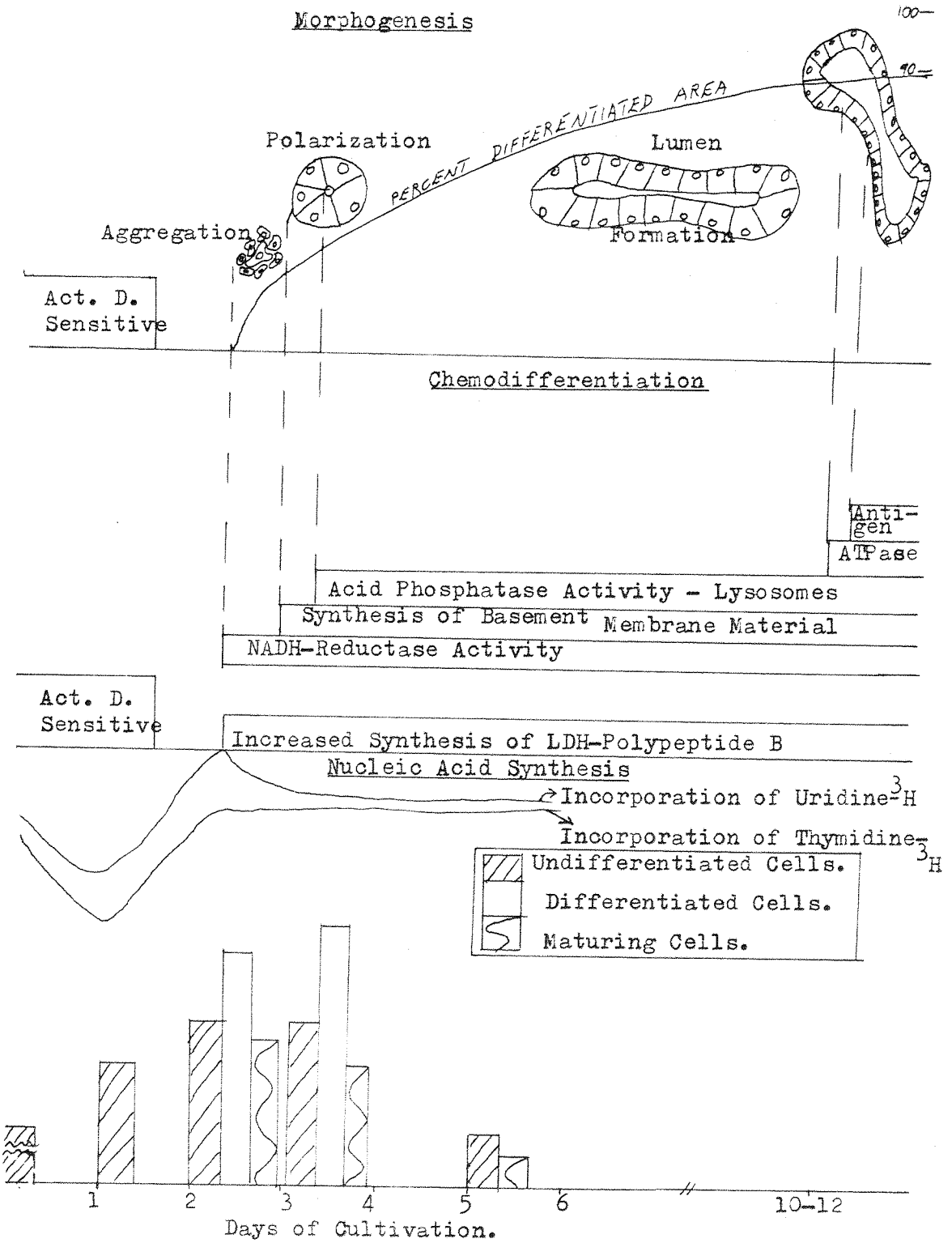
Both Grobstein's and Saxen's early transfilter experiments seemed to show that direct cell-cell interaction was not necessary for this induction. However, Nordling et. al. (1971) and Saxen (1971) used two Millipore filters (pore size 0.8μm, thickness of each 25μm) and showed that the longer induction time could not be due to a longer time for diffusion. Finally, Wartiovaara et. al. (1974), Lehtonen et. al. (1975), and Saxen, Lehtonen, Karkinen-Jääskeläinen, Nordling, and Wartiovaara (1976), using Nucleopore filters (which have straight pores rather than a meshwork system) and better TEM fixation techniques, found abundant cytoplasmic penetrations in filters with 0.2μm pore size or larger. Filters with a mean pore size of 0.13μm did not allow tubule formation in transfilter experiments. Saxen and Lehtonen (1978) went on to show that cellular contacts develop within 1hr of the cytoplasmic processes emerging through the filter. There is then a 16-24hr lag period before completion of induction. This lag is a function of the thickness and pore size of the filter. The intensity of the mesenchymal response was shown to be a function of pore size, pore density and duration of transfilter contact;

FIGURE 7.

Concise Diagram of the Developmental Events occurring in the Metanephric Mesenchyme induced with Spinal Cord.
(Taken from Saxen et. al., 1968.)

ACT.D = Actinomyosin D

See text and original reference for details.



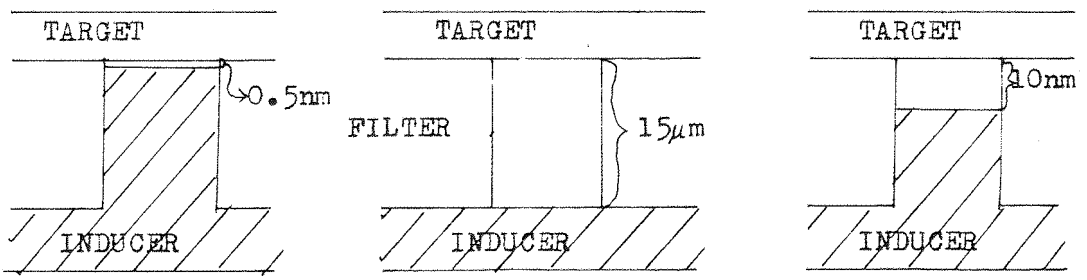
i.e. there is a positive correlation between the contact area between the tissues and the degree of induction (Nordling et. al., 1977). Weiss and Nir (1979) were able to show that direct cellular contact (0.5nm gap) is not essential to explain these results. A gap of 10nm will also give the same results, although diffusion over greater distances will not (Fig. 8).

There is some information as to the nature of the induction process itself. Nordling et. al. (1977) were able to show that DNA synthesis or cell division were not necessary in the inductor, but were essential in the mesenchyme for successful induction. The mesenchyme also needed to show RNA and protein synthesis. Charged polymers (e.g. Heparin) reversibly inhibit the cell-cell communication required for induction (Ekblom et. al., 1978). The effect being a function of the polymer's charge density. The charged polymers are known to alter cell surface characteristics and these workers suggest that a molecular barrier is formed between the interacting surfaces. Finally, Tunicamycin blocks induction in this transfilter system (Ekblom et. al., 1979). This seems to be due to its inhibitory effect on protein glycosylation. But, as both inductor and mesenchyme were exposed to it, either or both tissues could have been effected.

Recently, Saxen's group have been using immunological techniques to study the changes associated with tubule formation. Ekblom, Miettinen, and Saxen (1980) examined the formation of brush border antigens of renal tubules in vivo and in vitro. The antigens appeared c.30hr after lumen formation in both cases. Two types of tubule were formed in both cases; those which stained intensely and others that did not stain at all. Also, structures resembling glomeruli were

FIGURE 8.

Summary of Three Models for Transfilter Induction.
(Taken from Weiss and Nir, 1979.)



a) Penetration and Contact.

b) Diffusion without Penetration.

c) Penetration and Diffusion.

a). Shows penetration of a pore (diameter 0.2μm) by an inducer process, making physical contact with the target.

b). Shows no penetration of a filter pore (diameter <0.1μm); with induction by diffusion over the filter thickness (15μm). This is not found in the metanephric system.

c). Shows filter pore penetration to with 10nm of the target, followed by diffusion of a message.

seen *in vitro*. Ekblom, Miettinen, Virtanen, Wahlström, Dawnay, and Saxen (1981) were able to study the segregation of the metanephric nephron directly. They used fluorochrome-conjugated wheat germ agglutinin, which binds to the glomerular epithelial surface; an antiserum against brush-border antigens of the proximal tubules; and an antiserum against a glycoprotein found on distal tubules. *In vivo* these markers appear sequentially on days 13-15. The same sequence was obtained *in vitro* after a 24hr initial transfilter contact with the inductor. Thus, cultured mesenchymes can develop all three sections of the metanephric nephron in correct sequence over four days.

The ECM has also been studied. Ekblom, Lehtonen, Saxen, and Timpl (1981) have shown that undifferentiated mesenchyme before induction expresses types I and III collagens. Induction *in vivo* and *in vitro* leads to the loss of these proteins and the appearance of type IV collagen, which concentrates at the basement membrane. This change occurs before any apparent morphological changes. Ekblom, Alitalo, Vaheri, Timpl, and Saxen (1980) studied the formation of laminin, both *in vivo* and *in vitro*. It was first detected *in vivo* where the peritubular aggregates form. Later it was confined to the basement membrane of the tubules. *In vitro*, it was first seen after 12hr of culture, c.24hr before overt morphogenesis. After 48hr, it was only detected in cells destined to become epithelium. After 72hr, it was only seen in the basement membranes of the tubules. They suggest that the laminin is involved in the increased cell adhesiveness associated with aggregation. They also note that fibronectin formation is a property of the mesenchymal cells, which they loose if they are induced.

Finally, Ekblom (1981) used specific antibodies to laminin,

type IV collagen, basement membrane proteoglycan and fibronectin to study the development of the basement membrane (see also Roll *et. al.*, 1980). The undifferentiated mesenchyme expresses fibronectin but not the other three substances. These become detectable in the induced area, whilst fibronectin is lost.

In summary, Saxen's group has developed an *in vitro* system which closely resembles the situation found *in vivo*. They have examined tubule formation at the morphological and biochemical levels. To a certain extent they were able to correlate these changes. They have also found that a short-range cell-cell interaction of short duration can set in motion all these changes. However, a number of questions remain, including the exact nature of the "signal".

The last things to review before describing my own work are a short description of the normal development of the Urino-genital System (taken from Balinsky, 1975) followed by a summary of kidney tubule formation in *X. laevis*.

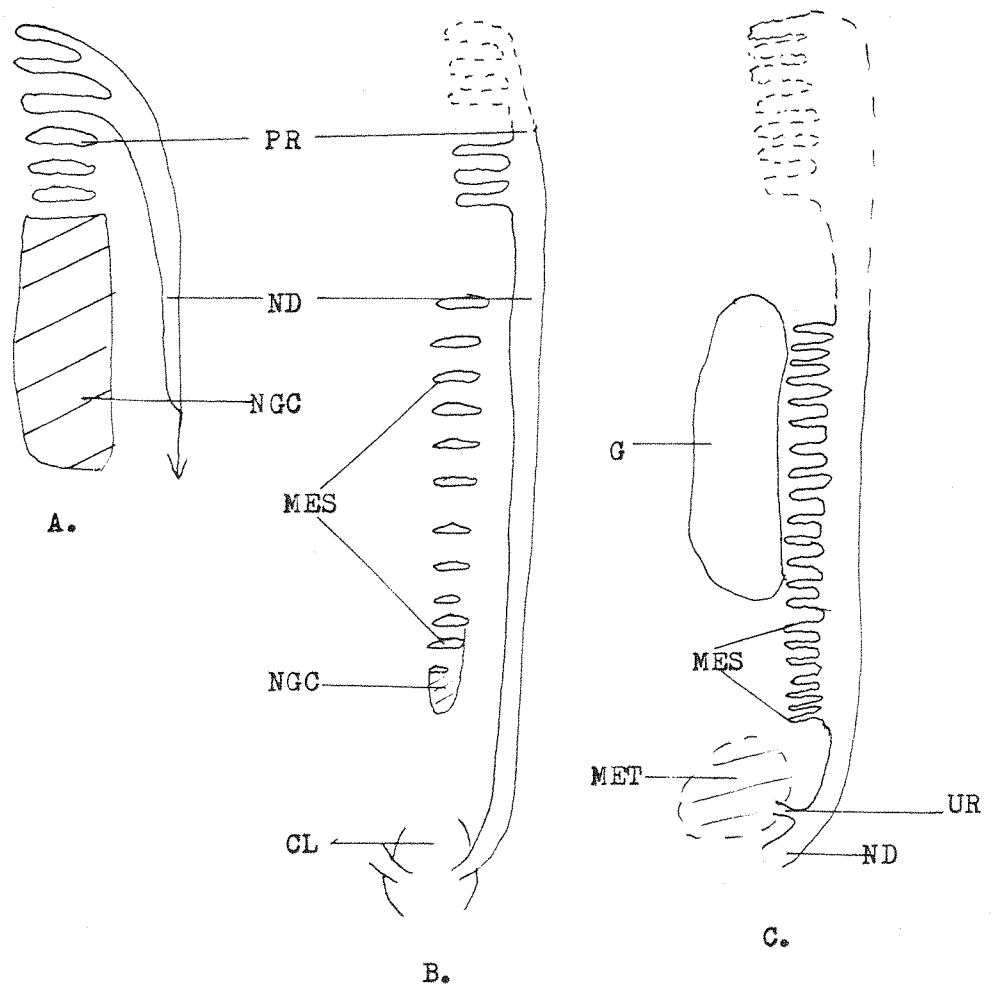
The nephric system of vertebrates develops as three units - Pronephros, Mesonephros, and Metanephros (Fig. 9) - occurring successively during development (but see Discussion).
The pronephric and anterior mesonephric tubules have a simple Metameric distribution. The posterior mesonephric and the metanephric tubules develop within an unsegmented cord of nephric material. The metanephros only develops in Amniotes (Burns, 1955).

Basically, the vertebrate excretory organs are aggregates of tubules, connected at their proximal ends with the coelomic cavity via ciliated funnels (the Nephrostomes; however, see Fig. 13 for the modern definitions) and communicating to the exterior by a system of ducts, which open into the

FIGURE 9.

Plan of the Development of the Nephric System of Vertebrates.

(Taken from Burns, 1955.)



A. Origin and Mode of Development of the Nephric Duct.

B and C. Its Relation to the other parts of the system.

PR . . . Pronephric Units.

ND . . . Nephric Duct.

NGC . . . Nephrogenic Cord.

G . . . Gonad.

MES . . . Mesonephric Units.

MET . . . Metanephros.

UR . . . Ureter.

CL . . . Cloaca.

cloaca in lower vertebrates. Both tubules and ducts are of mesodermal origin and develop from the stalks of the somites (Nephrotomes). This is a strand of cells connecting the somites to the lateral plate mesoderm. The cells become seperated into parietal and visceral layers. The cavity formed (the Nephrocoele) is for a while continuous with both the myocoele and the definitive coelom between the two sheets of the lateral plate mesoderm. The former connection is soon obliterated. In more primitive vertebrate excretory organs the latter connection forms the Nephrostome. The dorsolateral wall of the nephrotome becomes drawn out into a hollow tube, the cavity of which is an extension of the nephrocoele. This tube, which is in open connection with the coelomic cavity forms the renal tubule. The distal (outward) ends of the most anterior tubules soon turn backward and fuse together to form the nephric duct (or Wolfian duct or Pronephric duct; Fig. 10).

The renal tubules are associated with bunches of fine blood vessels, the Glomeruli. Here the blood plasma is filtered. Either the mass of blood vessels is invaginated into the wall of the renal tubule, which enlarges to contain the glomerulus and becomes the Bowman's Capsule. Or the blood vessels form a bulge on the wall of the coelom. In the latter case the structure is called an External Glomerulus, or if several segments are joined together, the Glomus (found in Xenopus). It is believed that the segment of the coelomic cavity into which the glomus projects is itself derived from an expansion of the nephrocoele, or several nephrocoeles (Fig. 11).

So much for the general description, now to move on to a description of the early development of the nephric system in Xenopus (taken from Nieuwkoop and Faber, 1967).

FIGURE 10.

Diagram showing the Relation between the Renal Tubules, the other parts of the Mesoderm, and the Nephric Duct.
(Adapted from Balinsky, 1975.)

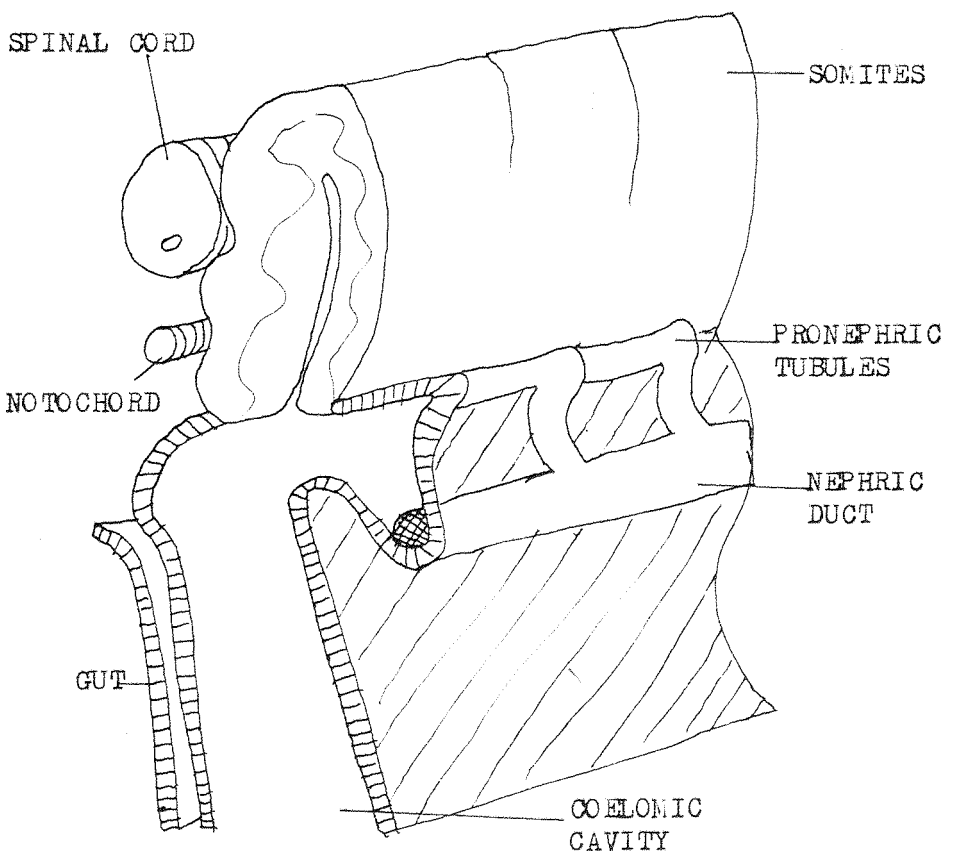
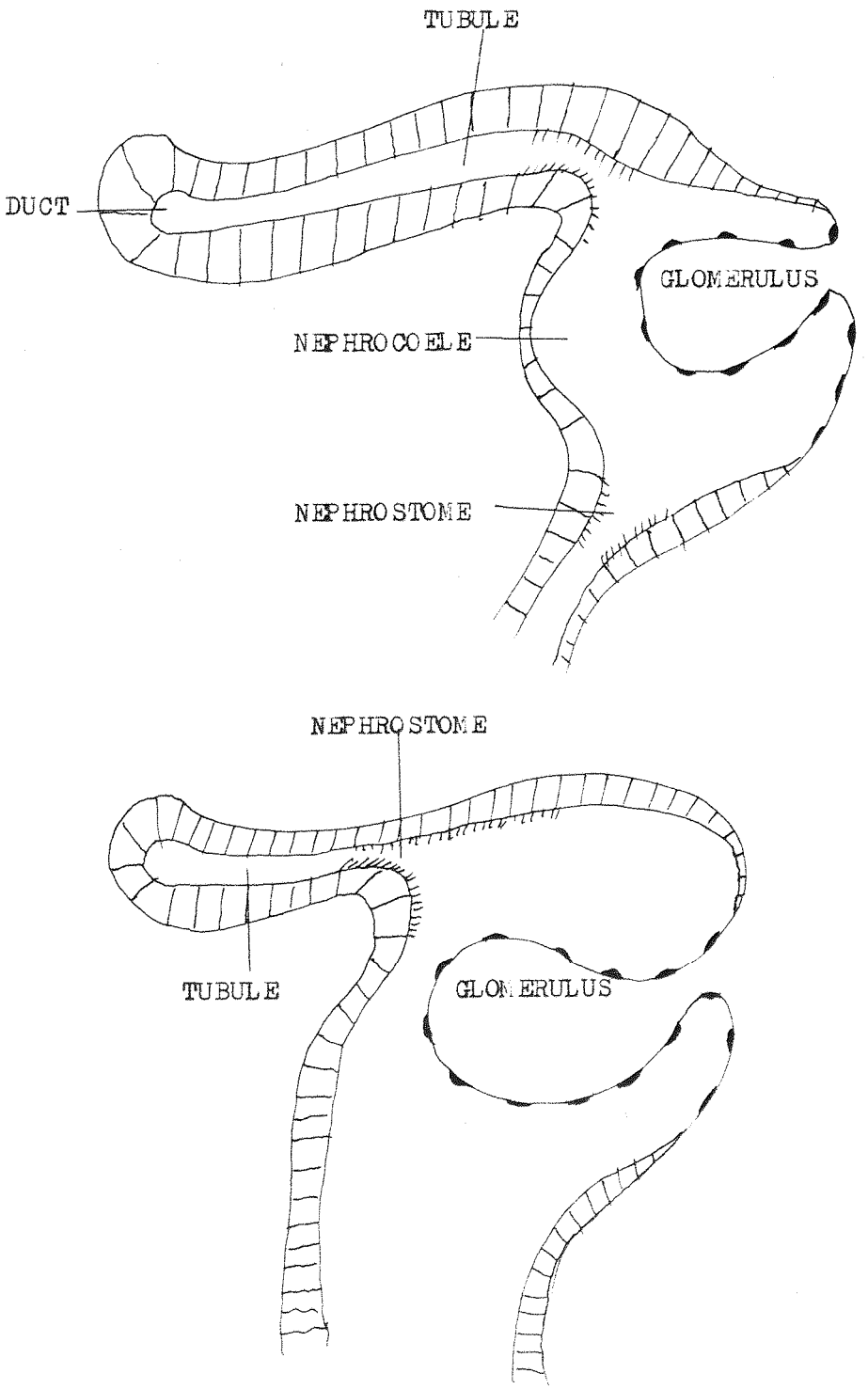


FIGURE 11.

Diagrams of Two Main Types of Excretory Unit, with Internal Glomerulus (top) and External Glomerulus (bottom).
(Taken from Fraser, 1950.)

(Compare with Fig. 13, for modern definitions of the Nephrostome.)



The pronephros is first seen as a slight thickening of the lateral mesoderm at stage 21 (c.22½hr, late neurula). It forms between the level of head somites III and IV and the 1st trunk somite. The 1st Nephrotome begins to segregate out at the anterior end at stage 23 (c.1 day). At stage 24 the 2nd Nephrotome and the Collecting Tube are segregating, the cells arranging themselves radially. A lumen starts to form at the anterior end of the collecting tube. By stage 32 (1 day, 16hr; tailbud stage) a central lumen has formed over nearly its entire length. At stage 28 three nephrostomes appear as small cell concentrations. By stage 29/30 the 1st Nephrostome has developed a central lumen. At stage 32, the 2nd Nephrostome has a lumen and the 3rd is about to open. The pronephric tubules, connecting the canaliculi of the nephrostomes with the collecting tube, are formed by elongation and subsequent coiling. At stage 33/34 the ciliation of the nephrostome funnels has formed, the lumen of the three nephrons is greatly enlarged, and the entire pronephros has become potentially functional. At stage 35/36 a rich blood supply has developed in the form of large blood sinuses derived from the posterior cardinal veins.

The Coelomic Filter Chamber becomes visible at stage 28. The Glomus appears at stage 29/30. They both increase in size slowly, so that they stretch along the entire pronephros by stage 35/36 (2 day, 2hr; hatching). At stage 37/38 (2 day, 5½hr) the pronephros is functional.

The Nephric Ducts are first visible at the level of the 1st and 2nd trunk somites at stage 23. They stretch caudad until they reach the Rectal Diverticulae, at stage 35/36. The cells begin arranging themselves radially, beginning at the anterior end, at stage 31. A small lumen forms anteriorly

at stage 32. This connects up with the rectal diverticulae at stage 37/38. At stage 42 (3 days, 8hr) the nephric ducts shift mediad, into the growing sub-aortic space between the endoderm mass and the aorta.

The pronephros starts to degenerate when the mesonephros is fully formed, aprox. stage 53 (c. 24 days).

Mesonephric cells are first seen at stage 39 (2 days, 8½hr), on both sides of the mid-line between aorta, myotome, medial posterior cardinal vein and the endoderm mass. A Mesonephric Anlage first appears most caudally, at stage 43. The migration of mesonephric cells (occurring caudally first) results in Mesonephric Cords along the nephric ducts at stage 45 (4 days, 2½hr; Feeding stage). Local thickenings appear in three or four areas, the number increases during development. By stage 47, 6-8 units are seen. At stage 46, the cells arrange themselves radially. At stage 47, a central lumen appears, while at their median ends the anlagen of the 1st Glomeruli appear. At stage 48 the first 6-8 pairs of mesonephric tubules extend rapidly and begin to coil. Two to three pairs have already connected with the nephric duct and become functional. The first nephrostomes and corresponding canaliculi appear at the ventral surface of the mesonephros. The nephrostomes open at stage 50 (c.15 days).

The mesonepric area extends both anteriorly and posteriorly. It reaches the posterior region of the pronephros by stage 47. At stage 49 the number of functional units has increased to 10-12 and the number of glomeruli to 6-8 on each side. Now a second generation of tubules begins to appear between the primary ones. They have narrower lumina, thicker walls and a more pronounced basophily of their cells. The number of

glomeruli also increases to about 20 on each side at stage 50. At stage 51 (c.17 days) a third generation of mesonephric tubules develops.

That is as far as I need to go. For a more complete description see Nieuwkoop and Faber (1967), whilst the early development of the nephric system is studied in some detail in this report.

Little work has been done on the induction of mesonephric tubules (the early work is summarised in Burns, 1955; see also the Discussion). But this work does appear to show that the nephric duct is necessary for tubule formation (as is the case with the metanephros). The most recent work appears to be that of Etheridge (1969). He worked on the California newt (Tricha torosa) and was able to show that various other embryonic tissues could act as inductors. He also showed that certain embryonic tissues could suppress tubule formation. Two of these "suppressors" are Neural Crest Cells and Neural Tube. The latter acting as a "suppressor" contrasts with other work (e.g. Saxen's) in which neural tube is a potent inductor. Etheridge also notes this point and produces a possible explanation in that older neural tube than that used in his experiments may act as an inductor. Other possibilties are that T. torosa could be a special case, or there may be more variation between species than is at present believed to be the case, or experimental manipulations may have greater influence than is commonly thought. In any case, this will be dealt with in greater detail in the Discussion.

Finally, I come to a brief description of my own project. The hope was to develop an in vitro system modelling kidney tubule development in Xenopus using an established cell line.

This cell line is an epitheloid cell line derived from adult Xenopus kidney by Balls and Ruben (1966). It shows overgrowth formation and the production of intercellular cavities (ICC) in the overgrowth of older cultures. This seemed to be very similar behaviour to that shown by sub-lines derived from Bennett's mammary stem cell line. Thus, it was hoped that this initial simple form of morphogenesis might indicate an ability to form organised structures, e.g. tubules, given the right conditions.

The cells were examined by light microscopy, TEM, and Scanning Electron Microscopy (SEM) to elucidate the mechanism of overgrowth and ICC formation, and allow a more detailed comparison with such cell lines as the MDCK cells. This might also allow some insight as to the type of environment and/or "stimulus" needed to produce organised morphogenesis. In fact, a wide variety of conditions were tried, e.g. collagen gels and various "inductors". The latter including brain, cell-free extracts of brain, neural tube, and nephric duct (taking Etheridge's work as a model). If any changes did occur in the XTC cells than the in vitro environment would facilitate study of the inductive influences involved (hence the use of cell-free extracts) and the nature of the changes themselves.

Concurrently with this work, in vivo kidney development was looked at by electron microscopy. This was to discover the behavioural and ultrastructural changes involved, so that these could be compared directly with the in vitro model. Explants were also taken to study the inductive influences involved. This was for two reasons; a). To make sure that the culture conditions used will allow tubule formation, b). To identify the inductor(s).

In summary, therefore, my project consisted of:-

1/. A study of the normal behaviour of the Xenopus cell line (examining the overgrowth and ICC's). For purposes of comparison, a salmon cell line was also briefly examined.

2/. Cytoskeletons of the 2 cell lines. A variety of techniques were used to see which gave the best resolution for my purposes. The best techniques provide a view of the whole cytoskeleton of the cell. This is of intrinsic interest, but also presents an extra parameter to look at during induction experiments. It was hoped that any overall and/or subtle changes would be easily detected.

3/. The effect of "inductors" on the XTC cells (including the effect on their cytoskeletons examined by techniques looked at in 2/).

4/. A study of the early development of the Xenopus nephric system.

5/. An examination of the inductive influences involved in Xenopus nephric system development.

It was hoped that the combined results would give a better idea of the mechanisms involved in kidney tubule formation.

Points 1/, 2/ and 4/ are mainly descriptive, whilst 3/ and 5/ form the main experimental area of the project. In the case of induction of the XTC cells, a negative result would present problems since there would be no indication as to why the cells failed to form tubules. Unfortunately, this was in fact found to be the case. No specific reasons can be given. Some form of genetic insufficiency cannot be eliminated. But the Discussion calls attention to the fact that some form of morphological polarity is often found in cells which do show organised morphogenesis in vitro. Insufficiency in the culture

conditions seem somewhat less likely, as pronephric explants did form tubules. This brings a second problem, however, since no mesonephric explants ever produced tubules. Again, no specific reasons can be given; though, in this case, some form of insufficiency in the experimental conditions is likely.

The Methods and Results sections give a detailed report of the experiments. The Discussion has seperate sections on the cytoskeleton and kidney development, as these were the most successful parts of the project. The latter section also tries to review why the mesonephric explants failed to show tubule formation. The last section of the Discussion presents a very simple model of overgrowth and ICC formation in the XTC cells. This leads on to similar models of morphogenesis and allows some insight as to why the XTC cells do not show organised morphogenesis.

METHODS

CELL CULTURING.

XTC Cells.

The Xenopus Tissue Culture (XTC) cells were obtained from P. Harris Ltd. They are a permanent cell line derived by Balls and Ruben (1966) from the kidneys of young adult Xenopus.

They are grown in flasks (Sterilin) in 5ml of 70% L-15 Medium (see Appendix A), with 8% Foetal Calf Serum (FCS; Gibco or Flow Labs.). (The FCS can be replaced by Newborn Calf Serum, NCS; Gibco; see Results.) No extra carbon dioxide or heating is required.

The cells are washed and fed once a week. They are washed in 5ml of Modified Steinberg's (MS) solution, sterilised by autoclaving (Appendix A).

The procedure for sub-culturing is as follows:-
The flask is washed once in 5ml MS solution, then 1ml Trypsin solution (Appendix A) is added. By shaking the flask gently, the cells are removed from the substrate within 5-15 min. 3-5 ml of Conditioned Medium (see below) is then added to the flask to stop the action of the trypsin. The resulting cell suspension is poured into a sterile plastic Universal (Sterilin) and centrifuged at c.100g for 1min. The supernatant is poured off and the cell pellet re-suspended in a suitable amount of medium (10-40ml, depending on the number

of flasks required).

Conditioned medium is here defined as medium used to feed a confluent culture of cells for one week, which is then millipored and stored at 4'C.

Salmon Cells.

These cells were obtained from Flow Labs. They were derived from Atlantic Salmon. (The reference Flow Labs give is Nicholson and Byrne, 1973. But these workers note that the cell line they isolated is fibroblastic, whilst Flow Labs describe their cells as Epithelial. Possibly the latter are a sub-line.) They are grown in 90% MEM (see Appendix A) with 8% FCS and added HEPES buffer rather than sodium bicarbonate. (This is so the cells can be grown in the laboratory without the need of a carbon dioxide-incubator.)

The cells are washed and fed once a week. They are washed in 5ml of Hank's (Appendix A).

Sub-culturing is as for XTC cells, except that the MEM medium replaces the L-15 medium, Hank's solution replaces MS solution, and 0.1% trypsin in Fish Disaggregating Saline (Appendix A) is used.

Photography.

The cells are examined under an Inverted Phase Microscope (Olympus or Microtec). All light micrographs of living cells are taken using the Olympus microscope and Olympus 1M camera.

KARYOTYPING.

(Ref:- Freed and Mezger-Freed, 1970.)

A confluent flask was trypsinised until half the monolayer had been removed, then it was re-fed. The following day 0.5ml of a 10^{-3}M colchicine solution was added to the flask, giving a final colchicine concentration of 10^{-4}M. Approximately 20hr later the following procedure was carried out:-

a). Contents of the flask into a Universal. 3ml of MS solution pipetted into the flask to wash it, this also poured into the Universal.

b). 3ml 0.5% trypsin solution added to the flask for 1 min, then discarded. Flask left for 20 min.

c). Spin down Universal at 500g for 10 min. Discard supernatant.

d). Add 5ml hypotonic (0.05M) KCl to the flask. Resulting suspension added to the Universal.

e). Gently pipette and treat for 15 min.

f). Equal volumes of fresh fixative (1 part Glacial Acetic Acid; 3 parts Methanol) added to the Universal, with stirring to avoid clumping. Leave for 10 min.

g). Centrifuge Universal at 500g for 10 min.

h). Draw off supernatant. Wash cells with 5ml fixative, 3 times.

i). Disperse pellet in 0.5ml fixative with gentle pipetting, to give a milky suspension.

j). A pre-cleaned slide from alcohol storage is washed in distilled water and drained to a thin film.

k). A drop of the cell suspension is added to the end of the slide and allowed to spread. The slide is passed through a bunsen burner to ignite the fixative.

l). The cell density is checked under a phase contrast microscope. If salt crystals appear, the slide is washed with fixative.

Staining:-

m). Dry slides are immersed in 1N HCl at 60'C for 7 min (to suppress cytoplasmic RNA staining).

n). Wash in cold distilled water. - 5 min.

o). Stain in 10% Giemsa blood stain. - 6 min.

p). Quick rinse in distilled water.

q). Two quick rinses in Acetone (do not destain).

r). Stand in dry Acetone:Xylene (1:1). - 2 min.

s). Two rinses in Xylene.

t). Coverslips with mounting medium (Depex) added.

COLLAGEN.

Collagen Solution. (Type I Collagen)

A wide variety of techniques can be found in the literature (e.g. Michalopoulos and Pitot, 1975; Schor, 1980). However, most of them conform to the following basic pattern.

Rat Tail Tendons are extracted and washed twice in distilled water. They are cut up into 1-3cm long pieces and placed in sterile 0.1% Acetic Acid. (About 1g of tendons for every 100-200ml of acid.) This is placed in the cold-room (4'C) and stirred with a sterile magnetic stirrer for at least 48hr. Then the suspension is left to stand at 4'C for at least 1 day.

The collagen solution can now be decanted off and can be used, being stored at 4'C. Or it can be further purified by centrifugation at 10,000g for 1-2hr at 4'C, under sterile conditions. This centrifuged collagen solution can be used

or again further purified. An equal volume of 20% NaCl is added to precipitate the collagen. This is spun down at 10,000g for 1hr at 4°C. The resulting collagen pellet is redissolved in 0.5M Acetic Acid. This solution is dialysed against 2-4 litres of distilled water, changing the water twice a day. Finally the solution can be further cleaned by centrifuging at 10,000g for 1hr.

All collagen solutions are kept at 4°C.

The most effective method of obtaining a collagen solution which formed gels consistently (see below) was obtained from J. Bard (pers. comm.). It varied only slightly from the above method. 0.5M Acetic acid was used to extract the collagen, at c.1g of tendons to 100ml acid. The suspension was spun down as above and used, i.e. no further purification occurred. The collagen solution obtained was quite concentrated (c.10mg/ml).

Collagen Calibration Curve.

(Ref:- Schor, 1980.) Collagen is precipitated out of a Centrifuged Collagen Solution with 20% NaCl. It is spun down on a bench centrifuge and the "mushy" pellet is then freeze-dried overnight. Several standard solutions are then made up from this collagen, in 0.1% acetic acid.

The optical densities of these solutions are measured on a PYE Unicam Spectrophotometer, set at 230nm, and a Calibration Curve then drawn (Graph 1).

Finally, the optical densities of the stock collagen solutions were also measured and their concentrations read off from the curve. (This was checked by drying out a sample of dialysed collagen solution in a weighed petri dish on a

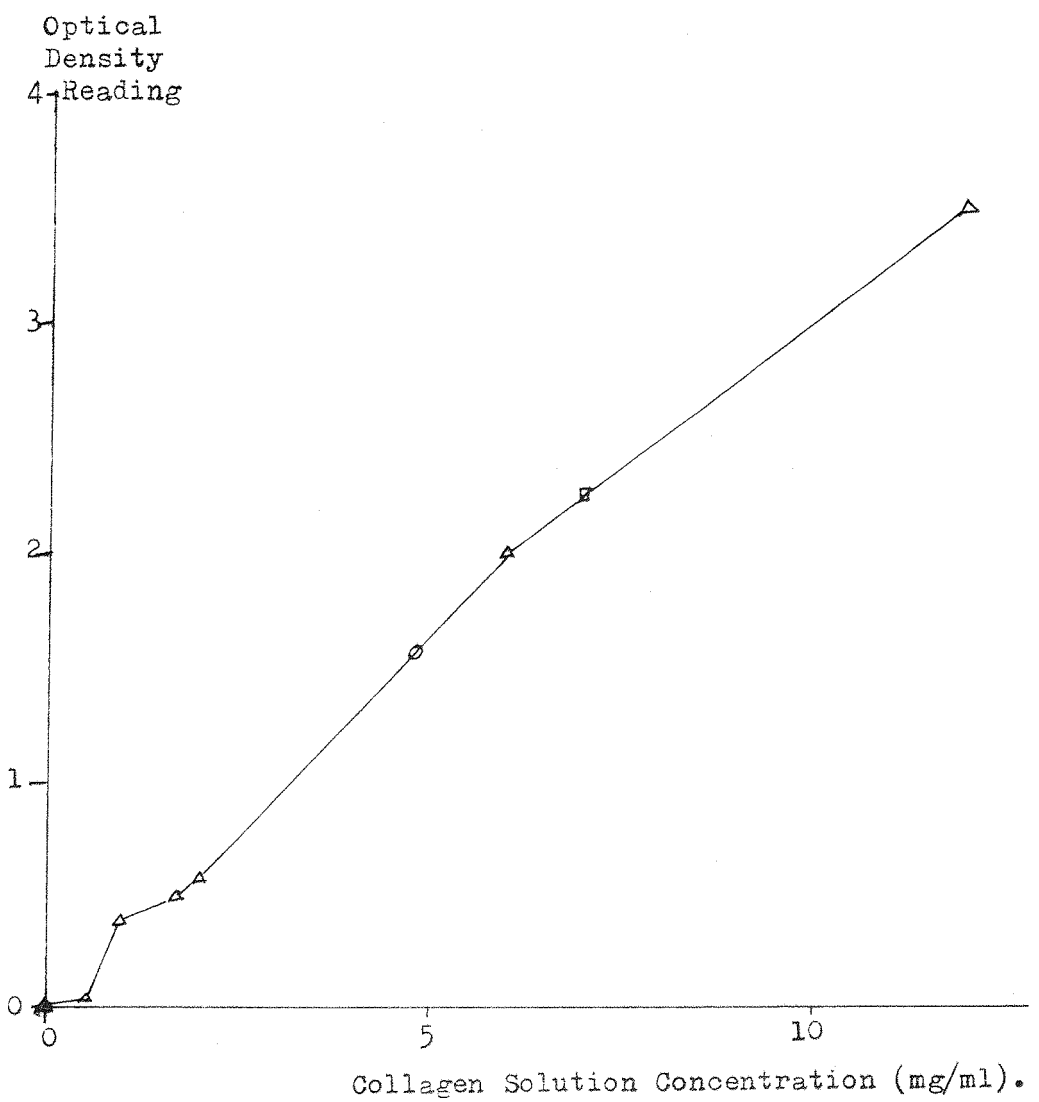
GRAPH 1.

Calibration Curve for the Collagen Solutions.

△. . . . Standard Solution.

○. . . . Dialysed Collagen Solution in 0.1% Acetic Acid.
(Conc. = c.5mg/ml)

□. . . . Dialysed Collagen Solution in 0.5M Acetic Acid.
(Conc. = c.7mg/ml)



hot plate, and then re-weighing.)

Collagen-Coated Dishes.

(Ref:- Michalopoulos and Pitot, 1975.) 0.5ml of a collagen solution (all the various types have been used) is poured into a 35mm-diameter tissue culture dish. (1.5ml being poured into 5cm-diameter dishes.) All such dishes are placed on a Dishwarmer set at c.40-50'C for c.40hr. On removal, the dishes are found to be coated with a thin layer of collagen. However, this is not a uniform layer, as the centre of the dish seems to have little if any collagen, whilst the edges have the most. The dishes are exposed to ultraviolet light for upto 1hr to ensure sterilisation. Then any remaining acetic acid is removed in 2-4 changes of sterile MS solution.

Collagen Gels.

Two methods are used depending on which collagen solution is used.

1st Method (Bennett, 1980):-

Alkaline medium is made by mixing 1ml of molar NaOH with 8ml L-15 (resulting in a final NaOH concentration of aprox. 0.11M). 1.5ml of this alkaline medium is added to 5ml of a collagen solution in 0.1% acetic acid and quickly mixed. 1.5ml of this mixture is pipetted into each of four 35mm-diameter tissue culture dishes. The resulting gels set in aprox. 3hr. To ensure sterilization, these were exposed to U.V. light for aprox. 1hr.

Points of difference with Bennett's (1980) method worth noting are as follows. -

a/. She uses 2x Dulbecco's Modified Eagles Medium rather than

1x L-15.

b/. Her gels apparently set in a few minutes.

2nd Method (Schor, 1980):-

The following are rapidly mixed.

8.5ml collagen solution in 0.5M acetic acid (c.12mg/ml or diluted down to c.2mg/ml with sterile distilled water).

1ml 10x ∝MEM or 1x ∝MEM or 1x L-15. (∝MEM is ∝ Modified Eagle's Medium.)

0.5ml 4.4% $NaHCO_3$

2ml aliquots of this are pipetted into 35mm-diameter tissue culture dishes. Again, the gels set within 3hr. However, gels made from 1x L-15 and c.2mg/ml collagen solution do <u>not</u> set. Whilst gels made with 10x ∝MEM (Flow Labs) and c.2mg/ml collagen solution often do not gel.

Points of difference with Schor's (1980) method are. -

a/. He uses 10x MEM medium.

b/. His gels set within 5 min.

The increased setting time for my gels may be due to the fact that I used 1x strength medium, whilst most other workers use a 10x or 2x strength medium. Too great a purification of the collagen may also have some effect (Bard, pers. comm.). Certainly, the quickest setting gels (c.½hr) were made using collagen solution obtained by Bard's method (see above).

CYTOSKELETONS.

<u>Light Microscopy.</u>

(Ref:- Pena, 1980; see also Paddock, 1982.)

The cells were grown on coverslips in tissue culture dishes. They were treated as follows (modified from Pena, 1980):-

1/. 0.1% Triton X-100 or 0.1% Saponin in Disaggregating Solution + 4M Glycerol, (i.e. Trypsin solution, Appendix A, but without trypsin). The type of disaggregating solution depended on whether XTC cells or salmon cells were being treated. - 2 min.

2/. 2.5% Karnovsky's (Appendix B). - 20 min.

3/. PAGE stain (see below). - ½-1hr.

4/. Wash several times with distilled water.

5/. Air dry.

6/. Mount in Permount or 5% PVA.

PAGE Stain:- 0.2% PAGE blue (Gurr) in Methanol, 46.5: Acetic Acid, 7: Distilled Water, 46.5

5% PVA:- 5% Polyvinyl Alcohol in distilled water.

Transmission Electron Microscopy.

Preparation of Carbon-Coated Grids (takem from Willison and Rowe, 1980, Pp. 63-64):-

"Prepare an c.0.6% (w:v) solution of Formvar in anhydrous chloroform. It will take several hours to dissolve completely. Fill a container, having a depth of about 20cm, to a depth of about 8cm with the Formvar solution and cover the container (e.g. with aluminium foil). Polish a previously unused microscope slide thoroughly, for at least one minute, using cotton cloth. The slide may be washed in soapy water, or treated with a commercial window-cleaning solution, before polishing. Scratched slides must be avoided since the scratches will bind the film making its subsequent release difficult. Dip the slide, held by one end with a pair of clean tweezers, into the Formvar solution. Don't dip the forceps into the

solution. Lift the slide gently from the solution and hold it for 15-30 sec. in the chloroform vapour above the surface of the solution. Remove the slide from the vapour and scribe its surface close to the edges using a needle or other sharp object. Slowly immerse the slide at an angle (c.45') into clean distilled water contained in a large dish. The Formvar film should lift readily from the surface of the slide and float onto the surface of the water. It is sometimes helpful, particularly when the humidity of the atmosphere is low, to breathe on the slide as it is immersed. If the film binds to the slide and does not release, polish the next slide more thoroughly. The interference colour of the film should be grey or silver. Gold or blue films are too thick and indicate that the Formvar solution probably needs to be diluted. Mount grids, shiny side down, on the Formvar film. The film, together with the attached grids, is readily removed from the water surface using a strip of typing paper, about 4-5cm wide, formed into an arc and pressed against the entire surface of the floating film. The paper is then lifted, starting from one end using a steady movement, and placed to dry with the grids uppermost. The grids may be stored still attached to the paper.

Formvar solutions readily become contaminated with water condensing from the atmosphere. Contaminated solutions will produce films with irregular holes which are unsuitable as support films. A fresh solution should be made monthly, or the solution stored with a suitable drying agent, such as molecular sieve."

The Formvar-coated grids were then given a light coating of carbon. (My thanks to Mr. Trevor Courtney for operating the AEI Carbon-Coater.) The grids were placed carbon-side up in

tissue culture dishes and exposed to U.V. light for 1hr to sterilize them.

Cells could now be seeded into these dishes and would settle and grow quite well on the carbon/plastic-coated grids.

The standard extraction process was as follows:-

Wash the grids in MS or Hank's, as appropriate.

" " " " the appropriate Disaggregating Solution (see Appendix A).

Treat the grids with 0.1% Triton X-100 or 0.2% Saponin in the appropriate disaggregating solution with 4M glycerol added - 2 min.

Fix in Karnovsky's - 1-2hr. (Post-fixation in 1% OsO_4, $\frac{1}{2}$hr, sometimes used.)

Dehydrate from 25% Alcohol to two changes of Absolute Alcohol - 20 min in each.

Critical Point Dry.

The grids are then examined under a Phillips EM300 Transmission Electron Microscope, set at either 80 or 100kV.

Two modifications of this basic treatment were used:-

a). KCl treatment to remove actin filaments. (Ref:- Schliva and van Blerkom, 1981.)

Washed and extracted as above.

60mM KCl in MS or Hanks, plus 4M Glycerol - 5 min.

600mM " " " " " " " " - 5 min.

60mM " " " " " " " " - 5 min.

Fixed, dehydrated, and critical point dried, as above.

b). Air-Drying and Negative Staining. (Ref:- Small, 1981.)

Washed and extracted as above.

Fix in 2.5% Glutaraldehyde in MS or Hanks - 1hr.

Wash in Hanks or MS.

Pass grid through 2 drops of distilled water.

" " " 4 " " Bacitracin (Sigma), 4μg/ml in distilled water. (Bacitracin is a wetting agent,)

Pass grid through 4 drops of 3% Sodium Silicotungstate (aq) or 3% Sodium tungstate (aq).

Air dry.

Finally, one more method of preparing cytoskeletons was used (Mesland, Spiele and Roos, 1981):-

Wash grid in MS or Hanks.

Fix in Karnovsky's - 1hr.

Wash and dehydrate as above.

Critical point dry.

Place Sellotape sticky-side down on the cells on the grid, then quickly rip it off. This literally tears the cells in half, see Results.

Specimens looked at under the SEM were grown on coverslips, otherwise treated as above.

Glycerinated Cell Models.

(Ref:- Owaribe et. al., 1981.)

Cells grown on grids are washed two times in cold MS or Hanks. Then placed in 50% Glycerol in MS or Hanks, at 4'C, for 1 day. Finally, wash grids with the appropriate disaggregating solution.

The cells could then be fixed in Karnovsky's, dehydrated, critical point dried and examined under the TEM. Or they could be treated with an ATP solution for 12 min.

(ATP Solution:- 3mM ATP, 5mM $MgCl_2$, 8mM Tris in MS.)

Then they are fixed, dehydrated, etc.

Or they could be Triton-extracted and then fixed, etc.
Or they could be Triton-extracted, washed, treated with the ATP solution for 12 min, washed, fixed, etc.

Thus, there are 4 basic treatments:-

1/. Glycerinated cells.

2/. " " exposed to ATP.

3/. Triton-extracted cytoskeletons of glycerinated cells.

4/. " " " " " " exposed to ATP.

These four treatments were also performed on cells which had been in the presence of "inductors" for 2 days. The "inductors" used were:- Brains extracted from stage 50 Xenopus embryos, and cell-free extracts of same. (The latter produced by homogenizing 50 brains in 10ml MS, centrifuging then Milliporing the supernatant.)

TRANSMISSION ELECTRON MICROSCOPY.

Table 5 lists all the methods used. Methods A, B, and C were used with cells grown on plastic. Method D was used on cells grown on collagen gels and on Xenopus embryos.

The formulae for all solutions will be found in Appendices A and B.

Of the methods A-C, C gives by far the better results. All are listed here, however, as the Results section includes photographs of specimens prepared by all 3 methods.

Method D was used on collagen gels and embryos as London Resin infiltrates specimens much easier and quicker than Araldite Resin. In accordance with the makers' recommendations, 1% OsO_4 was not used and 1% Phosphotungstic Acid was used as a

TABLE 5.

Transmission Electron Microscopy Preparation Techniques.

References:- Method A; Nelson and Flaxman, 1972.
Brinkley et. al., 1967.
Method B; Wollweber et. al., 1981.

Treatment	Methods			
	A	B	C	D
Wash 1-3 times, (MS, Hanks, or Phosphate buffer).	5min	5min	5min	5min
Karnovsky's Fixative.	-	-	1hr	1hr
1.5% Glutaraldehyde and 1.0% Formaldehyde in Phosphate buffer.	1hr	1hr	-	-
Phosphate buffer (2x)	5min	10min	-	-
Cacodylate buffer (2x)	"	"	5min	2min
1% OsO_4 in Cacodylate buffer	½hr	45min	1hr	-
Cacodylate buffer (2-3x)	5min	½hr	10min	-
1% Tannic Acid in Cacodylate Buffer	-	1hr	1hr	-
Phosphate buffer (3x)	-	15min	-	-
0.5% Uranyl acetate (aq)	-	½hr	-	-
MS (2-3x)	-	20min	5min	-
25% Alcohol	10min	10min	½hr	1hr
50% Alcohol	"	"	"	"
70% Alcohol	"	"	1hr-Over-night	Over-night
90% Alcohol	15min	15min	½-1hr	1hr
Absolute Alcohol	"	"	15min	"
" "	"	"	"	"
0.1% Toluidine blue in absolute alcohol	"	"	"	-
2:1; toluidine blue: araldite resin	"	"	1day	-
1:1; " " : "	10min	10min	"	-
1:2; " " : "	"	"	"	-
Araldite Resin	16hr	16hr	"	-
Araldite Resin	"	"	"	-

TABLE 5. (Contd.)

Transmission Electron Microscopy Preparation Techniques.

Treatment	Methods			
	A	B	C	D
Araldite Resin	16hr	16hr	1day	-
" " (Embedding	1-2	1-2	1-2	-
Oven, 60°C)	days	days	days	-
1% Phosphotungstic Acid in	-	-	-	1day
Absolute Alcohol				
London Resin	-	-	-	"
" "	-	-	-	"
" "	-	-	-	"
" " (Embedding Oven,	-	-	-	Over-
60°C)				night

The formulae for all solutions will be found in Appendices A and B.

stain.

Sections were cut on an LKB Ultramicrotome and stained as follows(Reynolds, 1963; N. Chegini, pers. comm.):-

1/. One drop of uranyl acetate (2% in 50% alcohol) on dental wax for each grid. Grid placed "section-side" down on a drop. Cover dental wax and leave for 15 min.

2/. Pick up grid with forceps and wash in distilled water.

3/. Take the grid through 3 beakers of distilled water.

4/. Dry on filter paper.

5/. Submerge grids in drops of 1% lead citrate (dissolved in NaOH) on dental wax, in a petri dish containing NaOH pellets. (These absorb the CO_2 from the atmosphere of the dish, preventing the precipitation of lead carbonate.) Replace lid of the dish, leave for 2 min.

6/. Wash in a drop of 1% NaOH (in petri dish) - 1 min.

7/. Wash twice in drops of distilled water (in petri dish).

8/. Pick up grid with forceps and wash with distilled water.

9/. Dry on filter paper.

Sections were examined under a Phillips EM300 Transmission Electron Microscope, set at 80kV.

Resin sections used for light microscopy were flattened onto slides by placing them on drops of 1% NH_4OH, at 60°C. They were stained by 1% Toluidine blue in 1% Borax, at 60°C for 1-2 min, and mounted in 5% Polyvinyl Alcohol.

SCANNING ELECTRON MICROSCOPY.

Fixation techniques A-C have been used here. Again, method C was by far the best. The only problem being that both tannic acid and uranyl acetate will cause precipitates which

obscure the specimen. This can be avoided by washing the specimen in MS before and after both treatments.

The only changes from the fixation methods given in Table 5 are as follows:-

a/. Three absolute alcohol changes were used, of 15 min each when the specimens were cells on glass, or ½-1hr each when the specimens were cells on gels or Xenopus embryos.

b/. Some embryos had their ectoderm removed when they were in 25% alcohol, others when in 70% alcohol.

c/. After dehydration, the following:-

Critical Point Dry. (Normally 2 washes of ½-¾hr each, with 3-4 flushes of 5-15 min each, before raising to critical point.)

Stick the specimen onto a stub with double-sided tape or silver paint. (If the latter is used, the paint has to be left to dry before continuing.)

Sputter Coat.

Examine under a JEOL JSM-P15 Table-Top Scanning Electron Microscope.

Photographs were taken on Ilford FP4 film.

LIGHT MICROSCOPY.

Xenopus embryos, explants, and cells grown on collagen gels were all embedded in paraffin wax and sectioned. The procedure used was as follows:- (Ref; Humason, 1972)

Bouin's Fixative (Appendix C; used with embryos) - several days

Buffered Formalin (Appendix C; used with gels) - several days

Running water (only for formalin-fixation) - Overnight

50% Alcohol - ½hr or longer.

1:1; NH_4OH: 70% Alcohol (only for bouin's fixation) - several washes

70% Alcohol - ½hr or longer

90% " - ½-1hr

Absolute Alcohol - ½-1hr

" " - "

" " - "

Toluene or Xylene - "

" " " - "

Paraffin Wax (60'C) - 1hr

" " " - "

Embed

Collegen gels were also embedded in Ester Wax, as this melts at a lower temperature. (The gels often shrank somewhat at 60'C.)

The blocks were sectioned at 5μ or 10μ, and Haematoxylin and Eosin stained (Humason, 1972). Slides were mounted in Depex (Gurr).

EXPLANTS.

Pairs of toads were induced to breed by injecting Chorionic Gonadotrophin (Pregnyl; Xenopus Ltd) into their dorsal lymph sacs (Gurdon, 1967). The male required c.150 i.u. and the female 200 i.u. Sometimes two injections on consecutive days were required.

The embryos were collected and the following procedure carried out when they reached a developmental stage just prior to that necessary for the explants. (With the younger embryos

by the time the washing procedure had been carried out, the developmental stage would be that required for the explants.)

Upto c.60 embryos were washed 9 times in sterile MS. This was done by passing them through 9 covered, sterile crystallising dishes containing sterile MS. The embryos and explants were always picked up with sterile wide-mouth pipettes. After the first set of washes the embryos were de-jellied if necessary. This was done by placing upto 5 embryos in sterile MS in sterile petri dishes and removing the jelly coats and vitelline membrane with flame-sterilised forceps. Then the embryos were washed again, as before, 3 times. (Extreme care has to be taken now, as the embryos are very delicate without their jelly coats and surface tension will literally tear them apart.) Then the embryos were placed in sterile agar-coated petri dishes, in sets of 3-5. These dishes contained either MS or 0.05-0.1% MS222 (Sandoz) in MS, depending on the developmental stage of the embryos. (MS222 is an anaesthetic.) The embryos were then operated on. The explants were cut out using U.V.-sterilised glass needles. The explants were left to heal for ½-1hr before being washed quickly twice, by passing them through sterile MS in 2 sterile petri dishes. They were then cultured in agar-coated tissue culture dishes containing the appropriate medium (see Results).

All washes were carried out in a flow cabinet. De-jellying and operations were carried out on the laboratory bench, under a dissecting microscope (all pre-cleaned with alcohol).

In 2 sets of experiments, the operated embryos were kept to see how the nephric system developed (see Results).

In those experiments where XTC cells were "induced", explants were produced as above but were placed in tissue culture dishes

containing a confluent layer of cells (see Results).

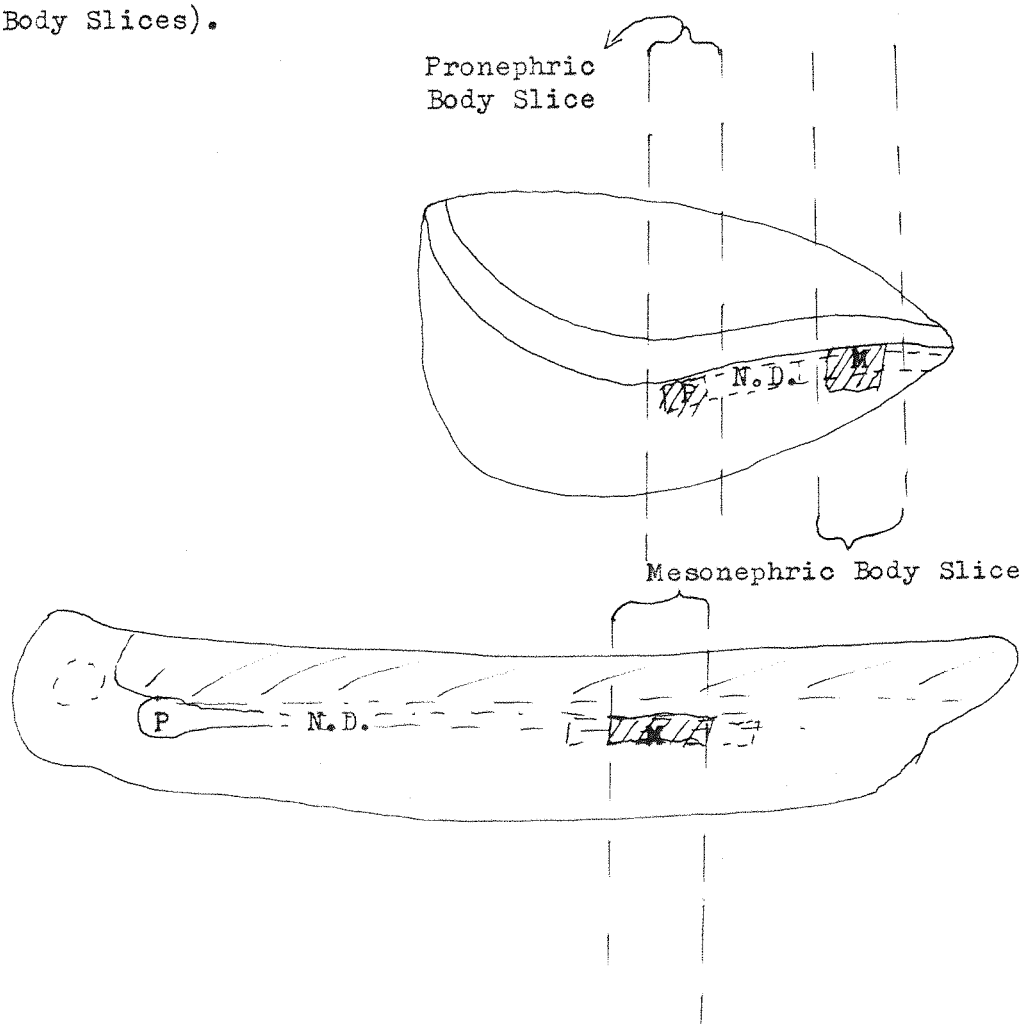
Explants were of 2 main types (Fig. 12); Body Slices and Explants proper. The former were simply produced by 2 cuts, one in front of the pronephric/mesonephric region and one behind. In the latter type, the appropriate mesenchyme was cut out with its attached ectoderm and either cultured alone (Control Explants) or combined with an "inductor" (Induction Explants). In the latter, both mesencyme and "inductor" were wrapped in ectoderm, either manually or by placing the 2 explants together and letting any associated ectoderm spread round both.

FIGURE 12.

Diagrams of the Positions of the Pronephric and Mesonephric Mesenchymes at Closing Neural Tube Stage (c.stage 17-19) and Tailbud Stage (c.Stage 26-28), also showing the Type of Explants taken.

(Adapted from Etheridge, 1969.)

Shaded areas show the areas cut out as Explants (rather than Body Slices).



P . . . Pronephric Mesenchyme.

N.D. . . . Nephric Duct.

M . . . Mesonephric Mesenchyme.

RESULTS.

XTC CELLS.

The cells have a fairly granular cytoplasm surrounding the nucleus. Nuclei normally have 2 nucleoli, but cells with 1, 3, or even 4 are sometimes seen, (Photo 9a-d). Vacuoles are sometimes seen, especially when the cells are kept in medium in which NCS replaces FCS. But extensive vacuolation indicates a contaminated culture. Binucleate cells are sometimes seen. These may be cells which have failed to divide after nuclear division, or cells which have actually fused. They are approximately twice the size of normal cells, taking up an area of aprox. 4.0-7.0 x $10^{-3}mm^2$.

Isolated (normal) cells take up an area of c.1.0-2.0 x $10^{-3}mm^2$.

Cells in monolayers " " " " " c.10.-8.0 x $10^{-4}mm^2$.

" " " , showing overgrowth,
take up an area of c.1.0-4.0 x $10^{-4}mm^2$.

Thus, there does seem to be a decrease in the area covered by a cell as monolayers form.

The cells take up a polygonal outline when in monolayer (Photo's 9b,d). This is characteristic of epithelial cell lines as opposed to fibroblastic cells which take up spindle-shape in monolayer (Photo 14c).

Single cells and cells at the edge of a monolayer often show ruffled membranes and filopodia (Photo's 9a,c and 10a).

The XTC cells are Aneuploid (i.e. have more or less than

the normal diploid number of chromosomes, which for X. laevis is 36; see Photo 12c). A survey of 2 slides of chromosome spreads gave the following results:-

Chromosome Number.	No. of cases seen.
32	3
33	3
34	8
35	11
36	11
37	8
38	0
39	2

Thus, there is a spread around the normal diploid number.

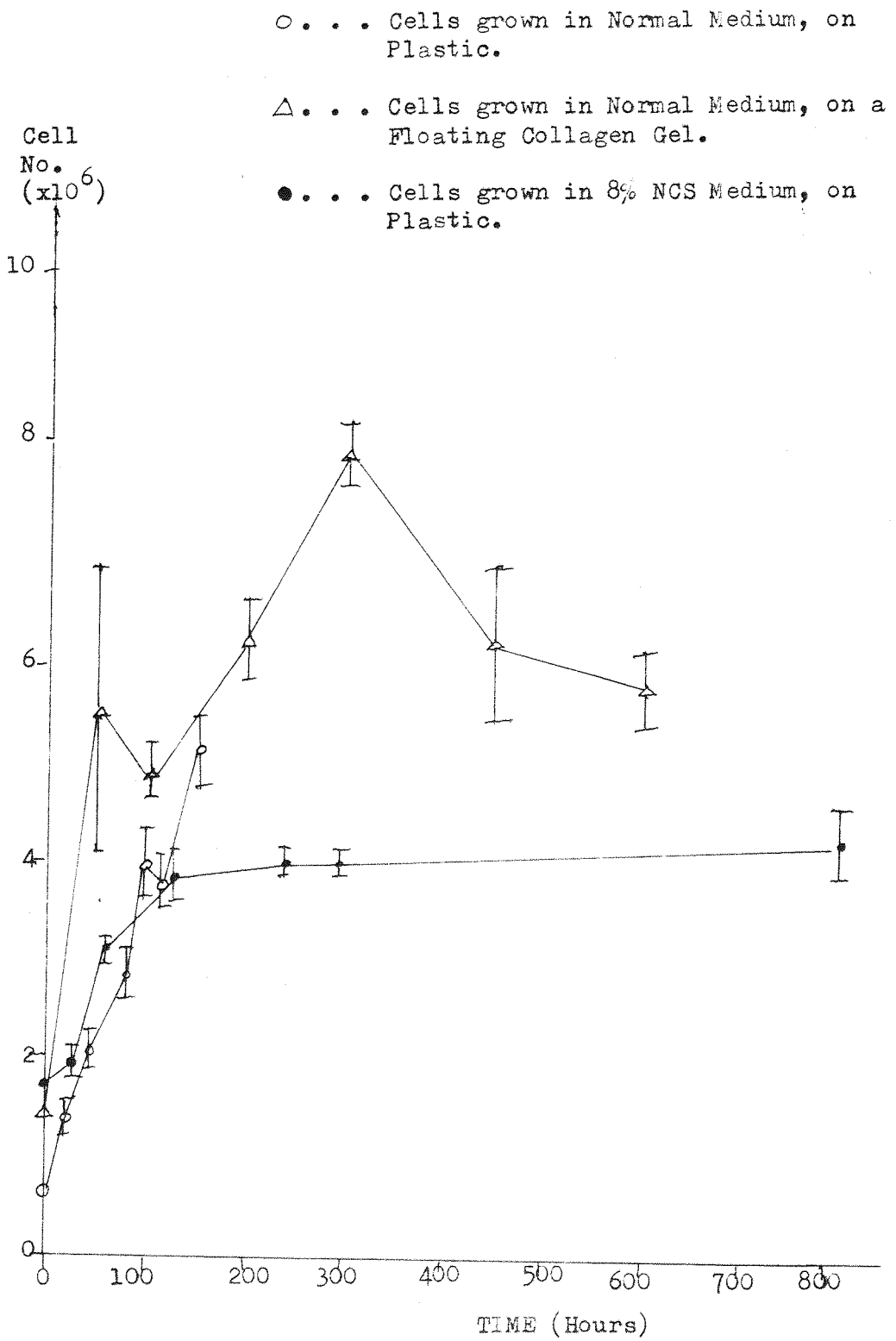
XTC cells grow and divide on glass, bacteriological plastic (Sterilin), tissue culture plastic (Falcon, Lux, Sterilin, or Nunc), collagen and collagen gels. They can be stored unfed at 4'C (at which temperature they stop dividing) for 2 weeks or more with little damage. In general, they are hardy cells and very easy to keep.

Growth curves have been made for XTC cells grown in a variety of conditions. Cells were counted in situ under the inverted phase microscope fitted with a squared eyepiece graticule of known area. Counts were taken from 10 areas of each flask or dish. Cell number per flask or dish was worked out from the mean of these 10 counts. The graphs (Graph 2) also show 90% confidence intervals.

Confluency is reached during the log phase of growth. After this point, overgrowth develops and a different procedure needs to be used to estimate cell number. The flask/dish has to be trypsinised and cell number estimated by haemocytometer

GRAPH 2.

XTC Cell Growth Curves.



count. By this method, each point on the curve is derived from different flasks/dishes. But this is valid as the different flasks/dishes were all plated at the same time with the same number of cells, such that their growth curves should be very similar.

The Mean Doubling Time (MDT) can be worked out from the following formula (Freed and Mezger-Freed, 1970):-

$$MDT = \frac{T_2 - T_1}{3.32(\log\chi_2 - \log\chi_1)}$$

Where χ_1 and χ_2 are 2 points on the growth curve at times T_1 and T_2 respectively.

The MDT's of the 3 curves shown in Graph 2 are as follows:-

XTC cells grown in normal medium, on plastic. MDT= c.56.7hr.

" " " " " " , on floating collagen gel.

(Log Phase) MDT = c.50hr.

(Resting Phase) MDT = c.300hr.

" " " " 8% NCS medium, on plastic.

(Log Phase) MDT = c.70hr.

(Resting Phase) MDT = c.300hr.

As can be seen, the log phase MDT in all cases is aprox. 50hr. Cell growth slows considerably after 100hr, but cell division continues even in these heavily overgrown cultures.

Confluency (i.e. c.2.0 x 10^6 cells/dish) is reached during log phase, but cell growth continues at aprox. the same rate for another 60hr or so. In 8% NCS medium, the population levels off at aprox. 4-6 x 10^6 cells (grown in flasks, which have a confluent density of c.5.0 x 10^6 cells/flask). In these cultures little overgrowth is formed. In normal medium, whether on plastic or collagen, the population levels off at

aprox. 6–8 x 10^6 cells/dish. These are heavily overgrown cultures. Cell division does not cease in these cultures, as indicated by the fact that the overgrowth will become even denser with time. Also, floating cells (c.50% of which are dead as indicated by trypan blue staining) are always found when the cultures are fed once a week.

Lastly, even in heavily overgrown cultures grown on plastic, areas of monolayer are always seen. This is not so if the cells are grown on collagen gels, where the cell peripheries become very difficult to distinguish.

Returning to cellular morphology, SEM studies show that single cells possess microvilli on much of their upper surface, except for their ruffled membranes (Photo 10a). The latter are seen to be wide and very flat areas, with ruffling activity at the cell's periphery. Ruffled membranes are no longer seen in confluent cultures (Photo's 9b,d and 10b). There seems to be a rough correlation between cell density and cell surface structures. The higher the density, the more surface structures. The rounded cells of patches of overgrowth are usually covered in microvilli (Photo 10b). To some extent this seems to be associated with the overall change in cell shape as cell density increases. The cells tend to round up in confluent monolayers and certainly in the overgrowth. Isolated rounded cells (possibly dividing) are also covered in microvilli and blebs (Photo 10a). The morphology of XTC cells conforms to this pattern whether grown on glass, plastic, collagen or collagen gels.

Overgrowth forms as irregularly-shaped bundles of cells within monolayers (Photo 9d). Within an isolated monolayered patch, overgrowth forms first in the centre. The overgrowth

finally forms a complex irregular meshwork pattern over the whole culture. When grown on plastic, patches of monolayer are always seen. However, on collagen gels such patches are not visible. Within the overgrowth are seen refractile spherical areas that seem to indicate some form of intercellular cavity. On sectioning, it was seen that this is exactly what they are.

As noted previously, cell morphology on collagen gels is the same as that on plastic. The XTC cells do not grow into collagen gels, whether settled or floating. They do not contract floating collagen gels, but do grow on both surfaces, producing overgrowth and ICC's on both surfaces. If grown between 2 gels, as a "sandwich", the cells do not infiltrate either gel. The upper gel may have mingled with the overgrowth before setting, giving the appearance of cellular infiltration into the upper gel. But this goes no further, no XTC cells actively move into either gel.

Ultrastructurally, isolated XTC cells are well flattened (Photo 11a). The cytoplasm is well supplied with Endoplasmic Reticulum, and Golgi bodies are often seen beside the nucleus. Stress fibres are not seen, but the cells often seem well attached to the substrate, apparently touching at a few points. However, in many cases, microvilli are seen on the under cell surface below the nucleus (Photo 11b). This indicates that cell attachment is probably very weak at the centre of the cell.

In confluent cultures (whether on plastic or gels) the cells are seen to be rounded and covered in microvilli (Photo 12b). Neighbouring cells make close contacts with each other over part of their opposing surfaces, although these also possess many microvilli. In sections fixed by technique C, these

close contacts are obscured by electron-dense staining material; presumably naturally-occurring ECM (Photo 11c). However, when fixed by technique A, a gap of at least 5nm is always seen.

Photo 12a shows a section through an ICC. The cells lining the cavity show no obvious specialisations. They are still covered in microvilli (there are fewer on the luminal cell surface, but this corresponds with the upper surface in monolayers). The only cell contacts are close contacts, no specialised cell junctions are seen. No specialisations of the cytoskeleton (e.g. stress fibres or apical MF meshworks) or other cytoplasmic changes are seen.

Photo 12b shows that the XTC cells do not extend into collagen gels. The furthest they get is to send in a few short microvilli. Again, no changes in ultrastructure or morphology are seen.

Cell Inductions.

A wide variety of "inductors" were tried:-

Neural Tube (Developmental Stage 32-34).

" " + Notochord (Developmental Stage 32-34).

Brain (Developmental Stage 48-51).

Mesonephric Body Slices (Developmental Stages 33-36 and 40-42).

Endoderm (Developmental Stage 33-36).

Nephric Duct (Developmental Stage 33-38).

Cell-free Extracts of Brains (Developmental Stage 50-51).

The nephric ducts were cut out using sterile glass needles and partially cleaned of surrounding matter by treatment with 0.2% collagenase in GMB (Appendix A) for ½hr.

These inductors were placed in dishes (5-15 in each) containing confluent monolayers of XTC cells, grown either on

plastic or collagen or collagen gels.

The explants usually settled within 2-3 days. The cultures were then kept for a minimum of 1 week and a maximum of one month.

The living cultures were examined periodically under the inverted phase microscope. In no case and at no time were any organised structures seen. The explants remained as comparatively dense structures, they did not spread out either over or under the XTC cells. Nor did the XTC cells appear to envelope the explants. Overgrowth was produced as normal, even up against the explant. ICC's were present, but showed no novel characters. No tubular structures were ever seen. Under the TEM, the contacts between XTC cells and explant cells were seen to be close contacts (Photo 8c). A lot of ECM was present, probably most of it derived from the explant. The explant's internal organisation breaks down; the cells arranged apparently haphazardly within a mass of ECM and sometimes dying cells (especially in the larger explants).

SALMON CELLS.

These are also epithelial cells (Photo 13a). However, these cells are so flat and have such clear cytoplasm that they were very difficult to distinguish under the inverted phase microscope. Isolated cells and cells at the edge of a monolayer can be seen to possess ruffled membranes. Faint stress fibres can also be seen in many of the cells.

SEM work confirms the flattened morphology of these cells (Photo 13b). Single cells are seen to have an irregular outline and very few surface features. Again, rounded cells

(often dividing) are seen to possess many blebs and retraction fibres, as with the XTC cells. However, in confluent culture these cells retain their flattened morphology, whether on glass, plastic, collagen or collagen gels. The cells overlap/underlap to a great extent. In old cultures this overlapping of flattened cells can reach 5-6 cells in thickness. This occurs evenly over the whole culture, such that individual cells are totally obscured when the culture is examined by phase contrast.

Ultrastructurally, such cultures are seen to contain overlapping flattened cells (Photo 14a). Neighbouring cells make close contacts at a few points, but no other form of cell junction. There is quite a lot of ECM, especially associated with the cell membranes and extruded from electron-dense vesicles. The cells are well supplied with endoplasmic reticulum. MF bundles are also often quite prominent.

These cells are able to contract a floating collagen gel. A population of 0.5×10^6 cells can contract a gel from 3cm diameter to 1.5-2.0cm diameter in less than a day. But, again, they do not infiltrate the gel (Photo 14b). They maintain their normal morphology on gels. But some form of specialised contact with the collagen fibres may occur as these are often aggregated at specific sites along the cell membrane. However, no internal cellular specialisations are seen. So, if valid, this appears to be purely a property of the cell membranes.

CYTOSKELETONS.

The PAGE staining technique reveals the XTC cell cytoskeleton

as a disorderly meshwork (Photo 19a). No stress fibres are seen. The nucleus and large organelles (e.g. mitochondria) occur near the centre of the cell and are seen as a large dark area, obscuring the cytoskeleton. The cytoskeleton at the ruffled membranes is a very fine, thin reticulate structure. All these features are also seen in SEM studies of triton-extracted XTC cells.

The cytoskeleton of Salmon cells, however, is seen to contain a large number of stress fibres by these methods (Photo 19b). These stress fibres are of various lengths and not necessarily in parallel. They usually cross the cell, but break up at the ruffled membranes. The nucleus is clearly seen, but the centre of the cell is not obscured by any other organelles.

Dry cleaving allows higher resolution, as the TEM is used. XTC cell preparations reveal a complex meshwork of fibres, 5–30nm in diameter. This meshwork also contains the nucleus, and a large number of vesicles, most of which are aprox. 20nm in diameter, but some are much larger. Individual fibres vary in width along their own length. Bends only tend to occur at "branch-points", where 2 or more fibres meet. It is impossible to clearly distinguish the 3 fibre systems of the cytoskeleton, although it is sometimes possible to distinguish a fibre of MT diameter. Salmon cells prepared by dry-cleaving show a similar structure, except that here stress fibres can be distinguished and add some regularity to the meshwork (Photo 18a). SEM studies of dry-cleaved cells reveal that the top of the cell has been removed, plus most of the large organelles except the nucleus.

Triton-extraction and TEM gives the best resolution, and

even a 3-Dimensional impression can be produced using stereo pairs (Photo's 15 and 16). Triton-extraction removes all cellular membranes and most of the cell contents (Photo 21a). The fibres of the XTC cell meshwork are seen to vary in diameter from c.4nm to c.20nm (Photo 15). They vary in diameter along their own length, bend only at "branch-points" and have c.20nm diameter particles attached to them. It is usually not easy to distinguish the 3 fibre types. But occasionally (especially if KCl treatment has been used to remove many of the MF; Photo 17a) things are clearer. It is then seen that "branch-points" can occur between both like and un-like fibres.

Things are usually much clearer with the salmon cell preparations (Photo's 16 and 17b). Here, the 3 fibre systems are clearly seen and "branch-points" between like and un-like fibres are very easily seen. MT fibres seem to "break-up" at their ends, either giving rise to or simply meeting smaller diameter fibres. Again, individual 12-20nm particles/vesicles are seen attached to individual fibres. KCl treatment in this case helps to provide a clearer view, especially of the "branch-points". But MF are still plentiful.

Air-drying and negative staining never produced good results with XTC cells. All that was seen was a coarse meshwork, with very bad resolution. However, with the salmon cells quite good preparations were sometimes obtained (Photo 18b). The 3 fibre types are seen; MT are clearly seen criss-crossing the meshwork, IF are also seen. The point to note is that both these 2 fibre types tend to be straight or smoothly curved. They do not show the "kinking" associated with the "branch-points". (Admittedly, "branch-points" are not clearly resolved by this technique.) MF are least well seen; they occur as a

meshwork indistinctly seen interconnecting themselves and the other fibres. No vesicles or particles are seen.

Saponin extraction produces the same results as triton-extraction, except that mitochondria are sometimes left intact and are seen to be enmeshed within the cytoskeleton.

Whole mounts of air-dried or critical point dried cells reveal a fine reticulate cytoplasm within which one can see the various organelles and stress fibres if present (Photo 19c). The only difference being that critical point dried cells may have a slightly larger "mesh-size" reticulum. The "mesh-size" is even larger with glycerol extraction (Photo 19d). Sections of glycerol-treated cells reveal that much of the cell contents, the cell membrane and the nuclear membrane have been removed (Photo 21b). But organeller membranes are still seen, though the organelles themselves appear to be disrupted.

Triton treatment of glycerol-treated cells again reveals the meshwork of the cytoskeleton (Photo's 20a,b). However, this time the fibres appear to be coated by some unextracted material. Sections of such cells reveal that whereas now most of the membranes have been removed, what remains of the cell contents is more diffusely spread than before (Photo 21c).

Both glycerol-treated, and triton and glycerol-treated cells will contract on treatment with ATP. The cells contract as individuals, retraction fibres being seen as the cells pull away from each other. Over the $\frac{1}{2}$hr of ATP treatment, the cells contract visibly (as seen under the inverted phase microscope). But the cells do not actually round-up or decrease in diameter dramatically. Whole-mount TEM preparations of such cells reveal the meshwork system as before, except that the cell periphery has pulled back leaving a number of retraction

fibres (Photo's 20c-f). The meshwork system can be seen to pass into the retraction fibres. In the salmon cells, stress fibres tend to end in the retraction fibres.

Non-glycerol-treated cells do not contract on treatment with ATP, whether or not they have been triton-extracted.

In section, contracted cells have a distorted outline, but otherwise their ultrastructure is the same as their non-contracted counterparts.

The effects of 3 types of "inductor" were tested:-

Brain Extract.

Brains (stages 48-51).

Mesonephric Body Slices (stages 36-38).

No change in the cytoskeletons were ever seen (e.g. Photo's 20a,c). Nor was there any change in the retraction behaviour of ATP-treated cells. They still retracted only slightly, and only as individual cells.

THE EARLY DEVELOPMENT OF THE NEPHRIC SYSTEM IN XENOPUS LAEVIS.

All three areas of the nephric system (i.e. pronephros, mesonephros and nephric duct) develop from the somato-pleural portion (i.e. the dorsal tip) of the lateral mesoderm.

Lateral mesoderm cells are flattened, irregularly-shaped, with thin filopodia touching neighbouring cells (Photo 1a). During the earlier stages they possess yolk platelets and lipid droplets.

Pronephros.

The pronephros develops between head somites III-IV and the 1st trunk somite (Photo 6a) as an aggregate of cells. These

cells soon take up a radial arrangement, with few filopodia and only close contacts between neighbouring cells (stages 23-27). Regional differentiation proceeds quickly. The proximal tubule develops a brush border, junctional complexes, and basement membrane (Photo 1b). Below the level of the junctional complexes, opposing cell membranes possess inter-coiling long microvilli (Photo 1d). The cells often possess a large lipid droplet at stage 35/36, but these have disappeared by stage 40. There are few yolk platelets even at stage 35/36, but a number of long thin mitochondria are present. A large nucleolus is often seen. Mesenchymal cells touch the basement membrane at a few sites, but never pass through to actually touch a tubule cell (Photo 1d).

The nephrostome is an open-ended funnel, well supplied with bundles of long cilia. It also has a basement membrane. But its cells have tight junctions rather than junctional complexes (at least at stage 35/36), and they do not have microvilli either between the cells or lining the lumen.

Nephric Duct.

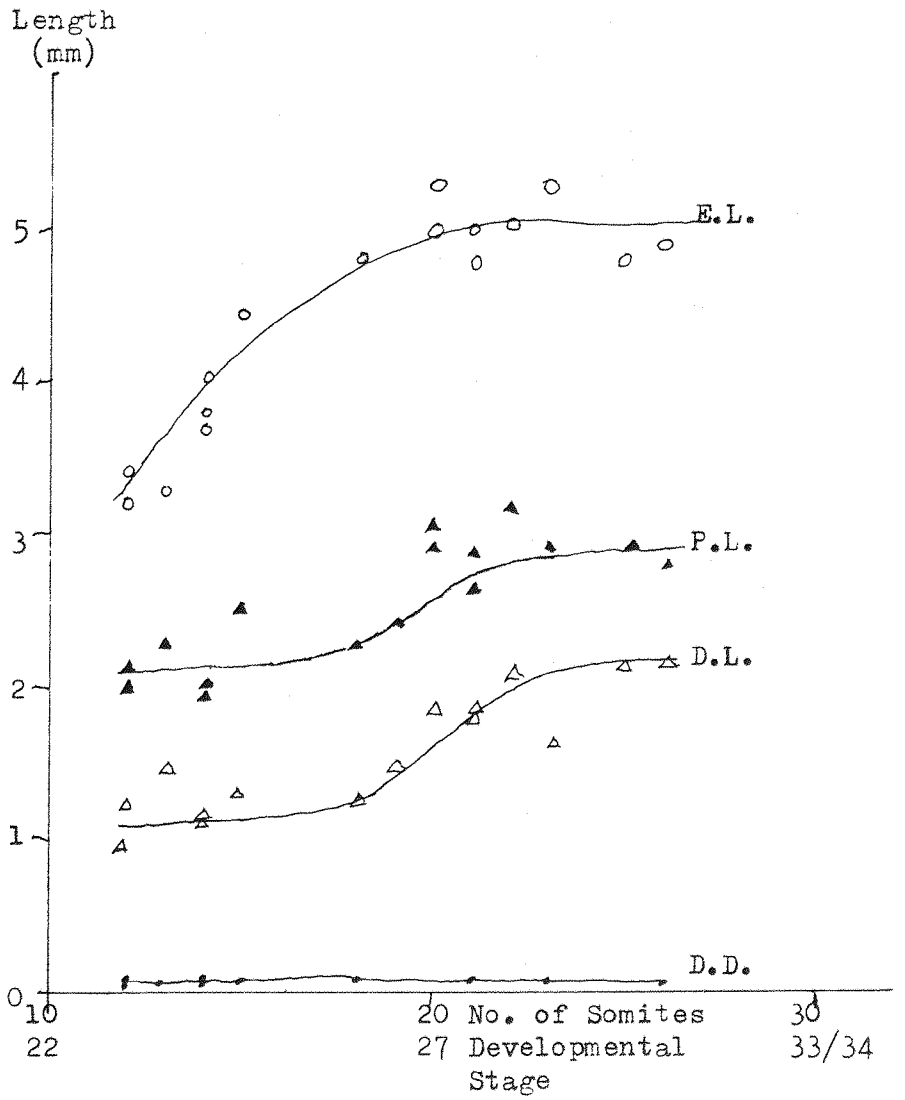
The nephric duct is first seen at stage 23. It forms against the pronephros, extending from the 1st trunk somite to the 2nd or 3rd. By stage 24, it has reached trunk somite 5 (Photo 6a). By stage 26 it has reached trunk somites 8-9. There it stays until aprox. stage 28 (Photo 6b), after which it extends to the rectal diverticula. By stage 39 its extension is complete (Photo 6c). It can be seen to extend to trunk somite 11-12, after which the view is obscured. The duct or rectal diverticula is apparently positioned below the level of the somites and lateral mesoderm in this posterior region.

Photo 7a shows that the nephric duct at stage 24 consists of an accumulation of cells, just below the level of the somites. These cells are clearly seperate from the somites, but cannot be clearly distinguished from the rest of the lateral mesoderm. The duct varies in width at this stage from aprox. 3-5 cells in diameter (aprox. 0.05mm). By stage 29/30 the cells of the duct have rounded up and formed a distinct tube of cells. This rounding-up has decreased the duct diameter somewhat, it now being 3-4 cells in diameter. The cells still have a large number of filopodia overlapping neighbouring cells, but they are very closely applied. The cells are now wedge-shaped, but no lumen has yet formed. By stage 33/34, the cells are very closely applied to each other, such that cell peripheries are very unclear (Photo 7b). The cells still seem to possess filopodia, but these are well flattened against neighbouring cells. The whole duct (especially its posterior region) seems to be sinking below the level of the somites and lateral mesoderm. Both somites and lateral mesoderm cells extend thin branching filopodia onto the duct cells. By stage 40 much of the duct's length has sunk into the body of the tadpole.

Graph 3 summarises much of this information. It shows that the embryo is increasing in length during most of this early development. The embryo length appears to level off, but this tendency is broken as the tail starts to lengthen (at stages past those shown). Path length (i.e. curvilinear distance from the end of the pronephros to the prospective renal diverticula) follows the same pattern as embryo length, as expected. However, actual duct length increases slightly to begin with, then levels off from aprox. stage 25 to stage 27, then increases again (as explained above). The duct diameter

GRAPH 3.

Dimensional Changes During Xenopus Nephric Duct Extension.



Embryo Length measurements (E.L.; ○) were recorded as the straight-line distance from tip of head to tip of tail.

Total Path Length (P.L.; ▲) and Duct Length (D.L.; △) measurements were curvilinear.

Duct Diameter (D.D.; •) is the linear distance from somite to lateral mesoderm across the duct at the level of trunk somite 6.

Dimensions taken from scanning electron micrographs.

is approximately constant throughout.

TEM shows that the initial aggregation and radial arrangement of the duct cells is associated with a decrease in the number of long filopodia, aggregation of cells leading to their wedge-shape, and close contacts between neighbouring cells.

By stage 40, the nephric duct has reached the rectal diverticula and a lumen has formed all along its length (Photo 2a). The cells are now free of yolk platelets, though lipid droplets are still present. The cells contain many mitochondria and are joined by junctional complexes. A basement membrane has also formed, which surrounding mesenchymal cells sometimes touch but do not pass through. A brush border is not present, the luminal edge of the cells possess only a few short microvilli.

By stage 46, the nephric ducts have shifted to the centre of the body, on either side of the medial post-cardinal vein. The lumen is occluded, as the cells meet at the centre of the duct (Photo 2b). In this region, the cytoplasm is granular and free of most large organelles. The cells are well supplied with mitochondria, and their basal cell membranes are beginning to form "intuckings", producing a "canal system" at the basal end of the cells. By stage 48-49, this "canal system" is very well developed (Photo 3b), with mitochondria inbetween them. Below the basement membrane is sometimes seen fibrous ECM.

Mesonephros.

The first mesonephric cells are seen on both sides of the mid-line at stage 39. By stage 45, definite mesonephric cords have formed along the nephric ducts. Photo 3a shows a region of

the embryo at stage 48-49 with accumulating mesonephric cells and the mesonephric cord. The accumulating cells are seen to be irregular in shape with a few long filopodia touching neighbouring cells. The cells of the cord are neatly arranged, though still irregular in shape. Only close contacts are seen, no specialised cell junctions. No lumen has formed. There are mesenchymal cells in the 3μm gap between the cord and the nephric duct. In general, very few mesonephric cells were ever seen to touch the basement membrane of the nephric duct.

Photo's 4a and 4b show another region of the developing mesonephros in a stage 48 embryo. (The mesonephros develops quickest in caudal regions, see the Introduction.) In this area, tubules have formed and coiled, and blood vessels have appeared. The tubule lumina possess brush borders. The cells of the tubules join at tight junctions, and possess microvilli on their basal surfaces and opposing surfaces, as well as their luminal surface. They are well supplied with mitochondria and rough endoplasmic reticulum. A basement membrane (and associated ECM) is also present, though obscured in section by the presence of the microvilli. The endothelial cells of the blood vessels are very long, thin cells, closely apposed to the basal microvilli of the tubule cells. The blood vessels contain both red and white blood cells. Note that the erythrocytes contain both a nucleus and mitochondria, and possess a marginal band of MT. (Only mammalian erythrocytes are anucleate.)

By stage 50, the mesonephros has developed into a large organ over much of the body length. The second generation of tubules is forming and the nephrostomes are open and functional (Photo 5). The whole system is well supplied with

blood vessels. The morphology of the tubule cells is becoming quite complex, including "canal systems" similar to that seen in the later nephric duct. The whole makes for very complex electron micrographs.

Explants.

a). Pronephros.

The explantation experiments show that pronephric mesenchyme is already committed to differentiate into tubules by stage 18 (Table 6). The body slices average 80% positive results, i.e. explants with tubules. The pronephric mesenchyme explants average 40% positive results (Photo 8a).

b). Nephric Duct.

32 embryos, stages 20-21, had the pronephros on one side removed (as part of the pronephric explant experiments). Seven of these survived to stage 30-32. On sectioning, it was found that in all 7 cases the pronephros and nephric duct were missing on the operated side. Except for one case, in which the anterior part of the pronephros was present, but not the rest of the pronephros nor the nephric duct.

In a similar experiment, involving 14 embryos operated on at stage 19-21, one embryo survived to stage 47. On sectioning, as above, the pronephros and nephric duct were missing on the operated side, as was the mesonephric cord (see below).

c). Mesonephros.

The explant and body slice experiments always gave negative results, i.e. mesonephric tubules were never seen (Table 7). In the body slices, there was a general breakdown of organization, although the neural tube, somites, notochord and

TABLE 6.

Summary of the Pronephric Explants.

Explant Type	Dev. Stage	Time in Culture	Medium	No. of Explants	Result (% Tubules)
Body	15	19-24 Days	70% L-15	5	100%
Slices	15-17	"	"	15	70%
	20	"	"	4	75%
	21	"	"	2	50%
Pronephric	18	9 Days	MS	3	100%
Mesenchyme	19-21	2 Days	"	6	40%
	20-22	9 Days	"	10	35%

TABLE 7.

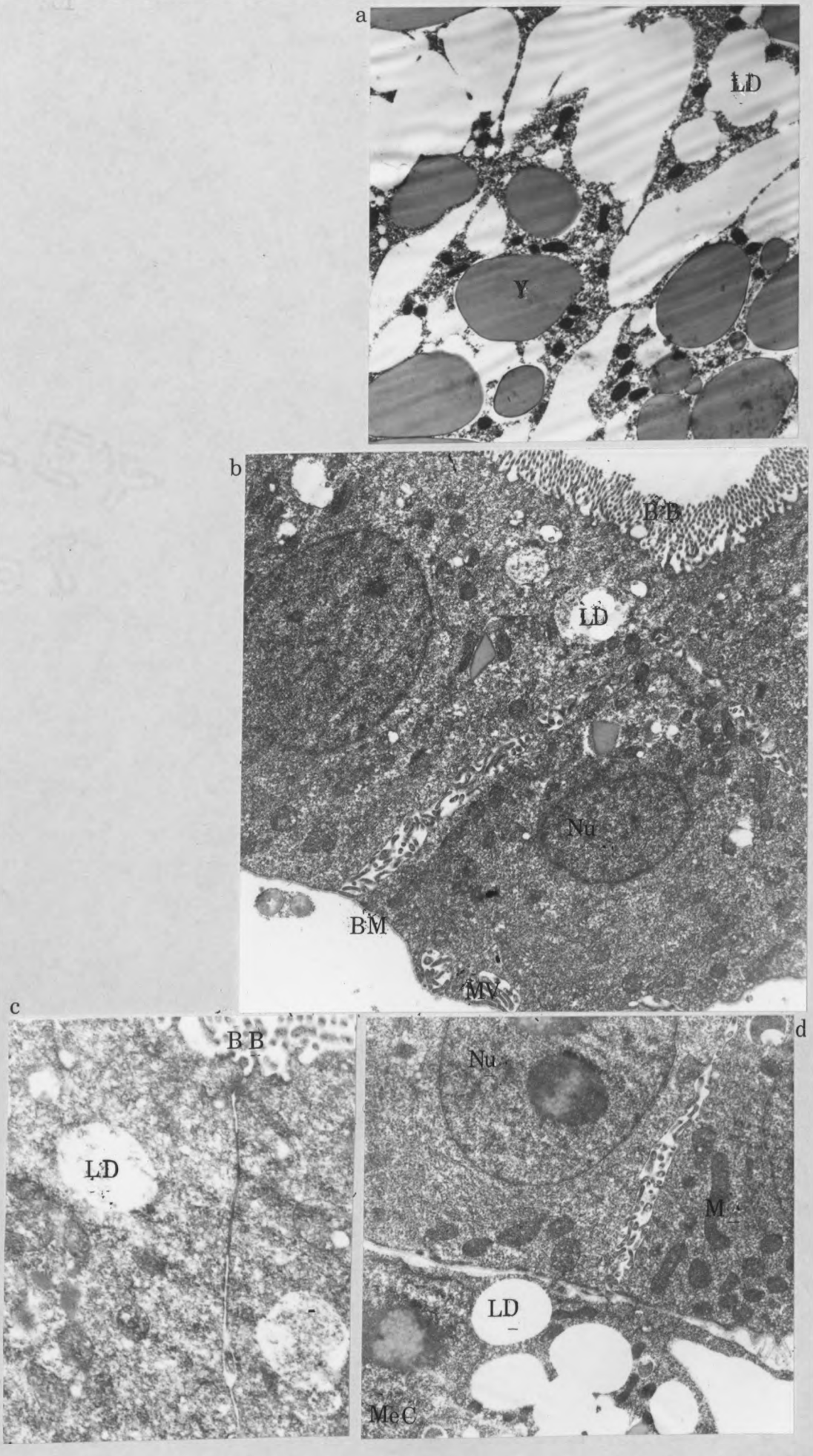
Summary of the Mesonephric Explants.

Explant Type	Dev. Stage	Time in Culture	Medium	No. of Explants	Results (% Tubules)
Body Slice	15–39	9 or 19–24 or 33–34 Days	MS or 70% L–15	85	0%
Mesonephric Mesenchyme (M.M.)	17–40	"	"	45	0%
M.M. + Gut	14–34	9 or 19–24 Days	MS	15	0%
M.M. + Muscle	18–41	19–24 or 33–34 Days	MS or 70% L–15	13	0%
M.M. + Neural Tube	14–41	19–24 Days	"	16	0%
M.M. + Brain (stage 48–50)	15–34	19–24 or 33–34 Days	"	44	0%
M.M. + Nephric Duct	33–39	15–16 Days	MS	8	0%

This Table gives a very brief summary of a series of experiments. At least 4 explants from any one developmental stage were used. The Table only shows the range of developmental stages from which the explants were taken.

endoderm differentiated almost as normal. The endoderm often tended to join with the ectoderm and/or form coiled tubes, but not a well organised gut and associated organs (Photo 8b). The somites tended to disaggregate, whilst both the neural tube and the notochord were often distorted in shape. The latter was probably due to the general distortion of the shape of the body slice or explant. Most importantly, no trace of mesonephric cords or tubules were ever seen. In explants and body slices including the nephric duct, this had disaggregated by the time the explant was sectioned.

In the experiment noted above, in which 14 embryos (stages 19-21) had one of their pronephri removed, one embryo survived to stage 47. In section, this embryo had the pronephros, nephric duct, and mesonephric cord missing on the operated side. Things were as normal on the unoperated side.


a
LD
Y
b
BB
LD
Nu
BM
MV
c
BB
LD
d
Nu
M
LD
MeC

PHOTOGRAPH ONE.

a). Electron Micrograph Through the Lateral Mesoderm, Stage 27.
Mag.= x5,000
1% Phosphotungstic Acid Stain.

The cells are irregular rectangles in shape, with long fine filopodia making close contacts with neighbouring cells. The cells are full of Lipid Droplets (LD) and Yolk Platelets (Y).

b). Electron Micrograph Through a Pronephric Proximal Tubule, Stage 40.
Mag.= x8,000
1% Phosphotungstic Acid Stain.

This section shows the Brush Border (BB) on the luminal cell surface and the Basement Membrane (BM) on the opposite surface. Opposing cell surfaces have interconnecting microvilli, and Junctional Complexes at their luminal end. The lower cell surface also has a few microvilli (MV) at some points. The cells have very few small Lipid Droplets (LD), but no yolk platelets. They have many mitochondria and rough Endoplasmic Reticulum; two Nuclei (Nu) have also been sectioned.

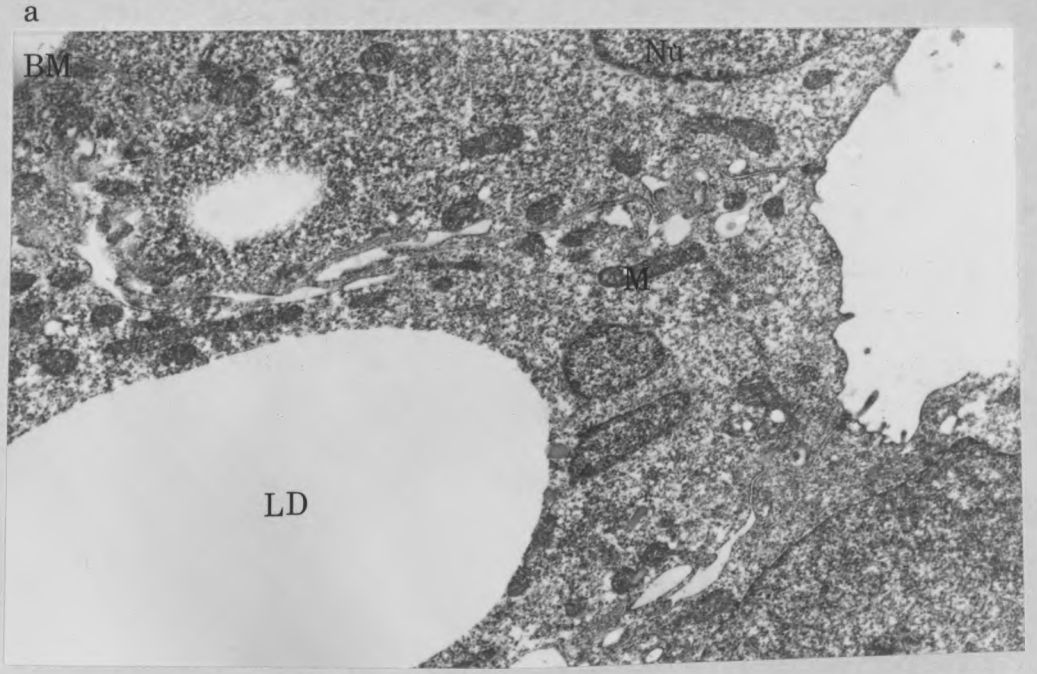
c). Electron Micrograph of the Junctional Complex of a Pronephric Proximal Tubule, Stage 40.
Mag.= x20,000
1% Phosphotungstic Acid Stain.

Compare with Fig. 2.

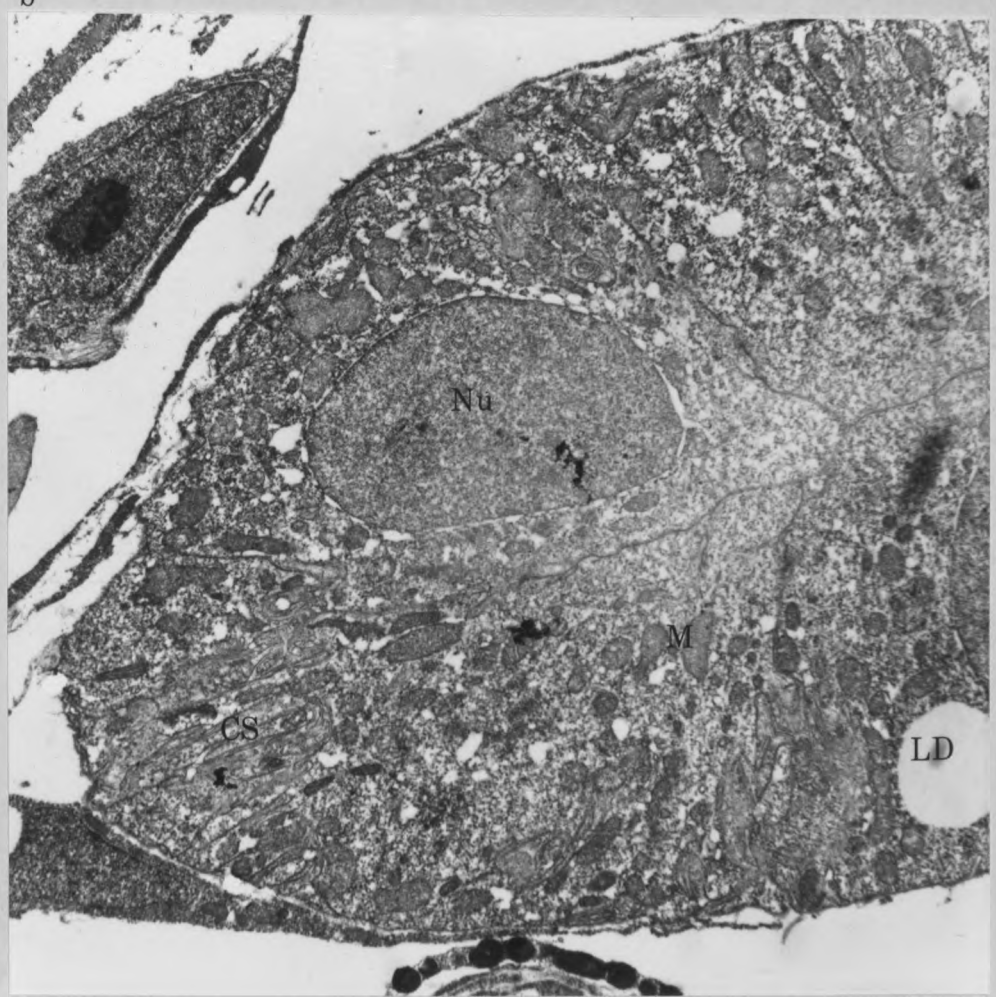
d). Electron Micrograph Through a Pronephric Proximal Tubule, Stage 40.
Mag.= x8,000
1% Phosphotungstic Acid Stain.

This section shows that Mesenchymal Cells (MeC) do actually touch the basement membrane, but do not pass through it. These mesenchymal cells also possess Lipid Droplets (LD). The tubule cells possess accumulations of Mitochondria (M), whilst their Nuclei (Nu) possess a nucleolus.

a



b



PHOTOGRAPH TWO.

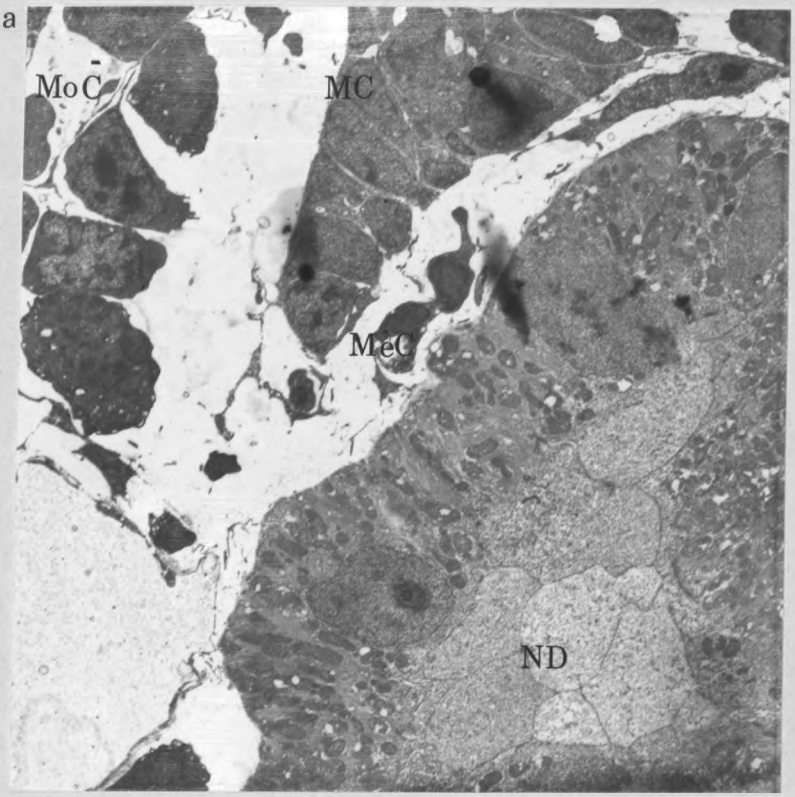
a). Electron Micrograph Through the Nephric Duct, Stage 40.
Mag.= x11,000
1% Uranyl Acetate Stain.

The lumen is seen to have few microvilli, and the cells are joined by Junctional Complexes. A Basement Membrane (BM) has developed and the cells still possess Lipid Droplets (LD), but no yolk platelets. The Nucleus (Nu) and Mitochondria (M) are also marked.

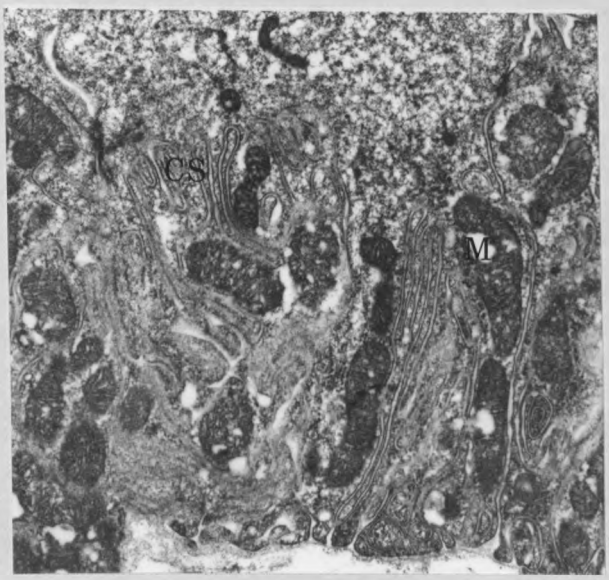
b). Electron Micrograph Through the Nephric Duct, Stage 46.
Mag.= x6,500
2% Uranyl Acetate Stain.

This section shows that the lumen is closed by the coming together of the cells. The cells have developed a distinct morphological polarity; at their luminal end the cytoplasm is granular-staining and clear of most of the large organelles. At the opposite end of the cells are concentrated the large organelles, including the Nucleus (Nu), and remaining Lipid Droplets (LD), and especially the Mitochondria (M). At this end, also, there appears to be developing a system of narrow "Canals" (CS) formed by "intuckings" of the cell membrane (see Photo 3b).

a



b



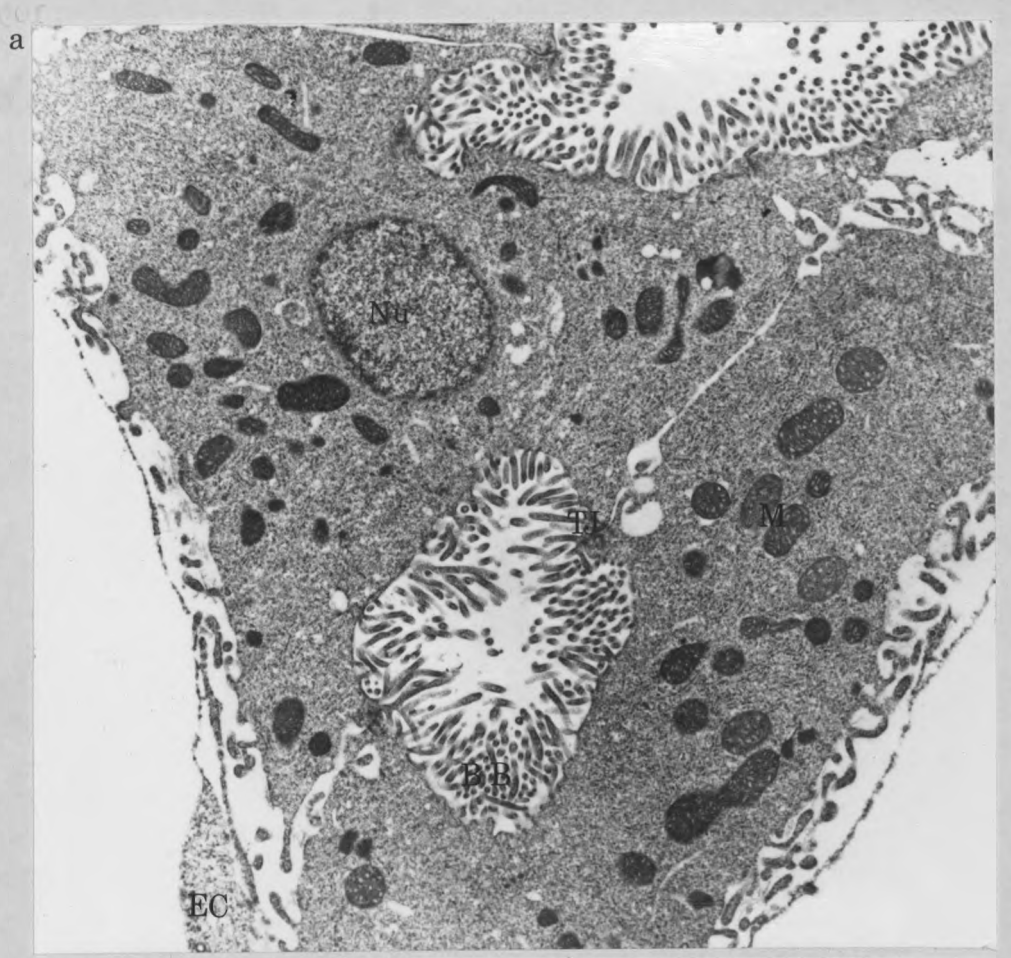
PHOTOGRAPH THREE.

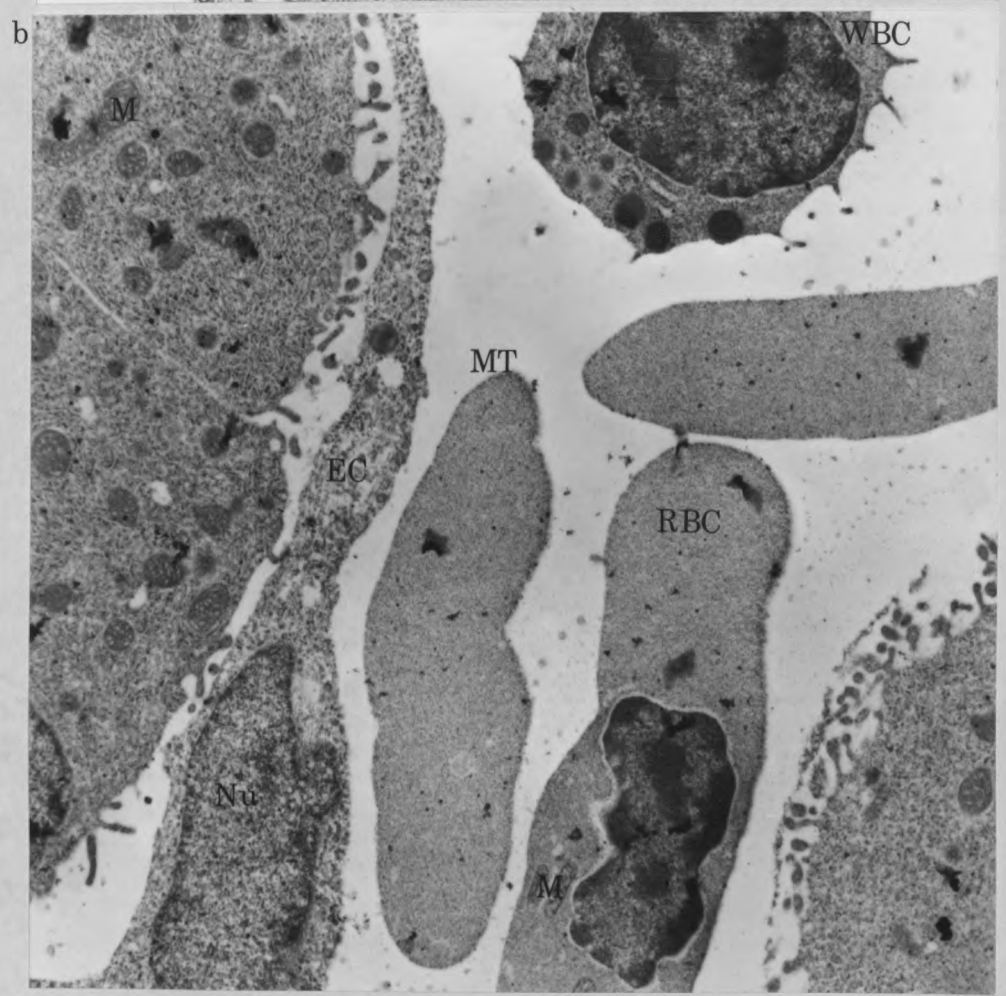
a). Electron Micrograph of the Nephric Duct and Developing Mesonephros, Stage 48-49.
Mag.= x3,000
1% Phosphotungstic Acid Stain.

The Nephric Duct (ND) is still closed. The cells of the Mesonephric Cord (MC) are still irregularly shaped, but are beginning to array themselves neatly. There is a 3μm gap between the nephric duct and the mesonephric cord, in which are found Mesenchymal Cells (MeC). Other Mesonephric Cells (MoC) appear to be aggregating near the mesonephric cord.

b). Electron Micrograph of the "Canal System" of the Nephric Duct Cells, Stage 48-49.
Mag.= x7,000
1% Phosphotungstic Acid Stain.

This is a high power of the "Canal System" (CS) showing that it does arise from the basal cell membrane. Note also the Mitochondria (M) arranged between the "canals".


a
Nu
M
BB
EC


b
WBC
M
MT
EC
RBC
Nu
M

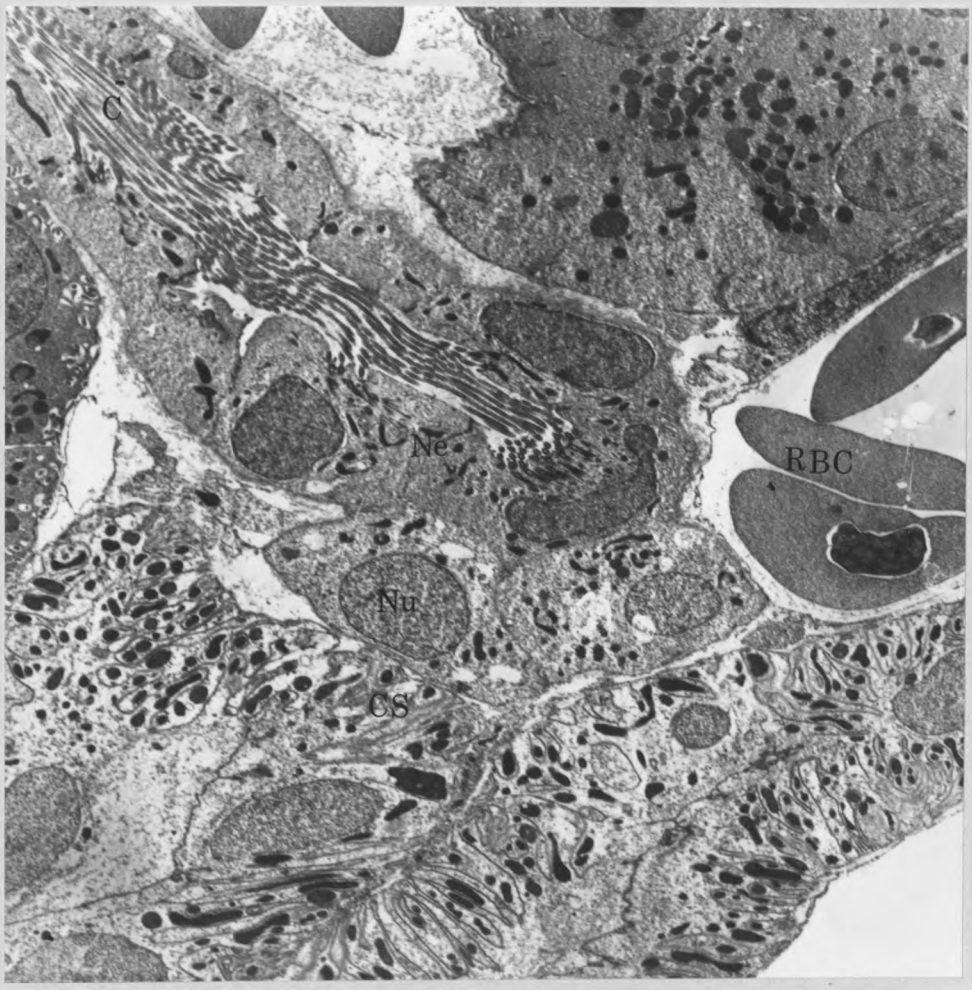
PHOTOGRAPH FOUR.

a). Electron Micrograph of the Mesonephros, Stage 48.
Mag.= x8,000
1% Phosphotungstic Acid Stain.

In this area of the developing mesonephros, tubules have formed and blood vessels are present. The cells of the mesonephric tubules are joined by Tight Junctions (TJ). The lumen of the tubules possess Brush Border (BB). The situation is complex as one cell can apparently line more than one tubule, but one should not forget that this is simply a section and that the tubules are coiled. The blood vessels are lined by thin Endothelial Cells (EC), and the tubule cells along these edges possess thick, coiling microvilli, a basement membrane and fibrous ECM. A Nucleus (Nu) and Mitochondria (M) are also marked.

b). Electron Micrograph of a Blood Vessel in the Mesonephros, Stage 48.
Mag.= x8,000
1% Uranyl Acetate and Lead Citrate Stain.

This shows a clearer view of one of the blood vessels, lined by Endothelial Cells (EC). This time, one can see the main cell body of one of these cells, with its nucleus (Nu). Note also the 3 Erythrocytes (RBC), one of which has been sectioned through its Nucleus and 3 Mitochondria (M). Finally, note also the Marginal Band of Microtubules (MT). A White Blood Cell (WBC) is also seen.

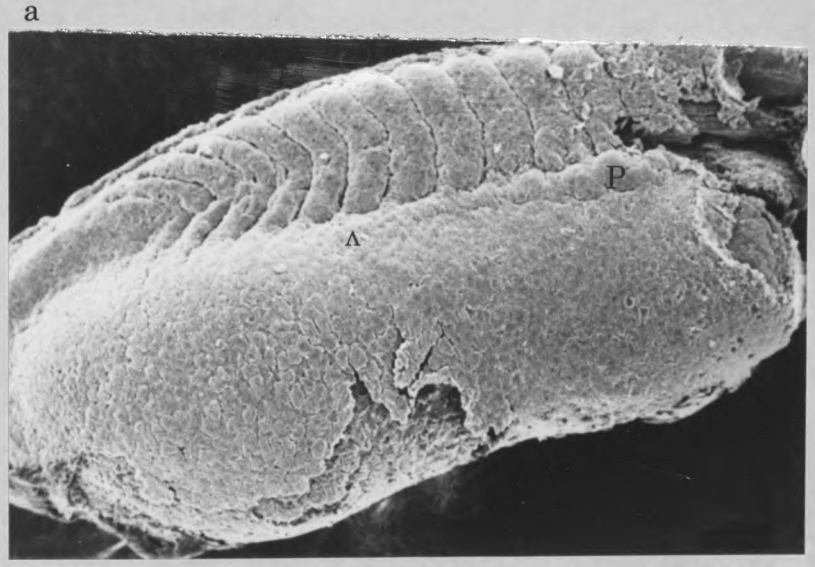

C
Ne
RBC
Nu
CS

PHOTOGRAPH FIVE.

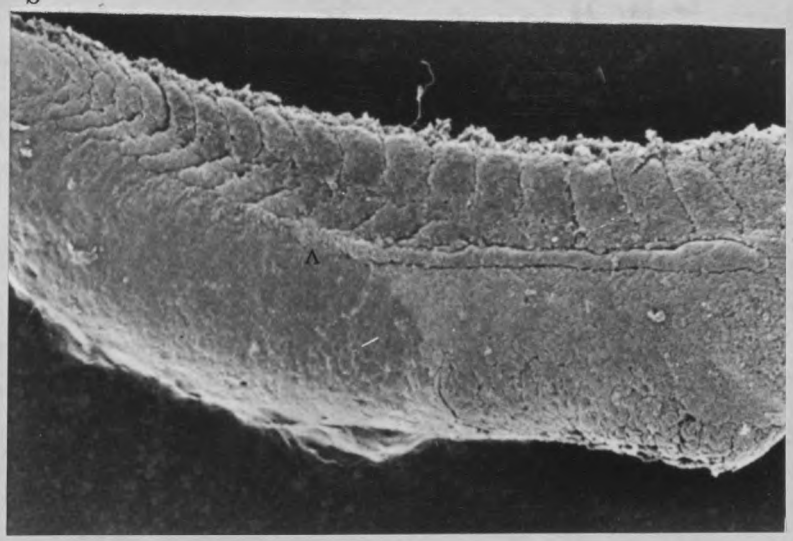
a). Electron Micrograph of the Mesonephros, Stage 50.
Mag.= x3,500
1% Phosphotungstic Acid Stain.

The situation is complex, with a variety of tubule and cell types. The Nephrostome (Ne) is seen with its abundant Cilia (C); a blood vessel with Erythrocytes (RBC) - note the nuclei - and tubules. The latter seem to have closed lumina. Many of the tubule cells possess a "Canal System" (CS), resembling the nephric duct.

a



b



c

PHOTOGRAPH SIX.

Scanning Electron Micrographs of *Xenopus* Embryos, with their Ectoderm peeled away.

Mag.= x50
Tilt= 0'

a). Stage 24.

Embryo Length = 2.5 - 3.0mm

The Pronephros (P) is seen as a bulge of cells under trunk somite 2. The nephric duct has reached trunk somite 7-8 (arrowhead) and is not clearly distinguished from the lateral mesoderm.

b). Stage 28.

Embryo Length = 3.8 - 4.0mm

The nephric duct has reached trunk somites 9-10 (arrowhead) and is easily distinguished.

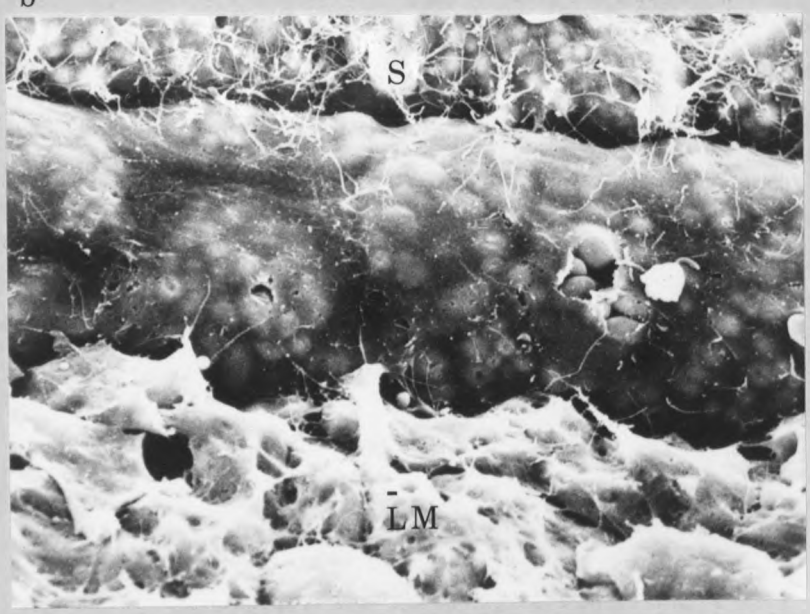
c). Stage 39.

Embryo Length = 5.5 - 6.5mm

The nephric duct is fully extended, but is obscured from view from trunk somite 11 onwards. The pronephros has coiled tubules.

a

b



PHOTOGRAPH SEVEN.

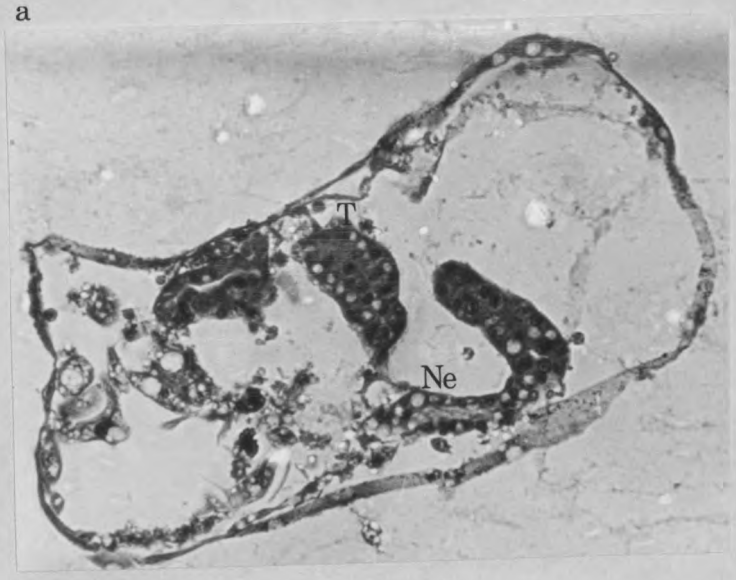
a). Scanning Electron Micrograph of the Nephric Duct (Ectoderm peeled away) at the level of the Trunk Somite 6, Stage 24.
Mag.= x700
Tilt= 0'

The duct is seperated from the Somites (S), but is only just beginning to seperate from the Lateral Mesoderm (LM). Morphologically, the duct cells are identical to lateral mesoderm cells, being flattened, irregularly shaped cells with fine filopodia touching neighbouring cells. The duct is aprox. 3 cells in diameter, i.e. aprox. 0.01mm.

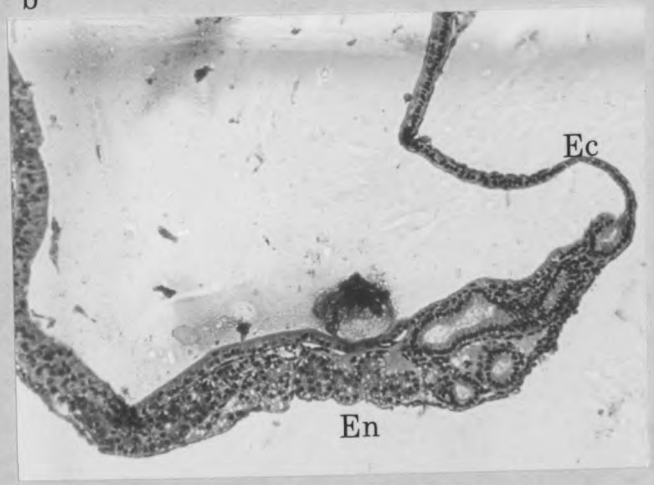
b). Scanning Electron Micrograph of the Nephric Duct (Ectoderm peeled away) at the level of Trunk Somite 6, Stage 33/34.
Mag.= x700
Tilt= 0'

The duct is smooth-walled and beginning to "sink" within the embryo. Both Somites (S) and Lateral Mesoderm (LM) have filopodia extended to the duct's surface. The duct is 2-3 cells thick, i.e. aprox. 0.008mm.

a



b



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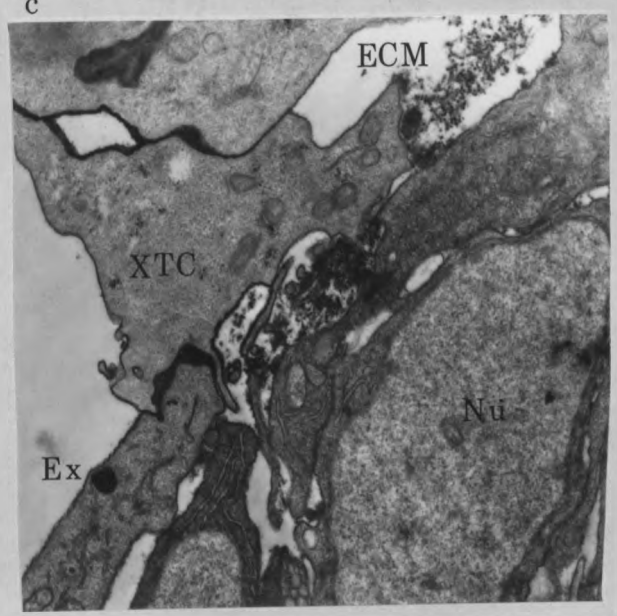


PHOTOGRAPH EIGHT.

a). Light Micrograph of a Transverse Section of a Pronephric Explant (see Text).
Mag.= x200
5μ Section of Paraffin Wax Embedded Material.
Haematoxylin and Eosin Stain.

Pronephric Tubules (T) have developed normally. Even a Nephrostome (Ne) is seen in this section. The whole explant has "blebbed" somewhat.

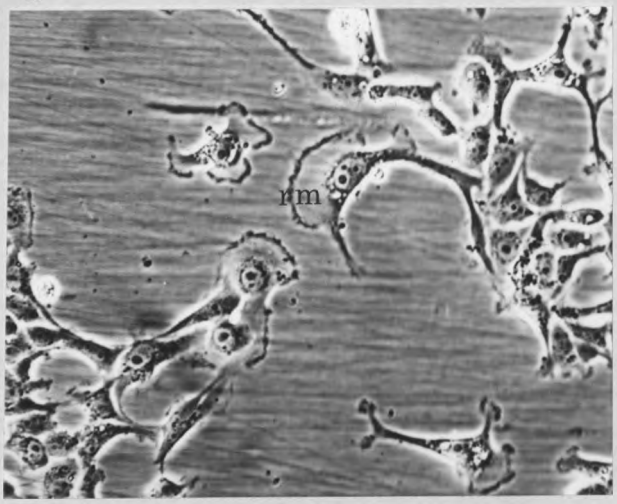
b). Light Micrograph of a Transverse Section of a Mesonephric Explant, with Endoderm as "Inductor" (see Text).
Mag.= x100
5μ Section of Paraffin Wax Embedded Material.
Haematoxylin and Eosin Stain.

The Endoderm (En) has joined with the Ectoderm (Ec) and produced lumina, but no mesonephric tubules are seen.

c). Electron Micrograph of XTC Cells with Endoderm as "Inductor" (see Text).
Mag.= x6,000
Araldite Embedded Material. Fixation Technique C.
Uranyl Acetate and Lead Citrate Stain.

The organization of the Endoderm Explant (En) has broken down. There is a lot of ECM present. The XTC cells have made contact with the endodermal cells, but no specialised structures have developed.

a



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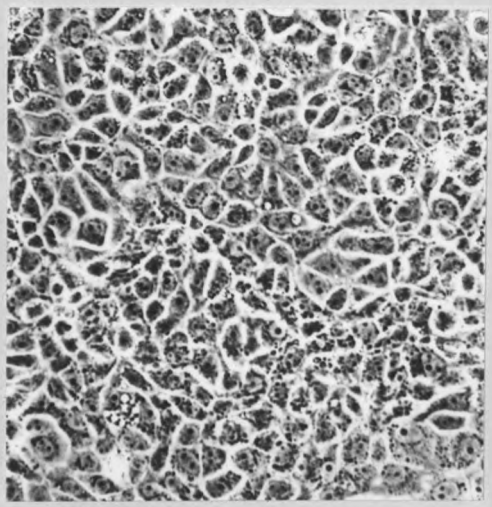

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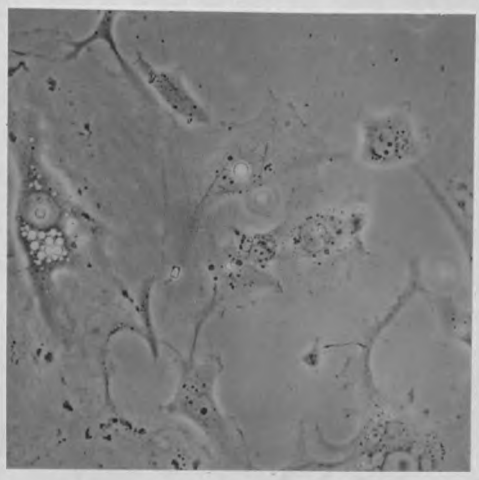

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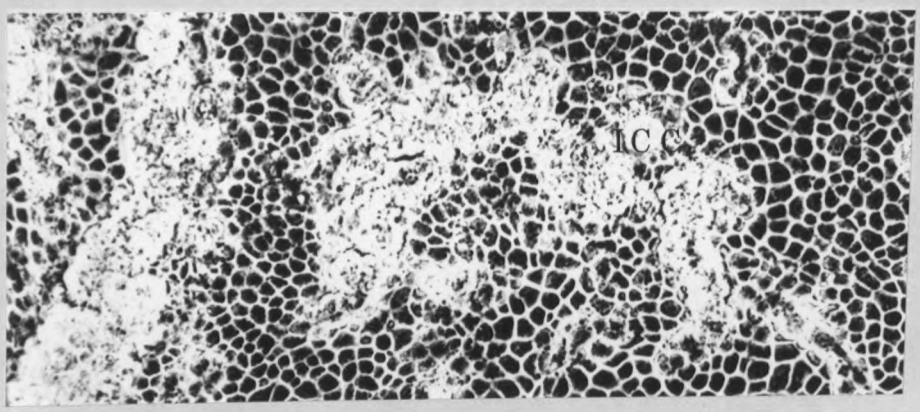


PHOTOGRAPH NINE.

a). Light Micrograph of a Non-Confluent XTC Cell Culture.
Mag.= x120

The nuclei are prominent, with 1-3 nucleoli and surrounding granular cytoplasm. Cells with free edges are often seen to have large Ruffled Membranes (RM) and even "tails", supposedly characteristic of fibroblasts (see Introduction).

b). Light Micrograph of a Confluent XTC Cell Culture.
Mag.= x150

The nuclei are prominent, with 1-3 nucleoli. The cytoplasm is granular and there are no ruffled membranes. The cells have a polygonal outline, typical of epithelial cells.

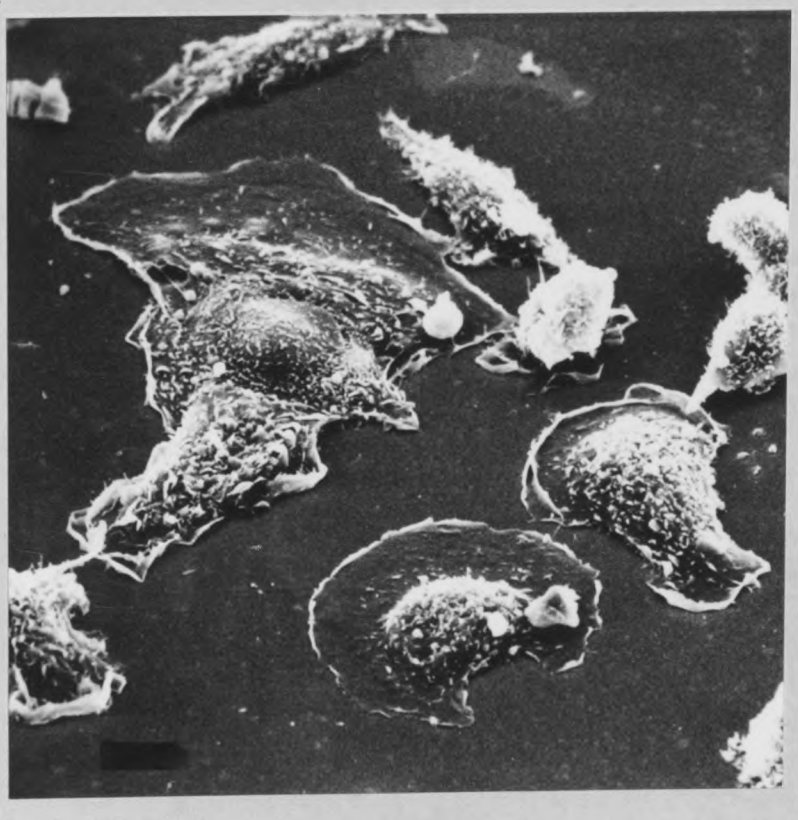
c). Light Micrograph of a Non-Confluent XTC Cell Culture grown in Medium containing "depleted" Horse Serum rather than Foetal Calf Serum.
Mag.= x200

The "depleted" Horse Serum was serum which had been kept in the freezer for over 1 year and had developed a slight precipitate. The cells did not grow in this medium. The cells have an irregular shape, with fewer ruffled membranes, but large lamellar areas of cytoplasm and filopodia (compare with 9a).

d). Light Micrograph of a Confluent XTC Cell Culture showing Overgrowth.
Mag.= x120

The cells in monolayered regions have the typical epithelial morphology of polygonal cells. The overgrowth has developed as as irregular mass and as a ridge. Associated with the overgrowth are a number of ICC's.

a



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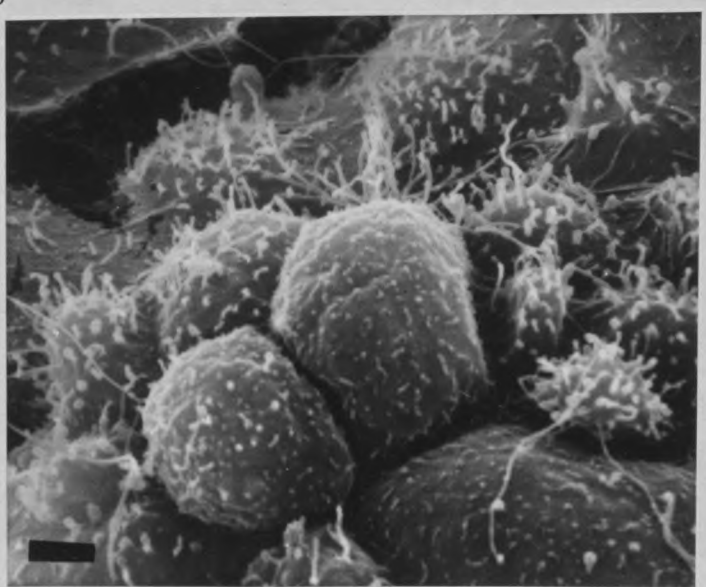


PHOTOGRAPH TEN.

a). Scanning Electron Micrograph of a Non-Confluent XTC Cell Culture.

Mag.= x1,000 Bar= 12.0μm

Tilt= 45'

The cells are seen to be covered in blebs and microvilli, except on their ruffled membranes. The more rounded cells tend to have more surface structures.

b). Scanning Electron Micrograph of XTC Cell Overgrowth.

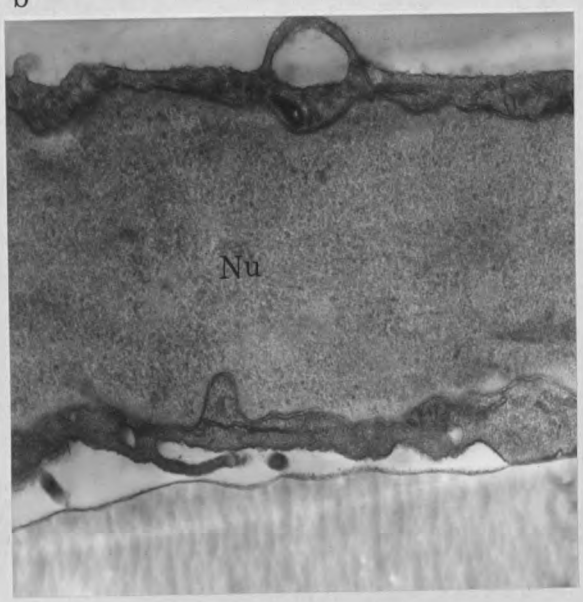
Mag.= x5,000 Bar= 1.8μm

Tilt= 45'

The overgrowth cells are seen to be rounded and covered in microvilli, and even a few long, thin filopodia. (Cell cracking has occurred to the top left of the micrograph, this is because fixation technique A was used, see Methods.)

a

b



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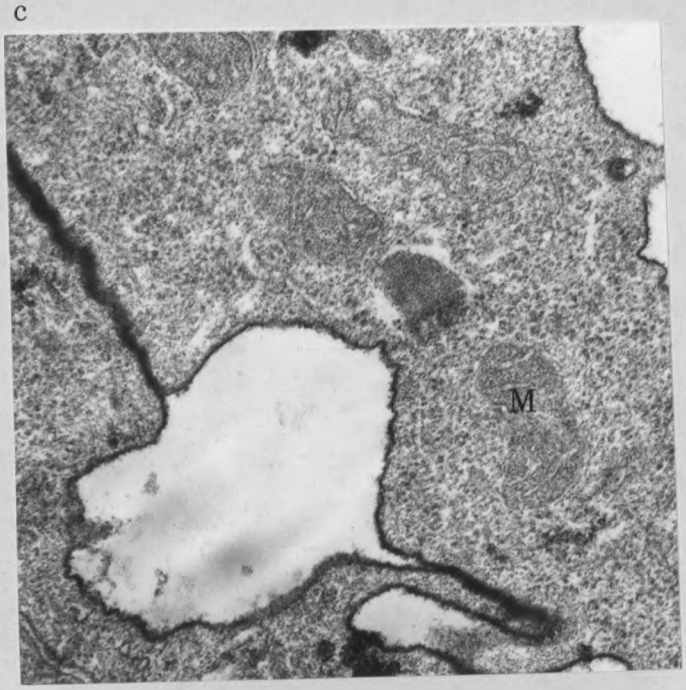


PHOTOGRAPH ELEVEN.

a). Electron Micrograph of an XTC Cell in a Non-Confluent Culture.
Mag.= x6,500
Araldite Embedded Material. Fixation Technique B.
Uranyl Acetate and Lead Citrate Stain.

Low power of a TEM section of an XTC cell, sectioned perpendicular to the substrate.
Height of the cell (over the nucleus) = 2.5μm
Height of the nucleus = 2.0μm
Diameter of the nucleus = 11.0μm
The only surface features are 2 blebs and a fold near the tip of the cell. The Nucleus (Nu) is featureless, i.e. no nucleolus was sectioned and there does not appear to be any heterochromatin. There are Golgi bodies at each end of the nucleus near the surface of the cell. Endoplasmic Reticulum (ER) can be seen passing down almost to the tip of the cell. The cell appears closely attached to the substrate (16-125nm).

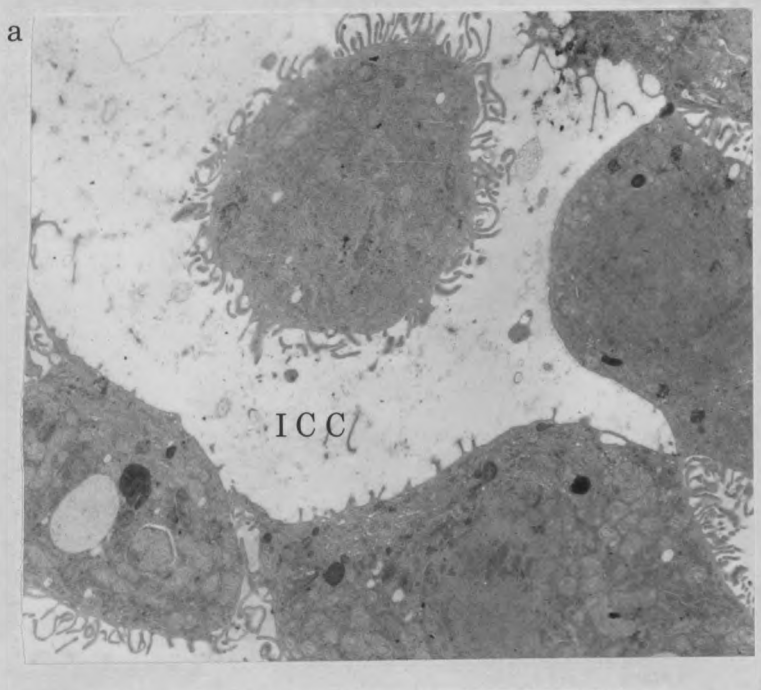
b). Electron Micrograph of an XTC Cell in a Non-Confluent Culture.
Mag.= x16,000
Araldite Embedded Material. Fixation Technique B.
Uranyl Acetate and Lead Citrate Stain.

TEM sections of yet another XTC cell. The centre of this cell is not so well attached to the substrate (10-500nm), microvilli and blebs being seen on both surfaces. Again, the Nucleus (Nu) is fairly featureless, but its double unit membrane can be clearly distinguished.

c). Electron Micrograph of XTC Cell Overgrowth.
Mag.= x38,000
Araldite Embedded Material. Fixation Technique C.
Uranyl Acetate and Lead Citrate Stain.

This micrograph shows the contacts between 2 cells. A fair amount of electron dense material is associated with the cell membranes, presumably ECM (but see Results). No specialised cell contacts are seen.

a



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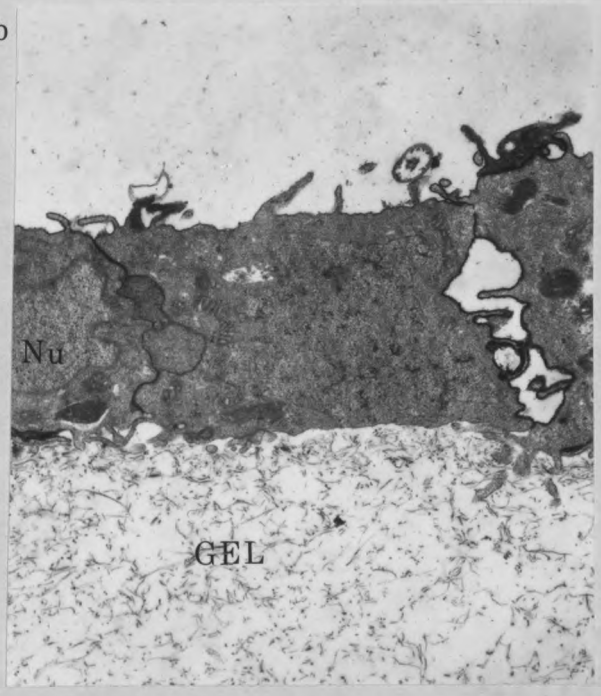

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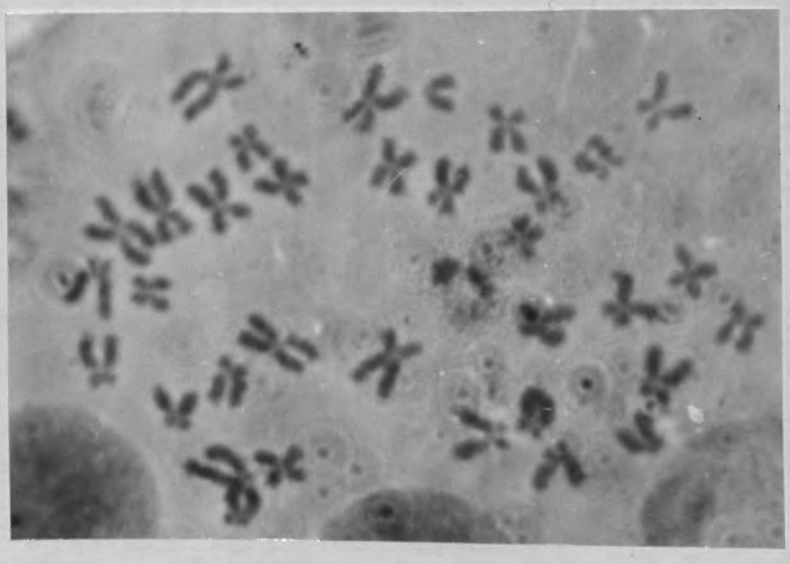


PHOTOGRAPH TWELVE.

a). Electron Micrograph of an ICC within XTC Cell Overgrowth.
Mag.= x6,000
Araldite Embedded Material. Fixation Technique A.
Uranyl Acetate and Lead Citrate Stain.

Note the lower number of microvilli on the inner cell surfaces and the areas of close contact at this edge.

b). Electron Micrograph of a Confluent XTC Cell Culture, grown on a Collagen Gel.
Mag.= x8,000
Araldite Embedded Material. Fixation Technique C.
Uranyl Acetate and Lead Citrate Stain.

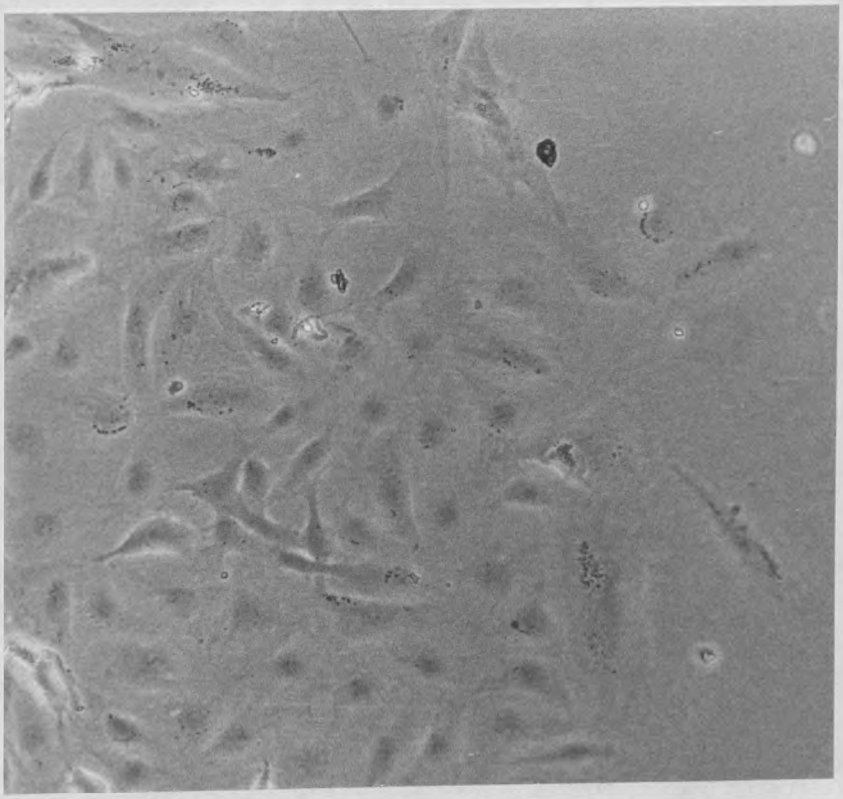
Height of the cell = 4.0μm
Diameter of the cell = 5.0μm
Height of nucleus = 2.0μm

XTC cells in confluent culture are no longer flattened. Microvilli are present on all cell surfaces, but do not extend into the gel very far. The cells themselves do not grow into the gel. Cells make close contacts with their neighbours, but no specialised cell junctions are seen. Nor are there any specialised contact points with the gel.

c). Light Micrograph of the XTC Cell Karyotype (see Text).
Mag.= x2,000
Oil Immersion, x100 objective.

Chromosome Number (2n) = 36

a



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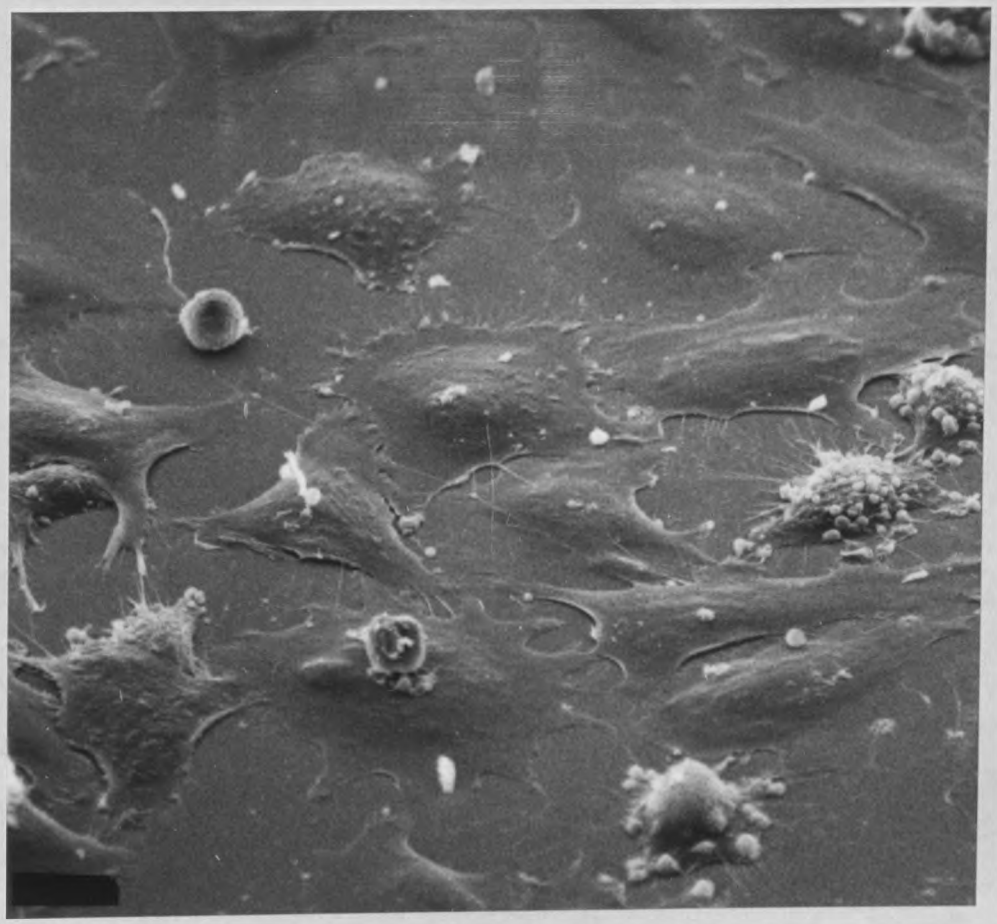


PHOTOGRAPH THIRTEEN.

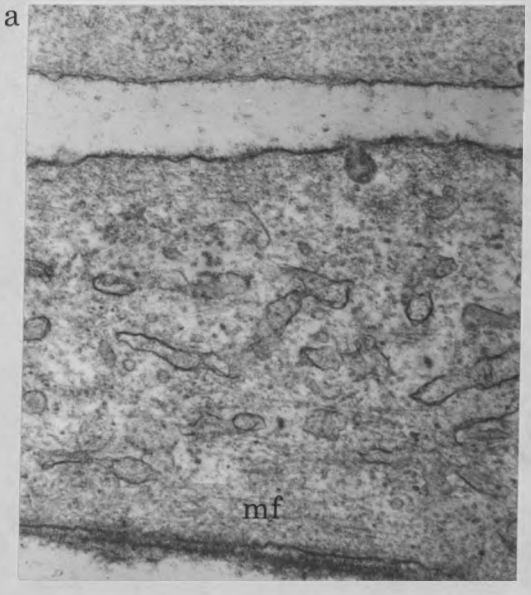
a). Light Micrograph of a Sub-Confluent Culture of Salmon Cells.
Mag.= x150

This shows the edge of a confluent patch of cells. The cells are not clearly seen as they are very flat and have a fairly clear cytoplasm. But one can see that they have the polygonal outline characteristic of epithelial cells. Cells at the edge of the monolayer possess ruffled membranes.

b). Scanning Electron Micrograph of a Sub-Confluent Culture of Salmon Cells.
Mag.= x500 Bar= 7.0µm
Tilt= 45°

These cells are well flattened with very few surface features and irregular outlines. Only a few have distinct ruffled membranes. Dividing cells are also seen, these have rounded up and are covered with blebs, and also possess long, thin retraction fibres.

a



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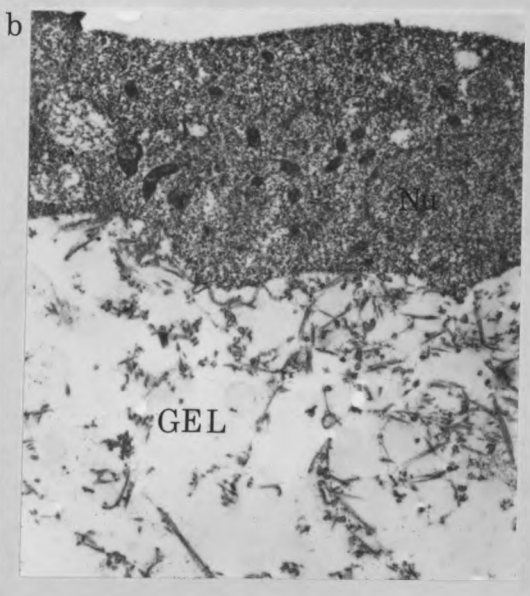

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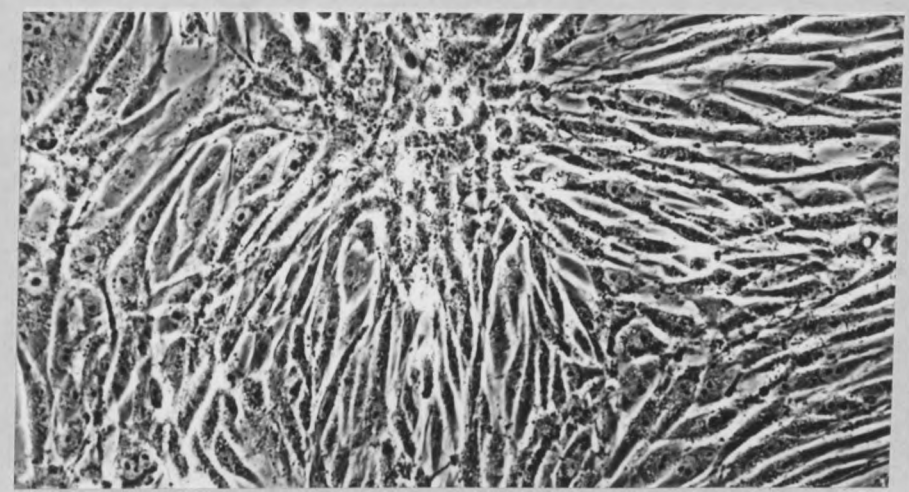


PHOTOGRAPH FOURTEEN.

a). Electron Micrograph of a densely crowded Salmon Cell Culture.
Mag.= x30,000
Araldite Embedded Material. Fixation Technique C.
Uranyl Acetate and Lead Citrate Stain.

In dense cultures the salmon cells overlap, going up to 6 cells deep, but remain flattened in shape. Note the ECM associated with the cell membranes. Note, especially, the Microfilament Bundles (mf).

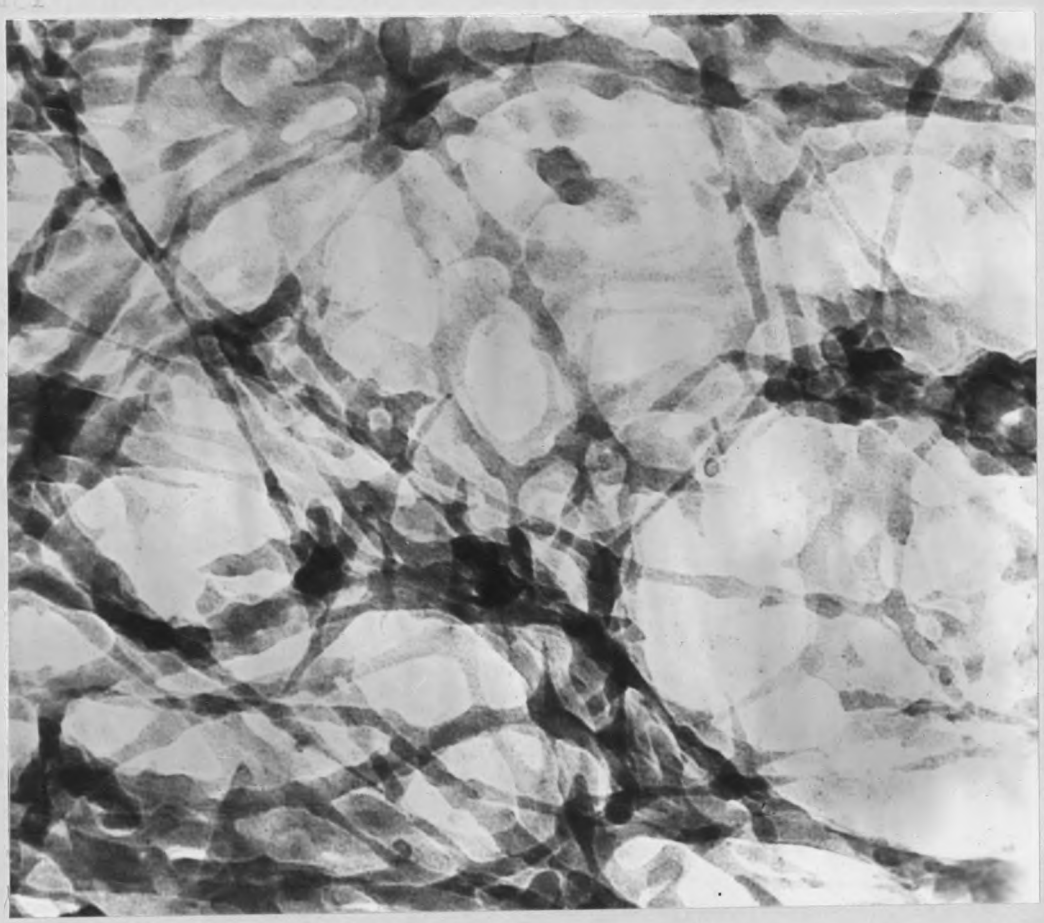
b). Electron Micrograph of Salmon Cells grown on a Floating Collagen Gel.
Mag.= x8,000
London Resin Embedded Material.
1% Phosphotungstic Acid Stain.

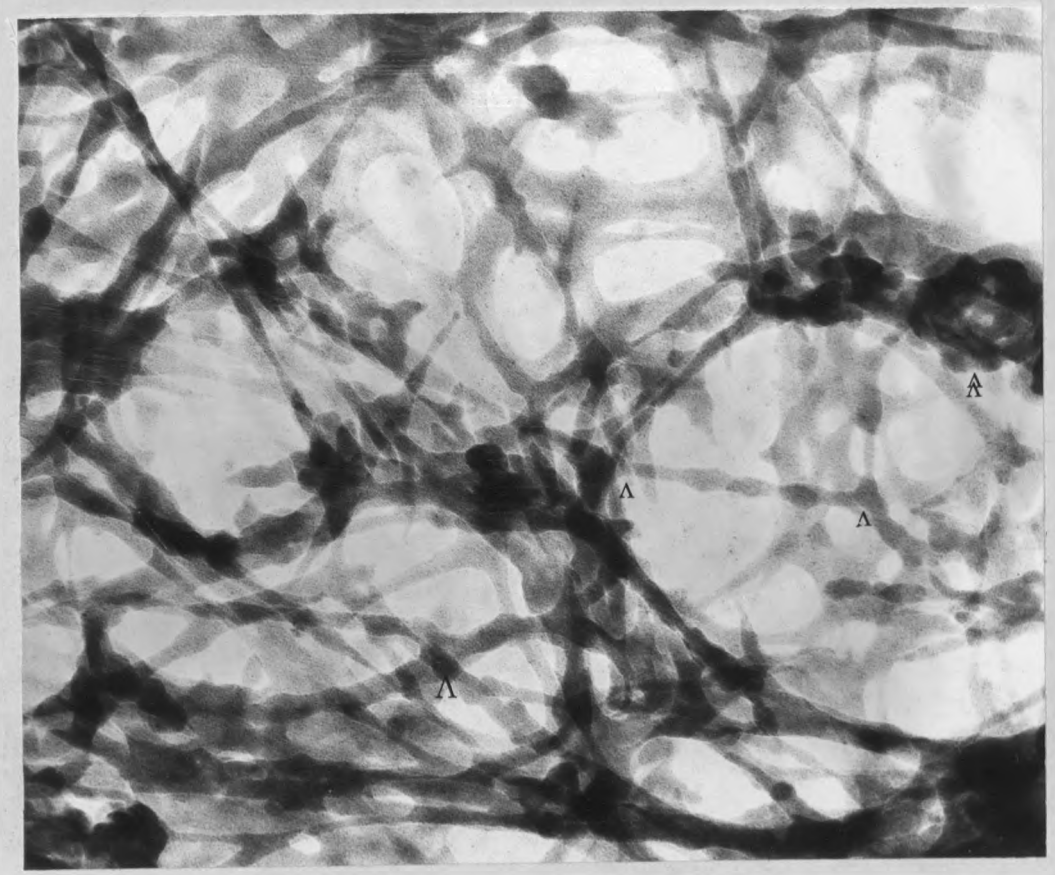
Height of cell = 3.0μm
Height of nucleus = 1.5μm

The cell (and Nucleus; Nu) is still flattened, even though this is a confluent culture. Although no specialised cytoplasmic attachment sites are seen, there are a few sites where collagen fibres have apparently aggregated against the cell membrane.

c). Light Micrograph of a Confluent Culture of Fibroblasts obtained from Xenopus Tadpole Tail.
Mag.= x160

These are fibroblastic cells derived from trypsinisation of Xenopus tadpole tails. They show the typical spindle-shape and parallel orientation of fibroblast monolayers. Compare with Photo's 9b and 13a.





PHOTOGRAPH FIFTEEN.

Stereo Pair of the Triton-Extracted Cytoskeleton of an XTC Cell.

Mag.= x125,000 80kV

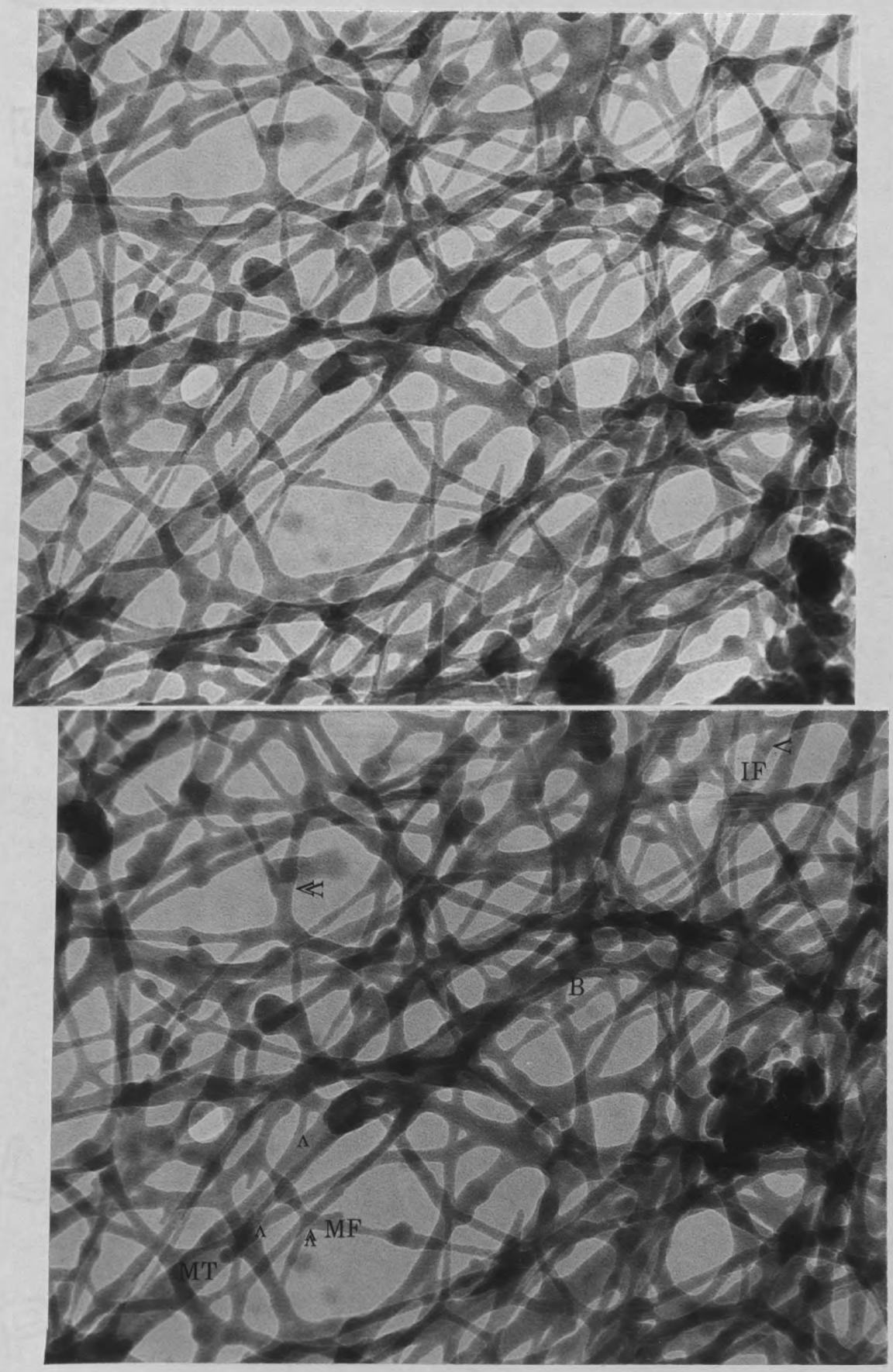
Stereo pairs should be viewed with a hand-held Stereo-Viewer (Agar Aids) held at about arms length from the centre of the page.

Fibres vary in diameter from aprox. 4nm to 20nm. Fibres vary periodically along their length, e.g. the arrowed fibre varies from 8nm to 12nm.

Groups of particles (c.18nm diameter) are apparently attached to the fibres at various points (e.g. double arrowhead).

Fibres split at irregular points, the 2 "daughter" fibres are often aprox. the same diameter as the "mother" fibre. Sometimes, one of the "daughters" is narrower (e.g. large arrowhead). The "branch-point" is often associated with a thickening of the fibre.

Individual fibres tend to be straight, bends mainly occur at branch-points.


IF
B
MF
MT

PHOTOGRAPH SIXTEEN.

Stereo Pair of the Triton-Extracted Cytoskeleton of a Salmon Cell.

Mag.= x125,000 80kV

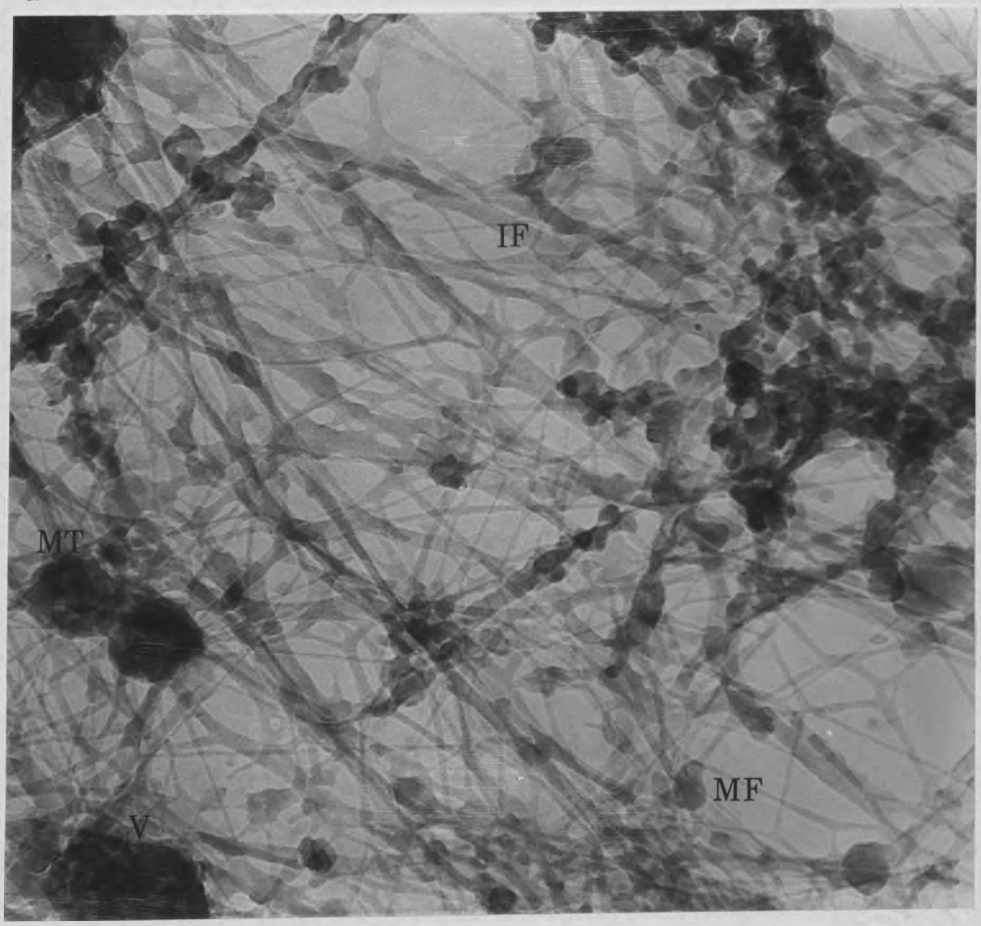
Stereo pairs should be viewed with a hand-held Stereo-Viewer (Agar Aids) held at about arms length from the centre of the page.

The fibres vary in diameter from c.4nm to c.20nm. Three types of fibre can be distinguished depending on diameter; MF - 4nm, IF - c.10nm, MT - c.18nm. They are taken to correspond to the 3 types of fibre in the cytoskeleton. Each fibre maintains its diameter, except for intermittent swellings produced by attached particles (diameter = c.12-30nm).

All 3 types of fibre show branch-points. MT branch-points result in an MT-diameter fibre and a thinner one (either MF or IF - arrowheads). MF branch-points result in 2 MF fibres (e.g. double arrowhead). IF branch-points can result in 2 IF fibres (large arrowhead), or one IF fibre and one MF fibre (double large arrowhead).

MT fibres also seem to finally "break-up" into smaller diameter fibres (marked by "B"). But at these points, there are so many fibres and particles it is difficult to see exactly what is happening.

a



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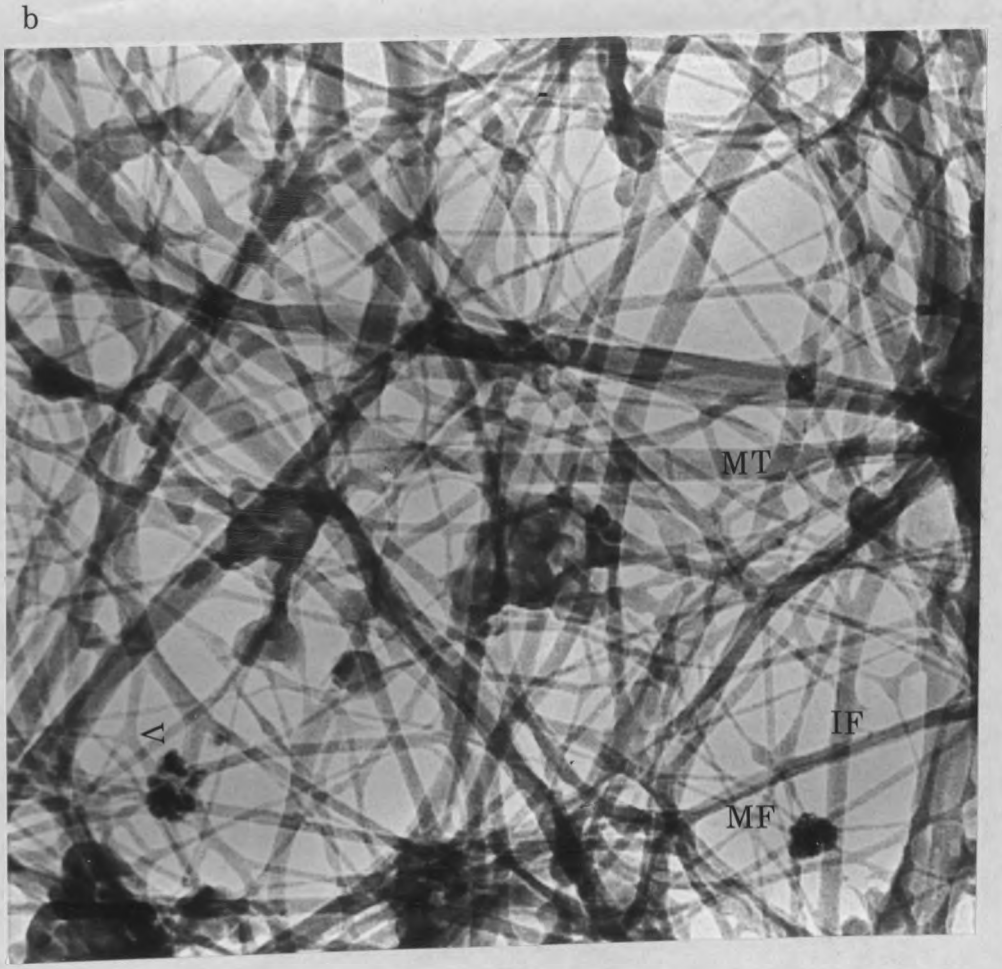


PHOTOGRAPH SEVENTEEN.

a). Triton-Extracted Cytoskeleton of an XTC Cell, treated with KCl to remove Microfilaments.
Mag.= x125,000 80kV

Three types of fibre can be seen, indicating that the KCl treatment was only partially successful. MF fibres (diameter c.4nm) can still be seen, IF fibres are also easily visible. But MT fibres are obscured by particles to the left of the micrograph.

Four large vesicles (V; diameter 40-90nm) are visible to the left. Smaller particles (diameter 9-20nm) occur throughout, especially to the top right, attached to the fibres.

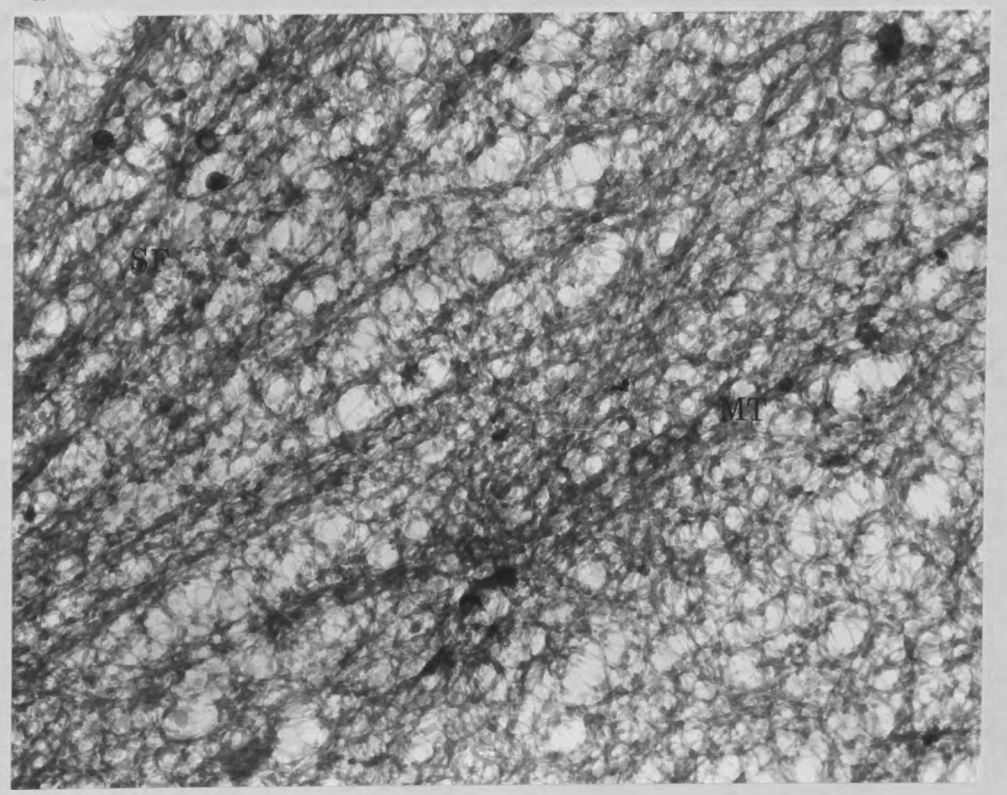
Fibres tend to be straight and of constant diameter, except where particles are attached. Branch-points occur irregularly.

b). Triton-Extracted Cytoskeleton of a Salmon Cell, treated with KCl to remove Microfilaments.
Mag.= x125,000 80kV

All 3 types of fibre are seen. Fibres tend to be straight between branch-points. Branch-points occur at irregular intervals. Few particles (diameter 8-30nm) are seen in this view.

Branch-points usually give rise to 2 new fibres, but sometimes 3 are produced (see arrowhead).

a



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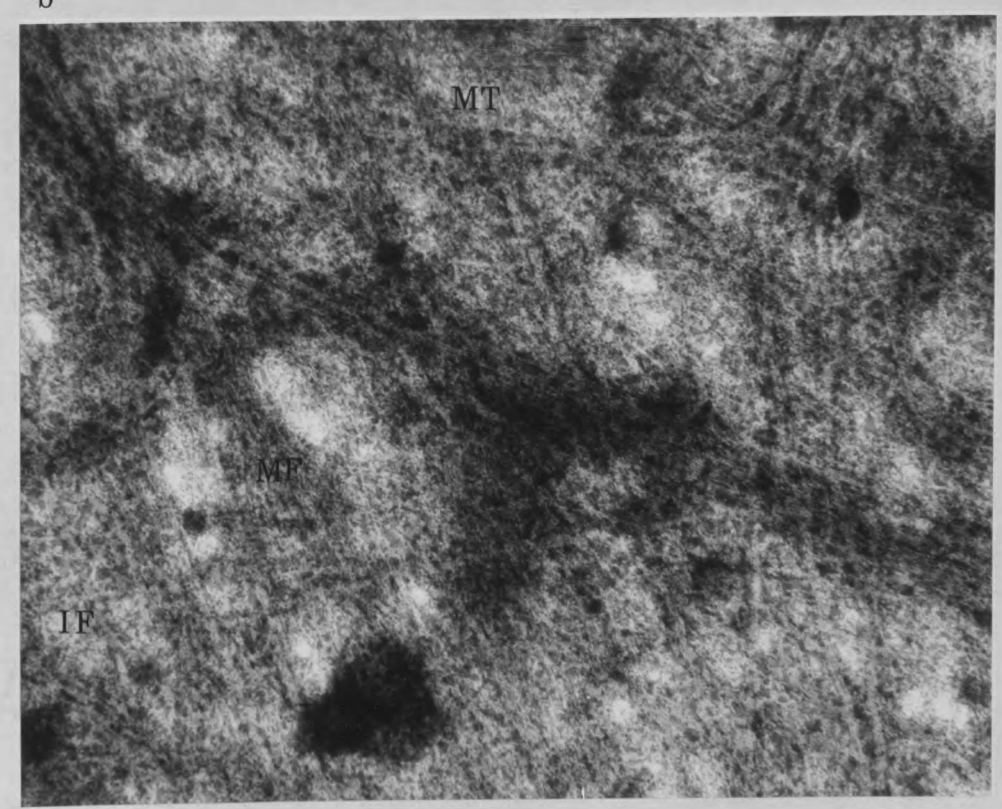


PHOTOGRAPH EIGHTEEN.

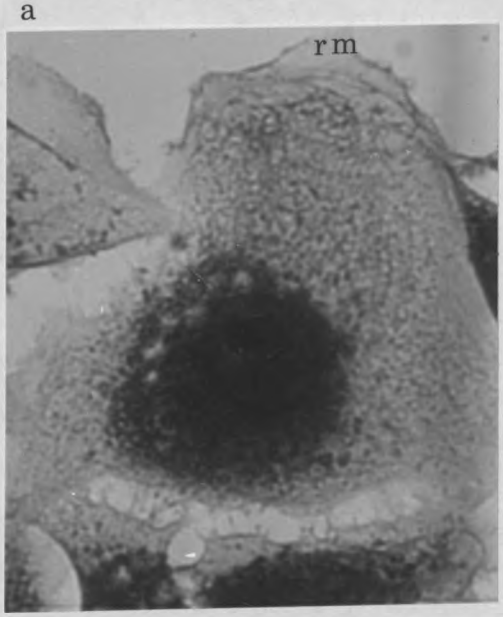
a). Cytoskeleton of a Salmon Cell, produced by Dry-Cleaving.
Mag.= x30,000 80kV

A number of Stress Fibres (SF) can be seen passing diagonally from the bottom left to top right. There are very few vesicles obscuring the few of the fibres. The fibres interconnect to form a complex meshwork. But the 3 individual fibre types of the cytoskeleton cannot be clearly distinguished. Fibre diameters appear to vary fairly evenly from c.5nm to c.35nm. Most of the fibres appear to be short (c.15nm) and interconnect to form the meshwork. The stress fibres (and associated 30nm diameters - possibly MT) are more or less straight, and pass diagonally across the whole micrograph.

b). Cytoskeleton of a Salmon Cell, Air-Dried and Negatively Stained with Sodium Tungstate.
Mag.= x50,000 80kV

20nm diameter fibres (MT) can be seen criss-crossing the micrograph. Aprox. 12nm diameter fibres (IF) are also seen. Both these 2 types of fibres are long and comparatively straight. Narrower fibres (c.4nm; MF) are also present as short straight fibres, forming a meshwork with each other and the other 2 fibre types.

a



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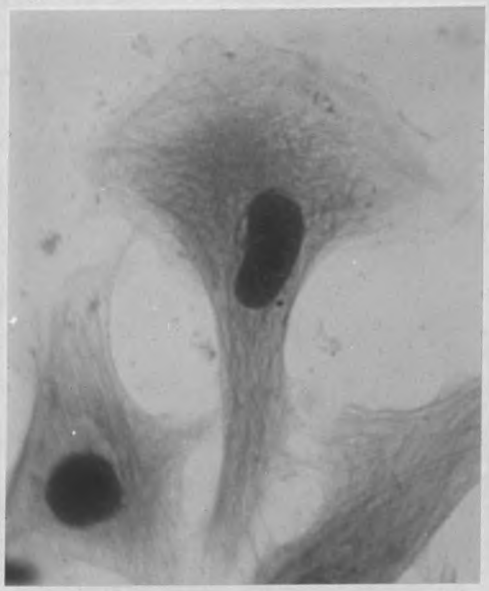
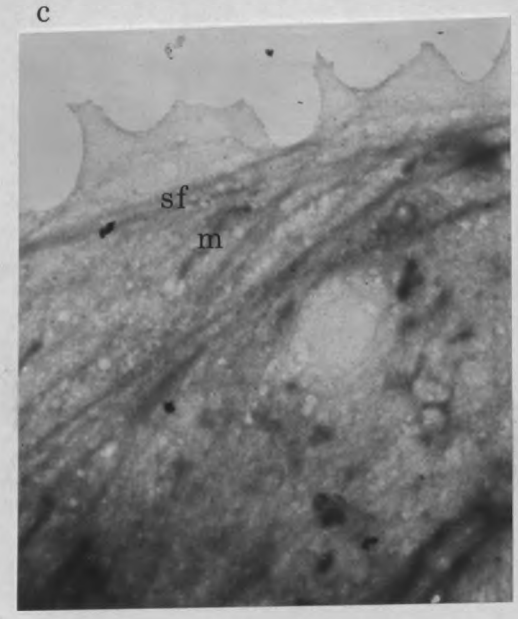


c



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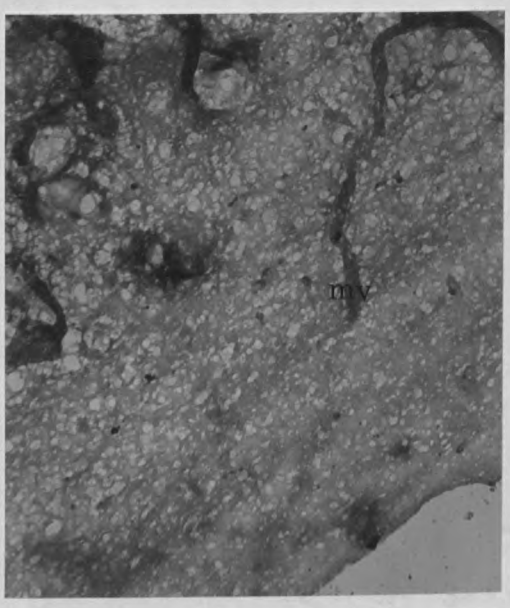


PHOTOGRAPH NINETEEN.

a). Light Micrograph of the Triton-Extracted, PAGE-Stained Cytoskeleton of XTC Cells.
Mag.= x1,600 80kV

This shows the XTC cell cytoskeleton to be a dense meshwork, without stress fibres. The meshwork becomes less dense near the periphery of Ruffled Membranes (rm) and is obscured around the nucleus by the large organelles.

b). Light Micrograph of the Triton-Extracted, PAGE-Stained Cytoskeleton of Salmon Cells.
Mag.= x700 80kV

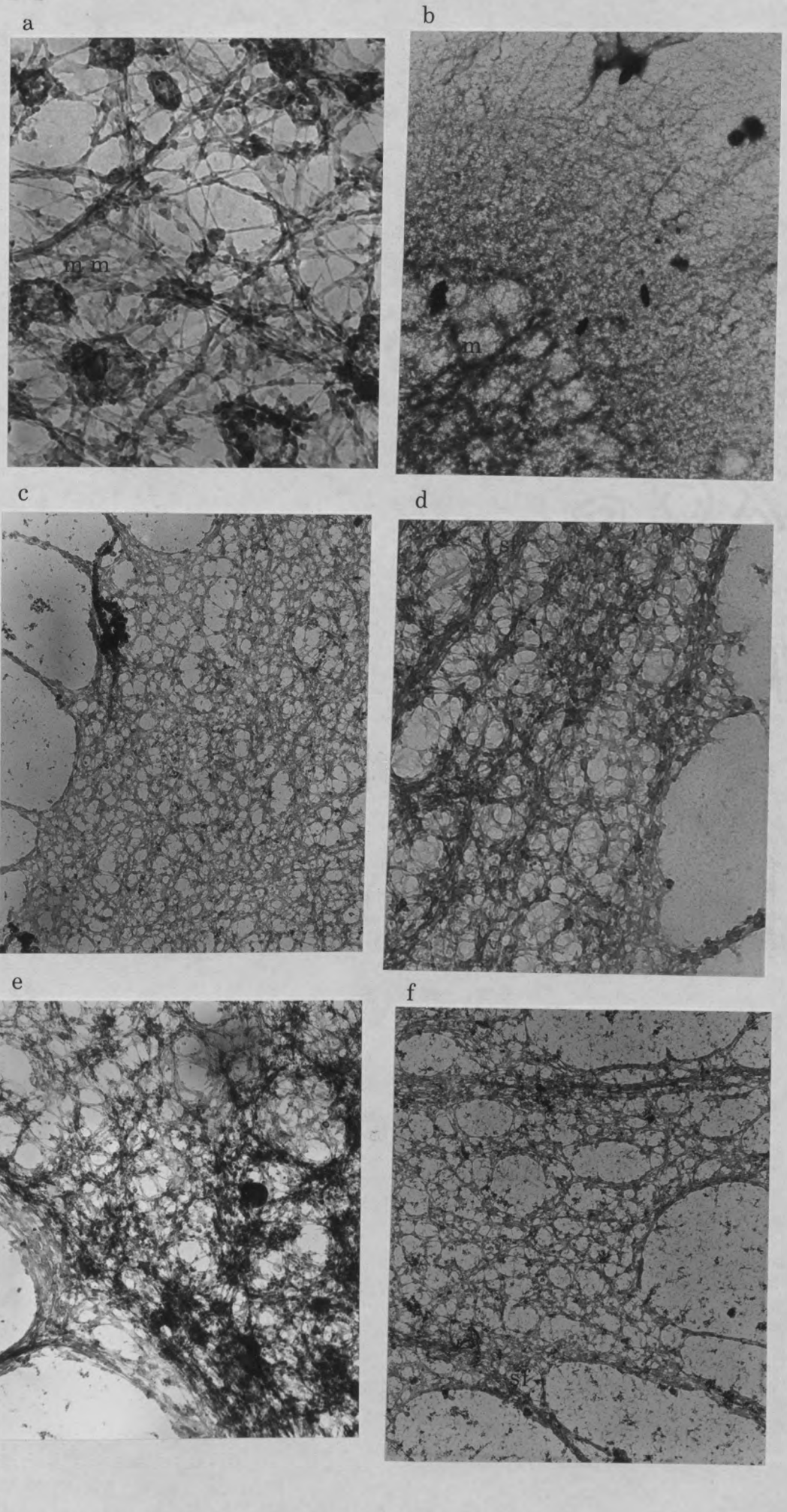
The salmon cell cytoskeleton is seen to be a meshwork of fibres, within which are clearly seen a number of stress fibres. These do not pass all the way across the cell, they intermingle with the rest of the meshwork before reaching the cell periphery. The meshwork is thinner at the ruffled membranes. The only large organelle seen is the nucleus.

c). Electron Micrograph of an Air-Dried Salmon Cell.
Mag.= x7,000 80kV

A number of stress fibres (sf) can be seen passing across the cell. Also, a number of mitochondria (m) are visible. The cytoplasm itself has a fine reticulate appearance, getting denser as one passes into the cell.

d). Electron Micrograph of a Glycerol-Extracted XTC Cell.
Mag.= x12,000 100kV

Microvilli (mv) are seen on the upper cell surface. The general cytoplasm has a reticulate appearance, but of coarser "grain" to a normal cell (see Photo 19c).


a
m m
b
m
c
d
e
f

PHOTOGRAPH TWENTY.

a). Glycerol- and Triton-Extracted XTC Cell (in the presence of Brain Explants).
Mag.= x100,000 80kV

The 3 fibre systems of the cytoskeleton can be distinguished and are associated with various vesicles, as normal. The only novel feature is that some form of membranous material (mm) appears to be coating many of the fibres.

b). Glycerol- and Triton-Extracted Salmon Cell.
Mag.= x6,000 100kV

The cytoplasm has a reticulate appearance, but fine stress fibres can be seen extending to the cell periphery. Mitochondria (m) are seen at the centre of the cell.

c). Glycerol- and Triton-Extracted XTC Cell, exposed to ATP, (in the presence of Brain Extract).
Mag.= x24,000 80kV

The cell has contracted somewhat, leaving retraction fibres connected to its previous position. The cytoplasm has the appearance of a meshwork of fibres.

d). Glycerol- and Triton-Extracted Salmon Cell, exposed to ATP.
Mag.= x35,000 100kV

Stress fibres (sf) are clearly visible, the rest of the cytoskeleton forms a meshwork interconnecting them. The cell has contracted slightly, and a retraction fibre can be seen to the lower right.

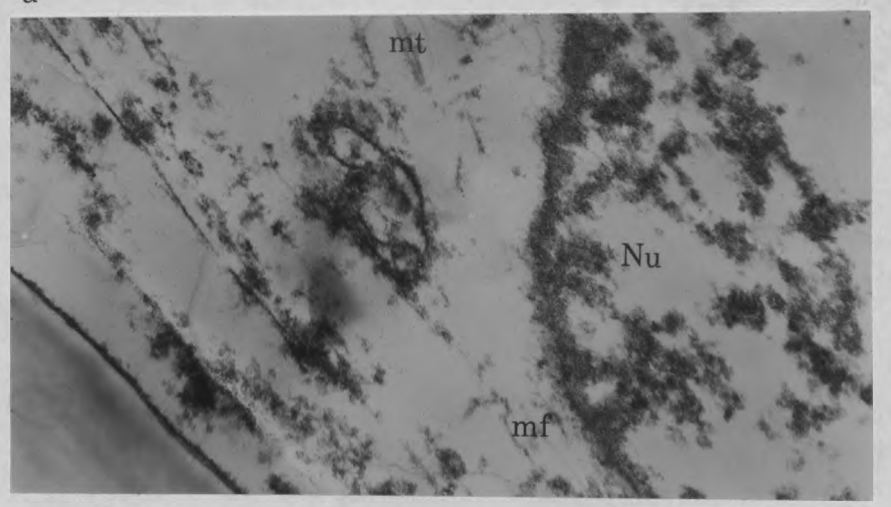
e). Glycerol-Extracted XTC Cell, exposed to ATP.
Mag.= x25,000 80kV

The cytoskeleton consists of a meshwork of fibres of varying diameter. One retraction fibre is partly visible, the cell having contracted somewhat.

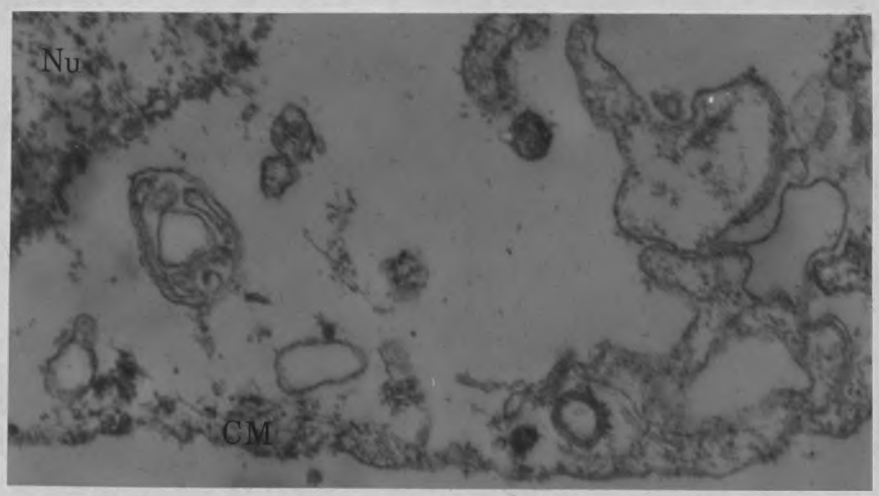
f). Glycerol-Extracted Salmon Cell, exposed to ATP,
Mag.= x18,000 80kV

This shows the periphery of the cell. The cytoskeleton is seen as a meshwork of fibres of varying diameter. Stress fibres (sf) are clearly seen, and they pass into retraction fibres (the cells having contracted somewhat).

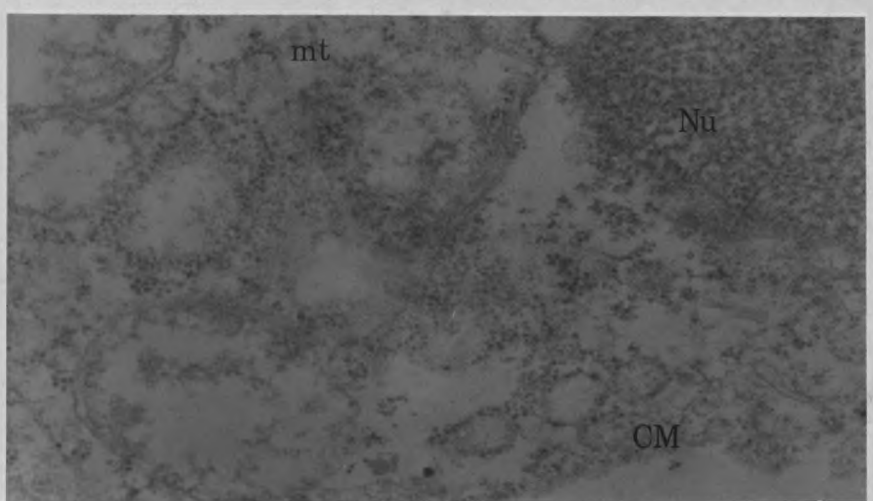
a



b



c



PHOTOGRAPH TWENTY-ONE.

a). Electron Micrograph of a Triton-Extracted XTC Cell.
Mag.= x50,000 Glutaraldehyde Fixation Only.
Araldite Embedded Material.
Uranyl Acetate and Lead Citrate Stain.

Triton treatment can be seen to remove most of the cell contents, including all membranes. But the outlines of the cell, nucleus and various organelles are still clearly seen. Also, MT and MF are visible.

b). Electron Micrograph of a Glycerol-Extracted XTC Cell.
Mag.= x50,000 Glutaraldehyde Fixation Only.
Araldite Embedded Material.
Uranyl Acetate and Lead Citrate Stain.

Most of the cellular contents have been removed. Both the nuclear and cell membranes (CM) have been partially removed, but other membranes appear to be more or less intact. Parts of the cytoskeleton appear to be associated with the remnants of the cell membrane.

c). Electron Micrograph of a Glycerol- and Triton-Extracted XTC Cell.
Mag.= x50,000 Glutaraldehyde Fixation Only.
Araldite Embedded Material.
Uranyl Acetate and Lead Citrate Stain.

Most membranes have been removed, but cellular contents are only partially removed. Parts of the cytoskeleton (e.g. MT) are seen, especially associated with the remnants of the cell membrane (CM).

DISCUSSION

In brief, the results seem to show that the nephric system develops by the aggregation of cells within which a lumen forms, rather than by the folding of an epithelial sheet. The induction experiments were very disappointing. Although the pronephric explants showed that tubule formation is possible under the culture conditions used, the mesonephric explants never showed any tubule formation. Only the result from the one surviving tadpole indicates that the nephric duct is an inductor of mesonephric tubule formation. The same "inductors" produced no changes in the XTC cell behaviour or structure. Their overgrowth and ICC formation can probably be explained by their continuing to divide past confluency.

These results will now be discussed in detail, The work on cytoskeletons and the electron microscopy work on nephric system development (points 2/ and 4/ in the list in the Introduction) gave the most complete results. They will be discussed seperately, before being combined with the work on the cell lines in a discussion of Models of Morphogenesis.

CYTOSKELETONS.

First, one needs a brief review of published work on whole-mount TEM techniques.

Light microscope techniques reveal the pattern of cytoskeletal elements in a whole cell. For example, Osborn

et. al. (1978) used immunofluorescent microscopy and were able to produce stereo pairs showing the 3-dimensional nature of the MF, MT and tonofilament bundles. They found straight, parallel arrays of MF bundles passing across the cell in various direction (also seen in the salmon cells, Photo. 19b), and actin "nets" around the nucleus and below the cell membrane. MT and tonofilaments were usually smoothly curved and interconnected to form complex webs, but surround the nucleus in a ring. Microinjection of fluorescent labels can also allow the examination of living cells and is therefore useful in examining the lability of the cytoskeleton (reviewed in Kreis and Birchmeier, 1982). Webster et. al. (1978) compared immunofluorescent microscopy with the whole-mount TEM technique. They found agreement as to the overall pattern of the cytoskeleton, but TEM gave far greater detail.

However, the detailed interpretation of the results obtained by the various TEM techniques is controversial at the moment. As my results have some bearing on this controversy, it is necessary to go into it in some detail.

Both Buckley (1975) and Wolosewick and Porter (1976) looked at whole cells without triton-extraction. They used glutaraldehyde and osmium tetroxide fixation before dehydration and critical point drying. The latter authors used a high voltage TEM (1,000kV). However, both authors obtained a meshwork system. To quote Wolosewick and Porter (1976) directly:- "At high magnifications a continuity of MT, MF, and membranous elements with thin (3-6nm) strands of the ground substance has been observed. These strands form a 3-D lattice or mesh that pervades all parts of the cytoplasm. The entire structure is referred to as a Microtrabecular

Lattice or Mesh, the strands being the trabeculae. The inclusion of MT, MF, ribosomes and vesicles of the endoplasmic reticulum within the material of the lattice makes them all part of a totally organised cytoplast."

Thus, they present the view that the microtrabeculae form a 4th filament system of the cytoskeleton. However, the possibility exists that the lattice is artifactual and this is the source of the controversy.

Wolosewick and Porter (1979) specifically looked at this problem. They compared various fixatives, including freezing. 2% glutaraldehyde (or 2% paraformaldehyde) in 0.1M sodium cacodylate and post-fixed in 1% osmium tetroxide (followed by 0.5% uranyl acetate staining and critical point dried) resulted in similar structures to those described above. Individual trabeculae varied greatly in length, reaching 200nm. Individual fibres also varied in diameter, from aprox. 10nm at their ends to aprox. 2-3nm at their midpoints. Stress fibres and MT were recognisable, as were various organelles such as endoplasmic reticulum and ribosomes. Osmium tetroxide as primary fixative yielded similar results, except that the trabeculae were less distinct and often terminated as free ends. One minute exposure to osmium tetroxide vapours gave similar results. But after 2hr exposure most of the trabeculae appear to have been removed. Freeze-drying (the specimen is frozen in liquid nitrogen, dried in a vacuum, warmed and coated) used as a fixative, or instead of critical point drying, resulted in a typical microtrabecular lattice, except that the trabeculae were 40-50% broader. Freeze-substitution (the specimen is frozen in liquid nitrogen then brought up to room temperature in 3 stages) also resulted in a

microtrabecular lattice. In this case, the filaments are more uniform in diameter. Lastly, they looked at sectioned material, both prepared conventionally and critical point dried. They found a great deal of "flocculent" material associated with the MT, MF, ribosomes, endoplasmic reticulum, etc. They equated this material with the microtrabecular lattice, concluding that any discrepancies between sectioned material and whole, critical point dried cells are due to the electron scattering properties of the embedding resin. To remove this problem Wolosewick (1980) used water-soluble Polyethylene Glycol as the medium. Thick (0.25μm) unstained sections, with the PEG removed, were examined. The ultrastructure appeared to be undamaged by the technique and filamentous structures were seen, which he interpreted to be trabeculae. (Certainly, the cytoskeleton survives, as Wolosewick and de Hay, 1982, were able to identify tubulin and actin immunologically.)

Schliwa et. al. (1981) also looked at the microtrabecular lattice. They found that treatment with 0.2% Brij 58 (a non-ionic detergent) before fixation resulted in the removal of the plasmalemma and some associated organelles, but left the microtrabecular lattice intact. If Brij-extracted cells were further extracted with triton X-100, many of the microtrabecular strands were dissolved leaving mainly the 3-fibre systems of the cytoskeleton. Two-dimensional polyacrylamide gel electrophoresis showed that many polypeptides were released by this triton-extraction. Thus, they conclude that the microtrabecular system is a "metastable association" of various proteins of the ground substance, probably associated with and stabilized by the cytoskeleton.

In support of this conclusion, Porter and Anderson (1980)

found consistent changes in the lattice to low temperature, different concentrations of Mg^{2+}, Ca^{2+} and to Cytochalasin. At 4'C, the lattice collapses into unstructured masses. Recovery at 37'C is rapid and involves the elongation of individual trabeculae from small spherical masses of lattice material. After 30min the former morphology is restored. Low Mg^{2+} concentration produces a meshwork in which large masses of lattice material are connected by fine trabeculae. 1mM Mg^{2+} produces a finely divided lattice. Incubation in 1-10mM Ca^{2+} at low temperature for 15 min, then 37'C for 1 min, produces a massive development of stress fibres. Cytochalasin produces a lattice of coarse strands, condensing at numerous points to form conical masses.

Thus, all these authors consider the microtrabecular lattice to actually exist <u>in vivo</u>. However, there is increasing evidence to the contrary. Ris (1981) seems to consider it to be a surface tension artifact due to residual water present in the CO_2 used in critical point drying. Taking care to prevent this, he obtains specimens with a meshwork of fibres of a more uniform diameter (5-10nm). Unfortunately, this is an abstract and no photographs were published. Bridgman and Reese (1982) also took precautions to avoid moisture. They used a glutaraldehyde-tannic acid fixative and/or "slam-freezing". In both cases, they obtained meshworks of fibres and granular material. The fibres could be identified as MT, MF, or IF. However, there were 4-5nm diameter fibres also present. Again, unfortunately, this is only an abstract. However, both these papers seem to indicate that any moisture present during critical point drying can result in a microtrabecular lattice.

Small (1981) notes evidence showing that osmium tetroxide

or organic solvents can disrupt actin filaments. (Certainly, purified actin filaments are destroyed by exposure to osmium tetroxide; Maupin-Szanaier and Pollard, 1978.) Thus, he used only glutaraldehyde fixation (with triton extraction), negative staining and air-drying. On examining the leading edge of the cells he found a highly organized, 3-dimensional network of actin filaments. MF bundles appeared to arise from the orderly coming together of individual MF or small, near parallel arrays of MF. More conventional techniques resulted in a disruption of this orderly arrangement, except that the MF bundles seemed unaffected. Thus, he concludes that the microtrabecular lattice is produced by the distortion of actin filaments. Certainly, Buckley et. al. (1981) were able to show that normal preparative procedures do produce close apposition of MF, which appear as IF-diameter fibres in whole mount preparations. Whilst, F-actin solutions examined by whole mount TEM resemble the microtrabecular lattice very closely (e.g. Condeelis, 1981).

Lehrer (1981) showed that glutaraldehyde could also damage pure actin filaments, when these showed extensive cross-linking. Tropomyosin protected against this damage. However, lysine used in combination with glutaraldehyde apparently preserves MF structure (Boyles, 1982), and tannic acid may have the same effect (Maupin and Pollard, 1983).

The conclusion seems to be that moisture present during critical point drying can affect the cytoplasmic matrix to produce a microtrabecular lattice. This possibly explains Schliwa et. al.'s (1981) results with Brij 58. Whilst osmium tetroxide or organic solvents can act on the MF in triton-extracted cells to produce a similar lattice. Normal

preparative procedures can suffer from both faults, especially if the initial triton-extraction is not very efficient. The consistent changes seen by Porter and Anderson (1980) thus become the distorted images of changes that actually occurred *in vivo*.

Where do my results fit into all this? I used a variety of techniques and comparing them might help in clarifying the controversy. The techniques used were:-

Whole cells and air-drying.

Whole cells and critical point drying.

Dry-cleaving.

Triton-extraction and critical point drying.

Triton-extraction and air-drying.

Both the whole cell techniques resulted in cells with a reticulate appearance (Photo 19c). The critical point dried cells may have a somewhat coarser "mesh". No precautions were taken to remove moisture during critical point drying, nor is overnight air-drying to be considered efficient. This suggests that any moisture present during critical point drying or when the specimen is introduced into the vacuum of the TEM will result in a "coagulation" of the cytoplasmic matrix. My results do not show distinctive filaments within a meshwork as reported by Ris (1981) and Bridgman and Reese (1982). The coagulation idea seems to fit better.

Dry-cleaving is the technique used by Mesland (e.g. Mesland Spiele and Los, 1981; Mesland, Los and Spiele, 1981). It literally tears the cell in half. Unfortunately, air-drying will not work as the cells presumably do not become dry and brittle. This method does give a view of the interior of the cell after critical point drying. The result is a microtrabe-

cular lattice. The fibres vary widely in length and vary in diameter along their length. There are a variety of vesicles and other organelles associated with the fibres. The 3 filament types of the cytoskeleton are not usually distinguished, though stress fibres (and sometimes MT) are seen in the salmon cells (Photo 18a).

Triton extraction removes most of the cytoplasmic matrix and cellular membranes (Photo 21a), leaving the cytoskeleton proper (Photo's 15-17). (Saponin, another non-ionic detergent, seems to have a somewhat weaker effect. Membranes of the cellular organelles may remain intact if a low enough concentration is used, less than 0.2%, for a short enough time, less than 2 min. This means that organelles, especially mitochondria, can be seen within the meshwork. This is in agreement with Heuser's, 1981, results.) The 3 filament types are usually clearly seen in the salmon cell preparations. However, with the XTC cells, a microtrabecular lattice-like appearance is often seen. This is probably an effect of the extraction medium used. Small and his co-workers (Isenberg and Small, 1978; Small and Celis, 1978; Small, 1981) use Ca^{2+}-free Hank's with added EGTA, Mg^{2+} and PIPES at pH 6.0. This is similar to the salmon cell extraction medium used here. However, a simpler medium was used with the XTC cells. This is presumably too simple a medium to produce optimum structural preservation. Although, Pudney and Singer (1979) used a similar medium and obtained results comparable with the salmon cell preparations. In fact, each set of authors have their own extraction media and there seems to be very few general rules for its make-up. Most do agree on the importance of removing Ca^{2+}. For example, Schliwa and van Blerkom (1981) used 10mM EGTA to achieve this, and do

not need Glycerol or PEG to stabilize MT. However, with the cells used here, such a concentration of EGTA removes most of the cell. Thus, a lower concentration of EDTA, plus glycerol or PEG was used.

Finally, the air-drying technique in my hands never gave useful results with the XTC cells. All these cells showed the microtrabecular lattice morphology. Again, this is probably due to the extraction medium used. Thus, the results are confined to the salmon cells. Negative staining reveals the MT and IF as long, straight or smoothly curved fibres (Photo 18b). This suggests that the "kinking" of these filaments seen in critical point dried specimens is an artifact, possibly associated with the distortion of the MF. This is the view of Small (1981). Unfortunately, in my hands, this technique did not give very good resolution of the MF. They appear as a faintly discerned irregular meshwork, interconnecting the other 2 filament types. Only MF bundles show at all clearly, and even here individual fibres are often difficult to distinguish. Small (1981) obtained very orderly systems of MF at the leading edge of his cells (the situation within the cell is too complex to be able to distinguish an orderly system). He interprets this as the normal situation. However, he used primary cultures of embryonic chick heart fibroblasts. Permanent cell lines are known to have distorted cytoskeletons (see Introduction). Thus, in previous papers (e.g. Small and Celis, 1978; Isenberg and Small, 1978) using similar techniques and permanent cell lines, no mention of an orderly arrangement was made. Nor did Edds (1977), working on coelomocytes of the sea urchin, find an orderly arrangement. Similarly, Heuser and Kirschner (1980), using their freeze-drying technique (see below), make

no specific reference to an orderly arrangement of MF at the cell periphery. Thus, it is possible that Small's (1981) result was due to the fact that he used a primary cell line.

In summary, therefore, it appears that moisture present during critical point drying or when the specimen is inserted into the TEM will result in a reticulate cytoplasm. Osmium tetroxide or organic solvents will distort the MF, resulting in kinking of the MT and IF. The extraction medium used with the triton may also affect the MF and the extent of matrix removal. Thus, all these factors can act together or seperately to produce a microtrabecular lattice. Optimum conditions of extraction should produce a clear view of the cytoskeleton, without kinking of MT and IF. This appears to have been achieved by Heuser and Kirschner's (1980) freeze-drying technique (see below) and to a lesser extent by Small's (1981) air-drying technique. (The latter does not allow the amazing high resolution achieved by the former.)

Bearing these points in mind, it is now necessary to examine the details of cytoskeletal structure obtained here. Only the triton-extraction procedures are relevant, as the others result solely in a microtrabecular lattice.

The PAGE staining technique reveals differences in the cytoskeletons of XTC and salmon cells. The salmon cells possess stress fibres, whilst the XTC cells do not (Photo's 19a,b). The XTC cell cytoskeleton is a dense meshwork, obscured near the centre of the cell by various organelles. TEM techniques allow high resolution examination of the cytoskeleton.

As already noted, triton-extraction removes most of the cytoplasmic matrix and the cellular membranes. This is in

agreement with Ben-Ze'ev *et. al.* (1979) and Lenk *et. al.* (1977). Many ribosomes are retained and are intimately associated with the cytoskeleton (seen as 20nm diameter particles). This association may be via mRNA (Lenk *et. al.*, 1977; Jeffery, 1982). It is known that mRNA is translated when associated with the cytoskeleton *in vivo* (Cervera *et. al.*, 1981). Whilst Traub and Nelson (1981) found a specific reaction between rRNA and the IF Vimentin. The shape of the nucleus is unaffected by the extraction; possibly supported by an internal protein "skeleton" as suggested by van Eekelen *et. al.* (1982). Finally, Ben-Ze'ev *et. al.* (1979) note that the remaining proteins associated with the cell membrane now form a lamina intimately associated with the cytoskeleton. Such a lamina seems to form only intermittently in my cells (Photo 21a) though it does appear to be present in glycerol-extracted cells (Photo 21b).

A number of similarities in the cytoskeletons of the 2 cell types are seen. This is especially true when most of the MF have been removed by KCl treatment. Both show complex interconnections between all 3 filament types. Filaments tend to be straight until they meet another filament at which point a change in direction may occur, these are called "branch points" in the legends to the photographs. (However, this change in direction may be an artifact of actin filament distortion, as noted above.) Schliwa and van Blerkom (1981) note similar "end-to-side" contacts in their detailed study of epithelial African green monkey kidney cells and chick embryo fibroblasts. (Associations between the 3 filament types are also indicated by immunofluorescent work, e.g. Daley and Eckert, 1981; Ball and Singer, 1981.) They specifically note such contacts between MT and MF (in which MT often change their direction)

and "Y-shaped" MF branches. Both types are also clearly seen in Photo 16. They can also be seen in the XTC cells, e.g. "Y-shaped" MF in Photo 17a. However, Schliwa and van Blerkom were also able to identify a new class of filament. These are 2-3nm in diameter, 30-300nm in length, and acted as linkers between the other filaments. They were distinguished from actin as they did not bind heavy meromyosin. Unfortunately, the heavy meromyosin technique was not used here, so I cannot confirm their result. But, Heuser and Kirschner (1980) using their technique of quick-freezing and deep-etching, obtained high resolution images of the cytoskeletons of mouse embryo fibroblasts and made no mention of any such fibres. Hirokawa (1982), using the same technique, found a cross-linker system of 4-6nm diameter fibres between neurofilaments, MT and membranous organelles in frog axons. However, he notes that these appear to be unique to axons. Similarly, a cross-linking system is found in the terminal web of the brush border of intestinal epithelial cells (Hirokawa and Heuser, 1981; Hirokawa et. al., 1982). But, again, this system appears to be unique to brush borders (with terminal webs of MF) and at least some of the cross-linkers appear to be myosin. Thus, it is possible that Schliwa and van Blerkom's 2-3nm diameter filaments are either remnants of the microtrabecular lattice, or actin filaments which have been damaged by the preparative procedures so as to no longer bind heavy meromyosin.

The suggestion has also been made that most, if not all, MT are associated with an MTOC (e.g. Solomon, 1980; Brinkley, Cox, Pepper, Wible, Brenner, and Pardue, 1981). A number of MTOC's may be found in some cells, so that they are not necessarily restricted to the centriole. Therefore, MTOC's

may control MT distribution. They do appear to have an organizing function in migrating endothelial cell sheets (Gotlieb et. al., 1981), in which 80% of the MTOC's are positioned in front of the nucleus, toward the direction of movement. However, my whole mount studies did not reveal any such structures and MT were not seen to connect to any centres. So, either my extraction media caused MTOC decay and/or fragmentation of MT, or the suggestion does not apply to all cells. The former is rendered less likely, however, as Schliwa et. al. (1982) did observe many MT centring on the centrosome in neutrophils in their TEM investigation of the cytoskeleton. Eckert et. al. (1982) suggests a similar sort of organizing centre for IF, this time associated with the nucleus. Again, my results do not support this, but the situation is too complex to be sure.

Therefore, triton-extraction and critical point drying can produce specimens in which the cytoskeleton is clearly seen, although the MF will be distorted by the critical point drying. A technique which seems to avoid any distortion and produces very high resolution images is quick-freezing and deep-etching, used by Heuser's group (e.g. Heuser and Kirschner, 1980). Extraction is as normal, but the cells are then frozen in liquid helium, then fractured and a replica made. This technique has been used on cultured cells (e.g. Gulley and Reese, 1981), axons (Hirokawa, 1982), hair cells of the chick ear (Hirokawa and Tilney, 1982) and a variety of other tissues. The results agree with most of the above conclusions, except that kinking of filaments is not prominent. Unfortunately, this technique has not been used on a developmental sequence, nor have specific staining procedures been tried to attempt to discover the

nature of interfilament connections or organelle-filament interactions (surely the next important extensions for this technique).

Triton-extraction can give a clear view of the cytoskeleton. But cytoskeletal-associated proteins are important for the cells, as indicated by glycerol-extraction. Untreated living cells or triton-extracted cells show no reaction if incubated in an ATP-containing medium. However, glycerol-extracted cells (whether or not they are further extracted) will contract in such a medium, though they will not then return to normal. (This is a technique apparently first used on muscle by Szent-Gyorgyi, and in non-muscle cells by Hoffman-Barling; original references in Allen and Allen, 1978; Taylor and Condeelis, 1979.) The cells used here contracted only slightly (adjustments to the ATP medium might have allowed slightly greater contraction); producing a scalloped periphery presumably because the cell periphery is more strongly attached to the substrate at a few points. Interestingly enough, the salmon cell stress fibres are usually associated with these points (e.g. Photo 20f). The results of glycerol-extraction and ATP-treatment are more impressive in other systems. For example, retinal pigmented epithelium will form "cups" due to the contraction of the apical circumferential MF bundles and the strong attachment between cells (Owaribe et. al., 1981). Whilst retinal cone cells elongate (e.g. Burnside et. al., 1982). (The contraction of brush border microvilli has been shown to be due to solvation of the core by high Ca^{2+} concentration. However, the brush border terminal web will contract; see Harris, 1983, for the original references.) Weber et. al. (1976) have shown that cytochalasin B-treated glycerinated

models can still contract. Cytochalasin B causes the rapid disorganization of MF into "star-like" patches in ordinary cells (see also Schliwa, 1982). It has no such effect on glycerinated models. My results show that cytoskeletal-matrix material remains in glycerinated cells, even after triton-extraction. It seems likely that this material includes the necessary elements of the contractile machinary in a state in which ATP and Ca^{2+} solutions can cause contraction. This almost certainly includes myosin, since this is found in other glycerinated systems (reviewed by Taylor and Condeelis, 1979), in which actin is cross-linked to myosin.

Having now discussed my results in relation to cytoskeletal structure, it is now time to move to the attempt to induce organised changes. There is some published work on cytoskeletal changes observed by the TEM technique. For example, Peng et. al. (1981) studied Xenopus myotome cells in culture, as they developed myofibrils. It seems as if MF bundles form the precursors to the myofibrils. Whilst Fulton et. al. (1981) looked at fusing chick myoblasts. They found that the "surface lamina" (see above) developed lacunae as the myoblasts prepared to fuse, but disappeared after the multinucleated myotube was formed. An associated reorganization of the cytoskeleton also occurs. Westermark and Porter (1982) show that treatment of a human thyroid cell line with the hormone thyrotropin or dibutyryl cAMP causes the loss of stress fibres and distinct changes in the microtrabecular lattice. (A similar loss is seen by immunofluorescent methods, Tramontano et. al., 1982.) The cells returned to normal after 24hr of treatment, i.e. in the presence of thyrotropin. Porter and McNiven (1982) observed changes in the microtrabecular lattice associated

with the aggregation and dispersion of pigment in erythropores. Finally, Stearns (1982) found changes in the microtrabecular lattice associated with organelle motion in axons.

It was hoped to elicit similar changes in the XTC cells by using collagen, collagen gels and/or "inductors", and hopefully associated changes in the contractile behaviour of glycerol-extracted cells. Unfortunately, no such changes were seen. (Failure to induce any changes in the XTC cells is dealt with in the last section of this Discussion.)

The negative results in this part of the work means that the cytoskeletal work tends to form a seperate section from the rest of the project. However, some of the conclusions from this work will be used in the discussion of Models of Morphogenesis.

In summary, the cytoskeletal work was started to give a clear view of cytoskeletal changes on "induction". A variety of techniques were looked at to see which gave the best resolution. This part of the work gave some interesting results. For example, the results are consistent with the view that the microtrabecular lattice is an artifact; the 3 filament types are intimately interconnected and appear to be associated with a variety of organelles (this suggests that there can be no clear seperation in the functions of the 3 filament types); and the regulation of structure associated with the idea of Organizing Centres is not supported as no trace of an MTOC was found. The part of the work involving "induction" produced negative results.

THE EARLY DEVELOPMENT OF THE NEPHRIC SYSTEM IN *X. LAEVIS*.

There are certain similarities in the early development

and differentiation of all three areas of the Xenopus nephric system. All start out as an accumulation of irregularly shaped mesoderm cells (admittedly, the mesonephric cells have to migrate to their area of differentiation). These cells possess no obvious ultrastructural specialisations; no specialised cell junctions, no basement membrane, nor indeed any excessive amounts of ECM. (On the latter point, however, the fixation and staining techniques used may not have stained all the ECM present; Szollosi, 1967. For example, both Luft, 1971, and Hay, 1978, used Ruthenium Red as a fixative and stain, which greatly enhances ECM contrast. Whilst Cato and Herker, 1983, found that ruthenium red tended to stain proteoglycans in the basement membrane, and tannic acid stained glycoproteins.) The cells then go on to arrange themselves radially; specialised cell junctions and basement membranes form. A lumen forms and associated structures appear, e.g. a brush border or cilia. Differences between the three areas are now apparent. But, in general one can say that all three areas begin to form before any obvious ultrastructural specialisations appear.

Strangely, there seems to be no published work on the development of amphibian nephric system at the TEM level. However, descriptions at the light microscope level seem to fit the pattern described above, (e.g. Jaffee, 1954, on the pronephros of Rana pipiens; Gray, 1930, 1932, and 1936, on the mesonephros of R. temporaria and Triton vulgaris).

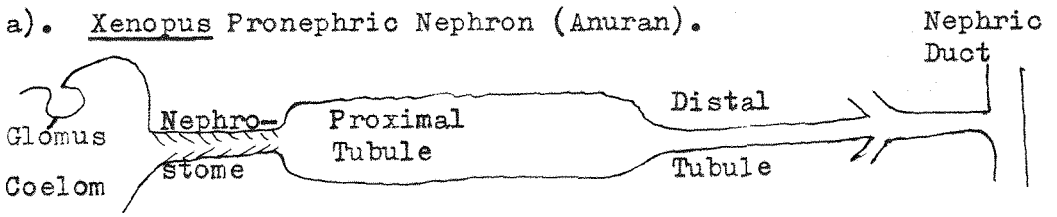
Figure 13 compares urodele and anuran pronephric nephrons, and compares both with the mesonephric nephron. There are three major differences between the two pronephri:- 1/. Xenopus has 3 nephrons (Ambystoma has 2 nephrostomes, but the 2 tubules soon fuse); 2/. The 3 Xenopus nephrons all open into

FIGURE 13

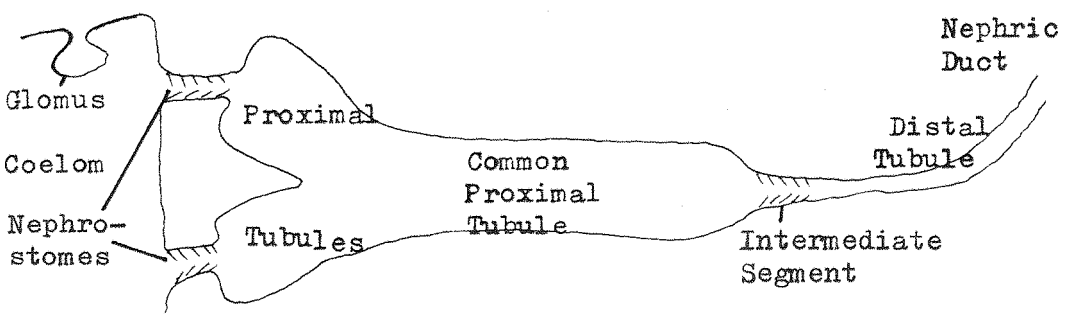
Diagrammatic Representation of the Xenopus Pronephric Nephron Compared with the Ambystoma (Salamander) Pronephric and Mesonephric Nephrons.

(13b and c are taken from Christensen, 1964.)

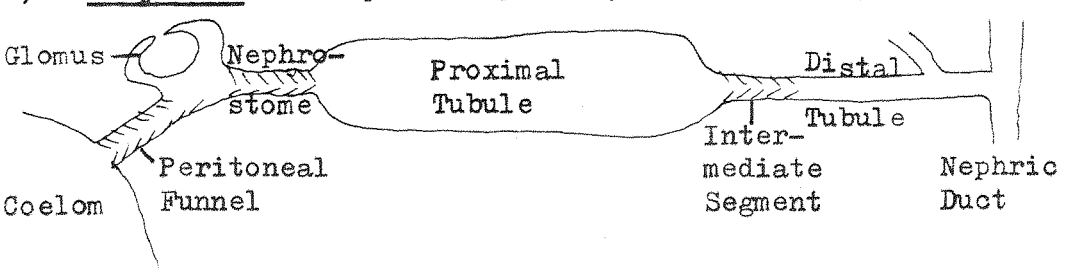
a). Xenopus Pronephric Nephron (Anuran).



b). Ambystoma Pronephric Nephron (Urodele).



c). Ambystoma Mesonephric Nephron (Closed Nephron, see text).



There is a basic similarity between all 3 nephrons, which goes down to the ultrastructural level, see text.

Note the Peritoneal Funnel in c), and compare with Fig. 11. The modern definitions are:- Nephrostome connects the nephrotome with the tubule. The Peritoneal Funnel connects the nephrotome with the coelom (Frazer, 1950). Compare with Balinsky's view summarised in the Introduction.

a collecting duct (the Ambystoma nephron opens directly into the nephric duct); 3/. Neither Nieuwkoop and Faber (1967) nor Fox and Hamilton (1971) mention a ciliated intermediate segment in Xenopus, although the latter do distinguish between proximal and distal tubules. However, Fox (1970) did find a ciliated intermediate segment in the anuran Rana temporaria, thus careful serial sectioning may find one in Xenopus. Fox notes that the ciliated intermediate segment has a similar morphology to the nephrostomes (see below).

Both pronephri have tubules of the Open Type, i.e. they open directly to the peritoneal cavity via a nephrostome. The mesonephros (and metanephros) have Closed Nephrons, opening to the coelom via Peritoneal Funnels (see Gerard and Cordier, 1934a,b).

In general, "in Anura, pronephros and duct rudiments are recognised below somites 3, 4 and 5 and are more condensed than in urodeles" (Fox, 1963). Whilst, "the pronephros arises as a continuous thickening of the somatic mesoderm on a level with . . . the 3rd and 4th somites in Urodela. From this the tubules are differentiated, whilst the duct develops immediately behind this level (somites 5-9) as a thickening of the somatic mesoderm serially equivalent to that of the pronephric region" (Fraser, 1950). Xenopus laevis is an Anuran, a member of the frog family Pipidae (Deuchar, 1972) and fits this scheme perfectly. The mesonephros develops from cords derived from the lateral mesoderm cells which migrate to lie beside the nephric duct (reviewed in Fraser, 1950, and Noble, 1931).

In phylogenetic terms, a large number of pronephric tubules appears to be the primitive state (Fox, 1962, 1963).

X. laevis has 3 nephrostomes with their associated tubules, as is typical for most Anura (Fox, 1963). But the 1st and 2nd nephrostomes fuse at stage 60, as the pronephros degenerates (Fox, 1962; Nieuwkoop and Faber, 1967).

There is some published TEM work on fully developed parts of the amphibian nephric system. These reveal that the similarities obvious from Fig. 13 extend to the ultrastructural level. Table 8 makes the similarities obvious. There are a number of points to note from this table:-

1/. Homologous regions in different species all have the same basic ultrastructure.

2/. Analogous regions of the pronephros and mesonephros are very similar, e.g. proximal tubules of both kidneys possess brush borders, whilst the distal tubules have only a few short microvilli.

3/. All areas possess a basement membrane, most seem to possess junctional complexes (or at least tight junctions). Many areas seem to possess a "canal system". Unfortunately, not all the authors shown in the Table mention all the features listed in the Table, nor do many of them include suitable electron micrographs.

a). Most areas of the mesonephros seem to possess either junctional complexes or tight junctions. However, this is one of the features the various authors tend not to mention. Whilst my brief review is not sufficient to be certain.

b). Where junctional complexes do develop, they are first seen as simple tight junctions.

c). The exact morphology of the "canal systems" is different in each region.

d). Presumably, the basement membranes of each region

TABLE 8.

A Comparison of the Ultrastructure of the Various Regions of the Nephric System in Various Amphibia (see Text for details).

BM= Basement Membrane. C= Cilia. MV= Microvilli.
BB= Brush Border. JT= Junction Type. TJ= Tight Junction.
JC= Junctional Complex. SF= Surface Features.
na= Not available (i.e. not discussed by the reference).

Organ	Region	Species	BM	SF	JT	Notes	Ref.
Pronephros	Nephrostome	X. laevis (Anura; Stage 32)	✓	C	TJ	Lipid + Yolk	This Report
	"	"	✓	C	JC	-	Fox + Hamilton (1971)
	" (+ Intermediate Segment)	Rana temporaria (Anura)	✓	C MV	JC	-	Fox (1970)
	"	Ambystoma (Urodele; equivalent to Xenopus stage 45)	✓	C MV	JC	Lipid + Melanin	Christensen (1964)
	Proximal Tubule	X. laevis (Anura; Stage 35/36)	✓	BB	JC	Lipid + Yolk	This Report
	"	" (stage 47)	✓	BB	JC	Canal System	Fox + Hamilton (1971)
	"	R. temporaria (Anura)	✓	BB	JC	-	Fox (1970)
	"	Ambystoma (Urodele; as above)	✓	BB	JC	-	Christensen (1964)
	Distal Tubule	R. temporaria (Anura)	✓	MV	JC	Canal System	Fox (1970)
	"	Ambystoma (Urodele; as above)	✓	MV	JC	"	Christensen (1964)

TABLE 8. (Contd.)

A Comparison of the Ultrastructure of the Various Regions of the Nephric System in Various Amphibia (see Text for details).

Organ	Region	Species	BM	SF	JT	Notes	Ref.
Nephric Duct	-	X. laevis (Anura; stage 40)	✓	MV	JC	Lipid	This Report
		" (stage 47)	✓	MV	JC	Canal System	Fox + Hamilton (1971); This Report
		Ambystoma (Urodele; as above)	✓	MV	JC	-	Christensen (1964)
Mesonephros	Proximal Tubule	R. pipiens (Anura; adult)	✓	BB	na	Canal System	Barch et. al. (1966)
	"	A. tigrinum (Urodele; adult)	✓	BB	na	"	Hinton et. al. (1982)
	Intermediate Segment	R. pipiens (Anura; adult)	✓	BB	na	No Canal System	Barch et. al.
	"	A. tigrinum (Anura; adult)	✓	BB	na	-	Hinton et. al. (1982)
	Distal Tubule	R. pipiens (Anura; adult)	✓	MV	na	Canal System	Barch et. al. (1966)
	"	A. tigrinum	✓	MV	na	"	Hinton et. al. (1982)
	Nephrostomes	R. pipiens (Anura; adult)	✓	C or BB	na	-	Barch et. al. (1966)
		A. tigrinum (As above)	✓	"	na	-	Hinton et. al. (1982)
	Collecting Tubule	X. laevis (Adult)	✓	MV	JC	-	Brown (1980)

differ in various ways (e.g. immunologically) but no ultra-structural differences are apparent.

e). The junctional complexes and intercellular regions all seem to fold irregularly late in development.

f). The number of mitochondria seems to increase during development, and become associated with the "canal system" at the basal ends of the cells.

g). Finally, there are regional differences in the mesonephric tubules (e.g. see Hinton et. al., 1982). Thus, the Table present a brief survey at best.

This ultrastructural similarity also seems to include the metanephros (e.g. Hinton et. al., 1982), with analogous regions showing analogous specialisations (e.g. Rhodin, 1958). This similarity is probably due to their similarity of function and development. In general, they all seem to function by fluid diffusing across the glomerular membrane and passing into the tubules. There, water and essential substances are reabsorbed, and only waste is excreted. The different regions of the kidneys presumably serve different functions. However, no details of this are known for amphibia. Amphibian larvae are known to be Ammontelic (i.e. excrete ammonia) whereas adults are Ureotelic (i.e. excrete urea). However, Xenopus remains ammontelic throughout life. But what these changes involve morphologically and physiologically is not known. (Deyrup, 1964, notes work on X. laevis which suggests that its tubules do not actively reabsorb water. Whilst, Dauca et. al., 1982, found differences in catalase activity - an enzyme involved in nitrogen excretion - between Xenopus and Rana kidneys and liver.) Finally, the mammalian metanephros possesses a loop of Henlé (not found in Amphibia; Noble, 1931) and are highly

tuned by the hormonal system, such that a urine hypertonic to the blood can be produced. Amphibian urine is usually hypotonic or isotonic. However, in amphibia the skin is also a major site of water and electrolyte exchange.

Now to turn to the inductive influences on development, starting with the pronephros. The pronephric explants were taken at late neurula to early tailbud stages. 40% of these showed tubule formation, which suggests that the pronephric primordium is determined by late neurula. (The fact that only 40% of the pronephric explants formed tubules as opposed to c.80% of the body slices is probably due in great part to the greater damage caused in producing the explants.) This conclusion agrees with earlier work summarised in Burns (1955), Fraser (1950) and Holtfreter and Hamburger (1955), which involved vital staining, transplants and explants. The transplant experiments led to the idea of a "Nephric Field". This area seems to stretch from the gill area to the caudal area, just below the somites. The anterior region of this field will tend to elicit pronephric tubule formation in responsive mesenchyme, whilst the posterior region will elicit mesonephric tubule formation. This hypothesis will have to be altered somewhat after discussion of mesonephric and metanephric induction.

The pronephric explants also show that the culture conditions used will allow tubule formation. However, the mesonephric explant experiments were very disappointing, as not even the body slices showed tubule formation. Both the explants and body slices developed swellings, which probably helped to cause the distortion in the tissue distributions seen in the body slices. However, such swellings also occurred in the

pronephric material, yet tubules still developed. (This swelling appears to be due to fluid intake by the explant, presumably through the ectoderm, and is not removed as no functional nephric system exists. Holtfreter, 1944, reports similar explant swelling and gives the same explanation. It can also occur in embryos - at least in Anura - if kidney function is blocked; Swingle, 1919; Fox and Hamilton, 1964.) But it is possible that the mesonephric cells are more sensitive than the pronephric cells. Or their migration to the nephric ducts (present in some of the explants and body slices) might be blocked. Or, finally, mesonephric differentiation in X. laevis may be solely dependent on the nephric duct, and this seems to degenerate in the explants and body slices. (Fox, 1956, also found nephric duct degeneration in Triturus tadpoles, after unilateral pronephectomy. Thus, its structure may only be maintained if it continues to function.) The only positive piece of evidence is the absence of a mesonephric cord in the single surviving operated embryo. This is far too weak to base any conclusions on, but it gives a provisional indication that the nephric duct is an inductor in vivo.

The early work on mesonephric differentiation is discussed in Burns (1955), Fox (1963), and Fraser (1950). The results varied greatly, even within the same individual, from the absence of tubules on duct excision to near normal development. The general conclusion that the three above authors seem to reach is that the mesonephric blastema has a variable power of differentiation, though the nephric duct is usually essential at some point. A possible explanation for some of the inconsistencies is Nieuwkoop's (1948) finding that only a very few duct cells are required to promote mesonephric

differentiation. This suggests that some of the experiments may not have involved complete removal of the duct. (This was not a problem in my case, since the pronephric blastema was removed so early, no nephric duct formed, see below.)

In higher vertebrates, however, the duct does seem to be necessary for full mesonephric development, e.g. in the chick (Boyden, 1927; Gruenwald, 1942; other references in Fraser, 1950).

Little work has been done on the effects of heterologous inductors on mesonephric development. Spinal cord can be used in Rana fusca (see Fox, 1963, for the original reference) and the chick mesonephros (Gruenwald, 1942). But most of the work has been done by Etheridge (1969; see the Introduction), and he found that spinal cord was a suppressor. He found that a combination of endoderm and notochord was almost as effective an inductor as nephric duct. However, even his controls (mesonephric mesenchyme in ectoderm) showed an irregular fluctuation of 20-50% tubule formation. But it is difficult to properly assess his results since no other extensive work has been done with amphibians.

Finally, early work on the salamander showed 90% of mesonephric transplants (as opposed to explants) from the tailbud stage showed tubule formation (reported in Burns, 1955). This was taken to mean that the mesonephric primordium is committed by late tailbud stage. Yet, the body slices from such stages (32 onwards) did not show tubule formation. Presumably, either something was wrong with the culture conditions used here, or commitment occurs later in X. laevis.

If one assumes that nephric duct is the normal inductor, micrographs such as Photo 3a, show that the mesonephric cord

develops at some distance from the nephric duct. Very few cells of the cord were ever seen to touch the basement membrane of the duct, none ever passed through. Thus, if a "message" exists, it must either pass through the ECM or be transmitted between mesonephric cells.

Comparing the mesonephros and the mammalian metanephros, one can say that the situation in the former is "messier". Mesonephric development is not rigidly dependent on the nephric duct as is the case with the metanephros. It is possible that the inductive interaction involved in the former may vary from long-range (ECM) to short-range (although it is of the Permissive type and may only require a short initial period to set all the changes in motion). It also seems possible that the effects of any heterologous inductors may vary with the species.

Finally, to turn to the nephric duct, my results show that unilateral pronephectomy result in the absence of the nephric duct on the operated side. (It is also interesting to note that the missing pronephros is not regenerated by the surrounding mesodermal cells.) This suggests that the duct is formed from or induced by the pronephric primordium. This conclusion is supported by earlier work on Triton and Ambystoma, summarised in Burns (1955). However, Holtfreter (1944) was able to produce nephric ducts in the absence of a pronephros. He used Ambystoma and Triton neurulae, bissecting them at the level of somite 6 or 7. Cuts any further back resulted in no nephric duct development. The answer to this apparent inconsistency is that the pronephric and nephric duct rudiments develop side by side. Excision experiments probably remove or at least damage both. Holtfreter bypassed this problem by using an early embryo and bissecting at various levels.

There are two views on the mechanism of nephric duct caudal extension.

1/. Outgrowth from the pronephric rudiment.

2/. *In situ* formation.

The early work is summarised in Burns (1955), Fox (1963), and Fraser (1950). These experiments involved duct blockage, bisection, or dorsal incisions. Fraser (1950) concludes that in Anura the duct forms *in situ* from the somatic mesoderm, whilst the situation in urodeles is less clear. (In both cases, the duct tip is important as its destruction stops further duct development, whilst mid-section damage regenerates. Whilst Bijtel, 1948, found that a developing nephric duct will grow towards an implanted proctodaeum in the urodeles *Triton* and *Ambystoma*.) My own work on the anuran *X. laevis* seems to support this general conclusion. But to make it clear why this is so, it is necessary to review the recent work by Poole and Steinberg.

Poole and Steinberg (1977, 1981, and 1982; Steinberg and Poole, 1981 and 1982) used the SEM and transplant techniques to study the development of the urodele *Ambystoma*'s nephric duct. They found that this extends caudally along the ventro-lateral border of the somites. There is a marked thinning of the duct and a reduction in cell number across the duct's diameter during its development. Vital dyes applied to the duct move caudally and elongate with the duct. They suggest that the duct develops by cell rearrangement, suggesting that the tissues can flow in the manner of a viscous liquid. This suggestion is based on the work of Phillips and Steinberg (1978) and Phillips *et. al.* (1977). These authors showed that certain cell aggregates behave as Elasticoviscous

Liquids on centrifugation. Flattening out quickly at first, but then cells slip past one another and reassume their original shapes. Thus, Poole and Steinberg suggest that a similar process of cell rearrangement and "tissue flow" occurs during nephric duct extension.

Poole and Steinberg (1982; and Steinberg and Poole, 1982) implanted pronephric primordia into the flanks of developing embryos. The implanted duct migrates dorsocaudally across the flank to fuse with the primary duct. They are able to eliminate chemotaxis (thus contradicting Bijtel's, 1948, result) and contact guidance by varying the age of the host embryo and the orientation, age and size of the implant. Instead they suggest a Craniocaudal Travelling Adhesion Gradient. In agreement with this they find that the cross-sectional shape of the duct varies along its length. It is circular and embedded in somite and lateral mesoderm at the anterior end; ovoid and partially embedded at middle levels; and "hump-shaped" at the posterior end. They explain this by suggesting that contacts and adhesion between duct cells remain constant, but adhesion of the mesoderm cells to the duct passes caudally during development in synchrony with the advancing wave of somite segmentation. (A clock-wavefront model has been developed for somite segmentation by Cooke and Zeeman, original reference in Cooke, 1981. Poole and Steinberg seem to suggest that nephric duct extension keeps pace with the "wavefront" and that there may be a causal connection.) It is the duct tip which is most important in adhesion to the lateral mesoderm, and the cells of the tip differ in morphology from the rest of the duct cells (see below). The Xenopus duct does not seem to show this variation in shape along its length. The duct tip does not keep in line

with the forming somites. Duct tip cells do differ somewhat in morphology from the rest of the cells, but in a different way to that seen in Ambystoma. Tip cells are always hard to distinguish from the lateral mesoderm cells, whilst the other duct cells round-up during development and become very smooth-surfaced (Photo's 7a,b).

Poole and Steinberg (1977) also studied the Xenopus nephric duct. In this case, they concluded that the duct forms in situ from the lateral mesoderm, except perhaps for the posterior end which derives from the rectal diverticulae. (This latter point seems to be generally true of the amphibia, O'Connor, 1940.) This conclusion varies slightly from Fox and Hamilton's (1964) view. By producing chimaeric embryos, in which one half is diploid and the other haploid, they were able to investigate duct make-up when it developed. They found both types of cell in the duct. Thus, they suggested that some cell rearrangement as well as in situ differentiation occurred, except for the posterior region which they too attributed to growth from the rectal diverticulae.

My own work agrees with Poole and Steinberg's (1977) description of duct growth in Xenopus. This stops at the level of aprox. trunk somite 9, from stage 23 to stage 26, after which it continues to meet the rectal diverticulae. This latter growth period is obscured as the duct has sunk somewhat into the tadpole's body. My graph 3 was modelled on Poole and Steinberg's (1981) Fig. 6. Their figure shows that the Ambystoma duct diameter decreases, graph 3 shows that the Xenopus duct maintains a constant diameter. Other differences are that the Xenopus duct length remains aprox. constant for a while (noted above), whilst the Ambystoma duct elongates

steadily. Also, the Xenopus duct is seen at the dorsal tip of the lateral mesoderm, whilst the Ambystoma duct extends along the side of the mesoderm. The Ambystoma duct is clearly differentiated from the somites and mesoderm throughout development. The Xenopus duct is not clearly differentiated from the mesoderm until aprox. stage 28, and the duct tip is never clearly distinguished. Finally, the Xenopus duct tip cells do not show the anteroposterior elongation and "fish-scale" overlapping of the corresponding cells in Ambystoma.

All this confirms the view that the Xenopus duct forms in situ (again, except for the posterior end) although some cell rearrangement may occur as suggested by Fox and Hamilton (1964). It also serves to confirm the general conclusion that Anuran nephric ducts develop in situ, whilst suggesting that Urodele ducts develop by extension from the pronephric primordium (possibly along a Travelling Adhesion Gradient).

To summarise this section, in Amphibia the nephric system seems to develop from a band of lateral mesoderm. At first, cells from any part of this region can be induced to form pronephros or mesonephros by implanting into the right position (work reviewed in Burns, 1955). But the pronephric primordium is committed by mid-neurula and the mesonephros primordium by the tailbud stage. The latter probably needs at least an initial inductive influence from the nephric duct for full normal development. (The failure of my explant experiments means that I can make no definite statement. However, the TEM work seems to discount an induction involving direct cell contact.) The nephric duct develops from the pronephric primordium, either by caudal extension (Urodeles) or by in situ differentiation (Anura). All three areas are first seen as an accumulation

of irregularly-shaped cells showing no ultrastructural specialisations. (Admittedly, the mesonephric cells have to migrate to their site of differentiation.) Cells then arrange themselves radially, a lumen forms and other ultrastructural changes occur. All this occurs similarly in all three regions, although there are obvious differences (see Table 8).

The structural and functional similarity of the metanephros has also been mentioned (see also Christensen, 1964). There are also developmental similarities; for example, Saxen (1971; Saxen et. al., 1968) note the increased contact between cells during initial aggregation. Whilst, Belsky and Toole (1983) found similar changes in hyaluronate and hyaluronidase activity during the development of the chick mesonephros and metanephros. Hyaluronate accumulates during the early and intermediate stages. It decreases in association with an increase in hyaluronidase activity, when most of the nephric mesenchyme has formed epithelium and the tubules are differentiating. They suggest that reduction in hyaluronate allows the formation of cell junctions and that it occurs in the basement membranes at later stages. These similarities also suggest that the immunological changes found during metanephros development by Saxen's group may have counterparts in pronephros and mesonephros development.

In general, Fraser (1950) is able to conclude that "the vertebrate excretory system is a Holonephros. The entire organ arises from one source, namely from the intermediate cell-mass which unites the somite with the lateral plate, and is originally segmental". This emphasizes the modern view that the division into pro-, meso-, and metanephros is artificial. A distinct pronephros is usually only found in

those vertebrates with a free-living larva. In many vertebrates, the mesonephros and metanephros develop as a continuous structure.

This section ends with a survey of the evolutionary relations between Anura and Urodeles. Their nephric systems are very similar, but there are significant differences, e.g. note the structural differences between pronephri in Fig. 13. The most apparent difference is the qualitative one concerning nephric duct extension. This may be connected to the fact that the Anura show atypical somite development and even vary amongst themselves (Youn and Malacinski, 1981a,b). The urodeles show typical vertebrate somite development; involving initial somite formation as a "rosette" of cells, which then elongate antero-posteriorly and then fuse. In X. laevis, however, somite cells first elongate mediolaterally, somite segmentation then occurs, then the cells rotate to a more normal orientation, and no fusion occurs. (Rana shows an intermediate condition, in which rotation occurs, but so does cell fusion.) However, the mechanism of somite formation is unknown.

M. Jacobson has developed a Compartmental Theory of Development for the Xenopus central nervous system. This is based on his fate maps, derived from his Horseradish peroxidase labelling experiments. He also repeated Spemann's classical "Organizer" experiments, making dorsal lip grafts, and found that the secondary CNS developed from the graft. There was no induction. Thus, he suggests that either Spemann's experiments on Triturus were misinterpreted or there is a difference between Urodeles and Anura. Smith (1983) reviews this work, but suggests that fate map data is not adequate for producing developmental mechanisms.

Nieuwkoop and Sutasurya (1976) note other differences in

anuran and urodele development. For example, they show differing modes of mesoderm formation. This is related to the early double-layered nature of the anuran blastula wall in contrast to its single-layered nature in urodeles. More importantly, they also differ markedly in the site and mode of formation of their primordial germ cells. In Anura (and apparently teleosts also) "germinal cytoplasm" can be traced back to the fertilised egg and is endodermal in origin (i.e. near the vegetative pole). In complete contrast, however, in urodeles the primordial germ cells are induced from the ectoderm by the ventral endodermal mass, and thus form part of the latero-ventral mesoderm of the blastula. This leads these authors to suggest that the two groups either split very early in their evolution or arose polyphyletically. However, since the underlying mechanisms for all these differences are unknown, we cannot be sure of the extent of the genetic differences involved. Thus, the best evidence for amphibian evolution rests with the fossil record. But there is disagreement in this field. The earliest fossil modern amphibia (i.e. subclass Lissamphibia) occur in the Permian age, aprox. 280 million years ago (McFarland et. al., 1979). However, both urodeles and anura are found. The majority modern view appears to favour a monophyletic origin from early amphibia, and a possible common ancestor (Doleserpeton) has been found in Lower Permian deposits.

MODELS OF MORPHOGENESIS.

This section will seek to combine the results on the cyto-skeleton, nephric system, and the behaviour of the XTC and salmon cells. The obvious connection would have been the work

on induction, both on the nephric system and the XTC cells. However, the XTC cells showed no novel behaviour in the presence of "inductors" or collagen, whilst the mesonephric explant experiments were very disappointing. Thus, the connection can only be made on a more general level, within a discussion of formal models of morphogenesis.

First, the behaviour of the XTC and salmon cells will be discussed, with special reference to overgrowth formation. This leads on to similar morphogenetic systems developed *in vitro* and allows suggestions as to some of the cell properties involved. This leads on to formal systems which use such properties to mathematically model aspects of morphogenesis. Finally, these model systems are compared to more qualitative models based on gland formation *in vivo*.

It seems that all permanent cell lines from *Xenopus* are aneuploid and epithelial in morphology (Arthur and Balls, 1971; Rafferty, 1969, 1976), although fibroblastic cells are found in primary cultures (e.g. Balls and Ruben, 1966). Rafferty (1969) seems to consider the likelihood of epithelial cells being derived from actual epithelia as quite good for amphibians in general. But *Xenopus* may be exceptional. Balls and Worley (1973) even report one permanent epithelial cell line which started off as predominantly fibroblastic. In any case, the kidney provides a wide variety of cells which might take up an epithelial morphology in culture, including the endothelial cells of the blood vessels. Thus, the exact tissue of origin of the XTC cells is unknown.

Xenopus cell lines may also show signs of differentiation. Rafferty (1976) briefly mentions a cell line derived from kidney which showed doming. Solursh and Reiter (1972) were

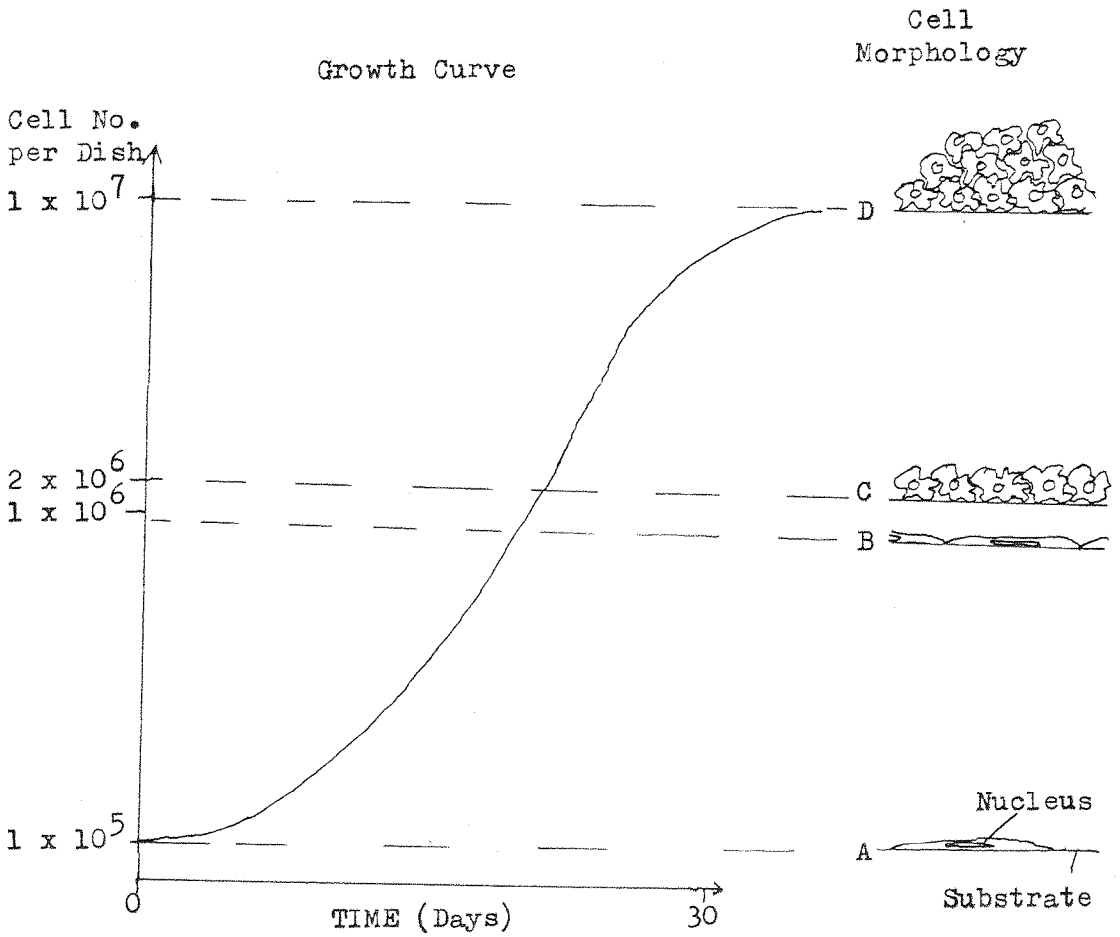
able to maintain long-term cultures of two clonal cell lines from liver. One was derived from larval tissue and showed the histochemical reactions of an hepatocyte. The other was derived from adult liver and was similar to a sinusoidal cell, since it phagocytosed trypan blue and stained positively for acid phosphatase. All this, plus the fact that the XTC cells show overgrowth with ICC's, raised hopes that more organised morphogenesis might be elicited by a suitable environment. This did not prove to be the case.

The XTC cells seem to be typical transformed cells, with many surface features and low adhesion to the substrate, as well as their lack of contact inhibition of cell division. (Locomotion and contact inhibition was not investigated, but Dr. Bell believes that individual cells can locomote and will stop on colliding, pers. comm.). Their MDT seems to be fairly long at 50-56 hr in log phase. In contrast, Rafferty (1969) calculates a value of 22-30hr for Rana and Xenopus cell lines. Whilst, Freed and Mezger-Freed (1970) found an average of 40hr for Rana cell lines. The long MD^T may in part be due to the osmolarity of the medium. Balls and Worley (1973) found that 50% L-15 media were optimum for growth in Xenopus cell lines. However, both they and Rafferty (1976) note that the effect is comparatively slight, as amphibians can stand and grow well in a wide range of osmolarity - aprox. 120-300 mOs. 70% L-15 is aprox. equivalent to 230 mOs; 50% L-15 is aprox. equivalent to 190 mOs.

The XTC cells grow past confluency in 8% FCS medium, with associated changes in morphology (Fig. 14). This will occur on plastic, glass, or collagen substrates. However, cell growth appears to stop or at least slow down at confluency if the

FIGURE 14.

A Summary Diagram of the Growth of the XTC Cells.



The cell morphologies correspond to the respective points on the Growth Curve.

A. Non-Confluent Culture. XTC cells are flat and comparatively well attached to the substrate. Though notice that there are no MF bundles.

B. Sub-Confluent to Confluent Culture. The cells are still broad, flat and well attached. However, cell division does not cease, leading to . . .

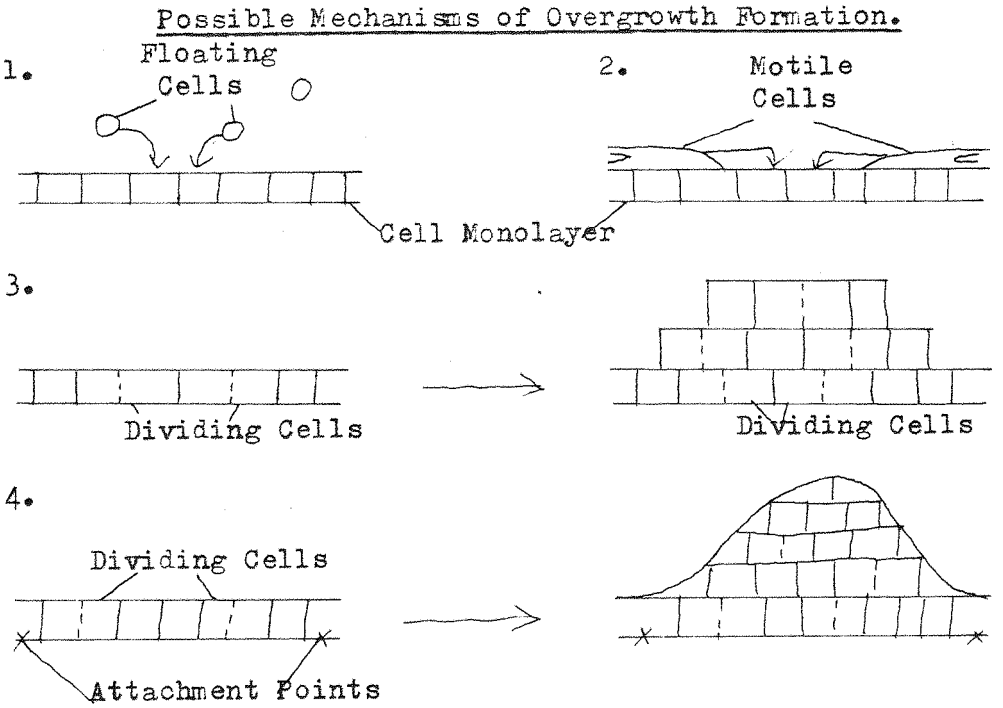
C. Confluent Culture. The cells are now spherical (as are their nuclei) and covered in microvilli. They are no longer closely apposed to the substrate. Growth in 8% NCS medium appears to stop at this point. However, cell division continues in 8% FCS medium, leading to . . .

D. Overgrown Culture. The cells are still spherical, but now large overgrown areas have been formed (see Fig. 15). The ICC's are also present (see text). Cell division has either ceased, decreased greatly, or daughter cells float away into the medium.

cells are kept in 8% NCS medium. It seems likely, therefore, that these cells do not show contact inhibition of growth (often associated with permeable cell junctions - which the XTC cells may not have - and metabolic cooperation; Loewenstein, 1979), but continue to divide as long as the medium allows it. This suggests that NCS either lacks or has fewer of the necessary factors, e.g. Growth Factors. (This is further supported by the fact that the cells did not grow in the depleted horse serum medium, Photo 9c.) It does not seem to be the case that either the substrate, pH, or ion concentration greatly affect growth. For example, pH in old overgrown cultures is quite acid, aprox. pH 6.0–6.5, yet the cells survive very well and still show signs of division. (Floating cells are always seen in such cultures when they come to be washed, yet the density does not decrease.) Thus, the XTC cells seem to have escaped the effects of any "kidney chalone" (Chopra and Simnett, 1969).

Overgrowth formation seems to arise from a combination of cell division and buckling of the monolayer (Fig. 15). Cells actively travelling over the monolayer to sites of aggregation can be discounted as no motile cells were ever seen on top of a monolayer. A conclusion also supported by the fact that cells from explants never spread over (nor under) the XTC cells. This seems to be in accord with Bard's (1979) conclusion that the dorsal surfaces of epithelial cells do not support locomotion (see the Introduction). (But the XTC cells themselves did not envelope the explants. They did not spread far either over or under the explants, even when a large amount of ECM was present. It is possible that the explants were not a suitable substrate due to the breakdown products,

FIGURE 15.



1. Floating cells in the medium settle on adhesive sites on the upper surface of the monolayer. This mechanism seems unlikely, as floating cells do not seem actively motile.
2. Cells move actively over the surface of the monolayer, collecting at adhesive sites. This mechanism seems unlikely as no motile cells have been seen on the monolayer, all such cells are rounded (see text).
3. Overgrowth forms at sites of (increased) cell division.
4. Overgrowth forms due to buckling of the monolayer as cells continue to divide (see text).

Method 3 seems unlikely, it would involve a dividing cell in some way stimulating it's neighbours to divide. This would, however, lead to overgrowth covering the whole culture. But areas of monolayer are always seen, at least on plastic substrates. To account for this would need some sort of mechanism involving 2 chemicals, Morphogens. One morphogen acting to stimulate division and one acting to inhibit it. Given varying rates of production and diffusion of these 2 morphogens, any number of patterns can be produced. (The general theory is elaborated by Meinhardt and Gierer, 1974, 1980; Gierer, 1977, 1981.) Such an elaborate mechanism seems unlikely, which therefore leaves method 4, and this is the method discussed in the text.

and the cell death that occurred in larger explants.) Thus, the net-like appearance of overgrowth in old cultures seems to be due to the fact that the XTC cells continue to divide in confluent cultures and are rounded in shape, with large numbers of microvilli and weak adhesion to the substrate. All this results in buckling of the monolayer, possibly against scattered sites of greater adhesion to the substrate (Dipasquale, 1975a,b). A somewhat similar mechanism is invoked by Bard and Ross (1982a,b) to explain the development of folds in the ciliary body of the avian retina. They conclude that these folds are caused by the rapid growth of the retina being constrained by the slower growth of the pupillary ring.

Therefore, the ICC's either arise "accidentally" during this buckling (and may then be stabilised by the close contacts that only seem to develop at a free cell surface), or by an osmotic effect similar to doming, or by a combination of both. A doming-like effect seems unlikely since the spaces and close contacts between XTC cells do not look as if they could obstruct the passage of water or various solutes.

This mechanism of simple morphogenesis in culture is in contrast to that given by Elsdale and Foley (1969) for fibroblasts (see the Introduction). The latter seems to be dependent on the spindle-shape of fibroblasts and localised ECM production. The salmon cells (and perhaps most epithelial cell lines that can multilayer) seem to have a similar ECM-based multilayering behaviour. These cells also grow past confluency, but they maintain their flattened shape and seem to produce ECM all over their dorsal (and ventral) surface. This presumably allows cell movement both over and under other cells, resulting in a uniform multilayer rather than

the ridge-pattern seen with fibroblasts.

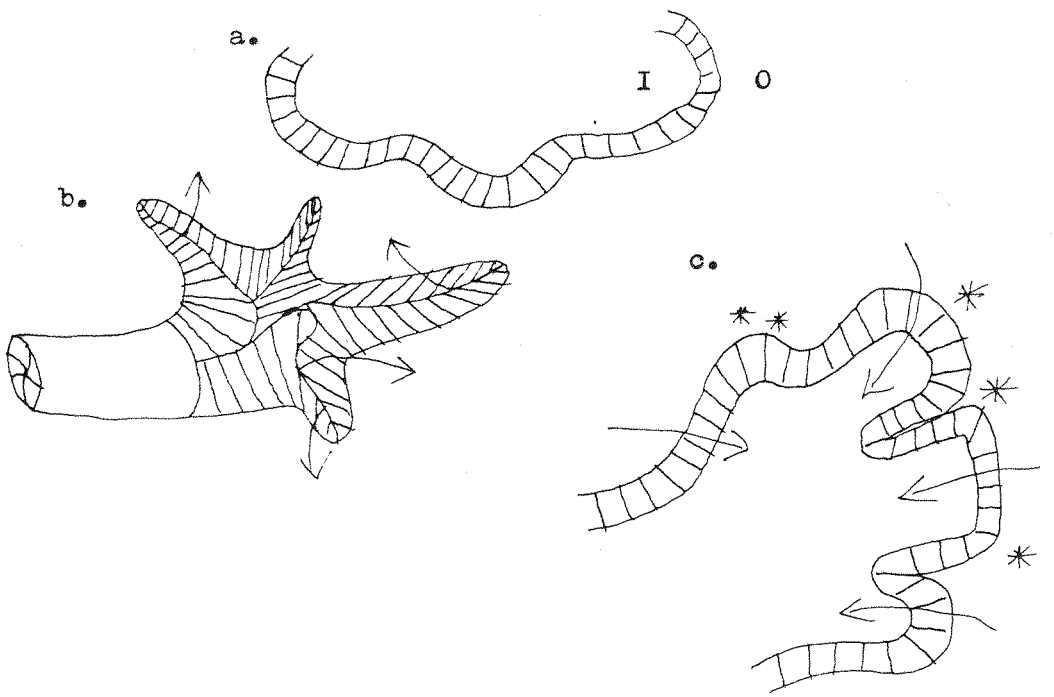
The maintenance of the flattened morphology may be associated with the possession of stress fibres (Lloyd et. al., 1977; Greenspan and Folkman, 1977). Thus, the difference in cytoskeleton (and ECM production) between salmon cells and XTC cells may explain their difference in behaviour. Similarly, transformed cells may show changes in adhesion and production of lamellar cytoplasm, which can explain their different morphologies in dense culture (Cherny et. al., 1975; Bell, 1977; Erickson, 1978). Lloyd et. al. (1977) also suggest that the spindle-shape of fibroblasts may be due to the presence of MT mechanically restricting the sites of the ruffled membranes (either by interaction with membrane components or with MF). Epithelial cells, lacking such cytoskeletal specialisations, have no dominant site for ruffling and, therefore, produce the characteristic polygonal outline in monolayer. (This is reminiscent of the shape of soap bubbles, but see the note by J. Bonner on pp. 124-125 in Thompson, 1977.)

Overgrowth and ICC formation in XTC cells does not seem to involve any morphological or genetic specialisations. It simply seems to be due to their continued growth in confluent cultures, weak adhesion to the substrate, and rounded shape covered in microvilli. This, unfortunately, makes it less likely that organised morphogenesis can be induced. To clarify why this is so, a very brief review of models of morphogenesis is now presented.

The first model Bennett used to explain tubule formation in her cell line was derived from Gierer's mathematical models of morphogenesis (e.g. Gierer, 1977 and 1981; Fig. 16). It depended on the formation of a stable, deformable, impermeable

FIGURE 16.

Hypothetical Set of Properties allowing Cells of a Simple Type to Form Tubules and Alveoli.
(Taken from Bennett, 1980.)



a: (1) Cells cohere to form a membrane. (XTC cells do not have strong cohesion.)
(2) The membrane has limited thickness, for example, a single cell layer with an upper limit to cell height. (XTC cell monolayers show overgrowth.)
(3) Cell volume has a lower limit. (Probably true of XTC cells.)
(4) Cells are readily distorted. (Probably true of XTC cells.)
(5) The membrane remains continuous (cannot fragment). (Probably untrue of XTC cell monolayers.)
(6) "Inside" (I) and "outside" (O) surfaces are different. (Not true of XTC cell monolayers, except that close contacts tend to be at the free surface.)

b: (7) Processes exist which keep the inside area at a minimum (zero). The interior is thus 1-dimensional; a point for very small membranes or a line which can branch. The membrane then forms an occluded sphere or (branching) tube respectively. It can grow while maintaining this form.
(7a) (Optional) If the membrane is relatively impermeable (probably untrue of XTC cell monolayers) and seperates 2 fluid compartments, a strong force contributing to (7) can be net outward transport of fluid by the cells (arrows). The resulting pressure difference tends to collapse the interior.

c: (8) Cells respond to an environmental signal (*) by transporting fluid inwards, causing inflation.

monolayer of cells. The XTC cells do not form a stable, impermeable monolayer, so such models of sheet deformation are not valid. However, Bennett's model changed with further work, which showed that fibroblastic cells were required to initially invade the gel, then the epithelial sheet deforms to form the tubules (Bennett et. al., 1981). This model, in its turn, has been challenged by Ormerod and Rudland (1982). They worked at the TEM level, and considered that only epithelial cells were sufficient to invade the gels, whilst the tubules formed by cell death. A similar idea is feasible for ICC formation in XTC cells, but no cell death was seen.

Bennett's first model stressed cellular polarity to stabilize deformation of the sheet. Cellular polarity has also been stressed in another system, but one which does not involve sheet deformation. Hall et. al. (1982) consider the tubules and acini produced by MDCK and NMuMG cells in collagen "sandwiches" to be due to the upsetting of epithelial stability (see Introduction). This stability is restored by lumen formation. Similar behaviour, and a similar explanation, was found in thyroid cells cultured in collagen "sandwiches" (Chambard et. al., 1981).

On a theoretical level, cellular polarity appears to be essential in the formation of hollow structures (Gierer, 1977). The polarity in the position of the close contacts in XTC cells is presumably enough to allow the formation of ICC's, but do not produce the required stability for tubule formation.

Odell et. al. (1981) and Oster et. al. (1980) have produced a mathematical model of morphogenesis based on the idea of the cell as a viscoelastic solid. (Due, in part at least, to the

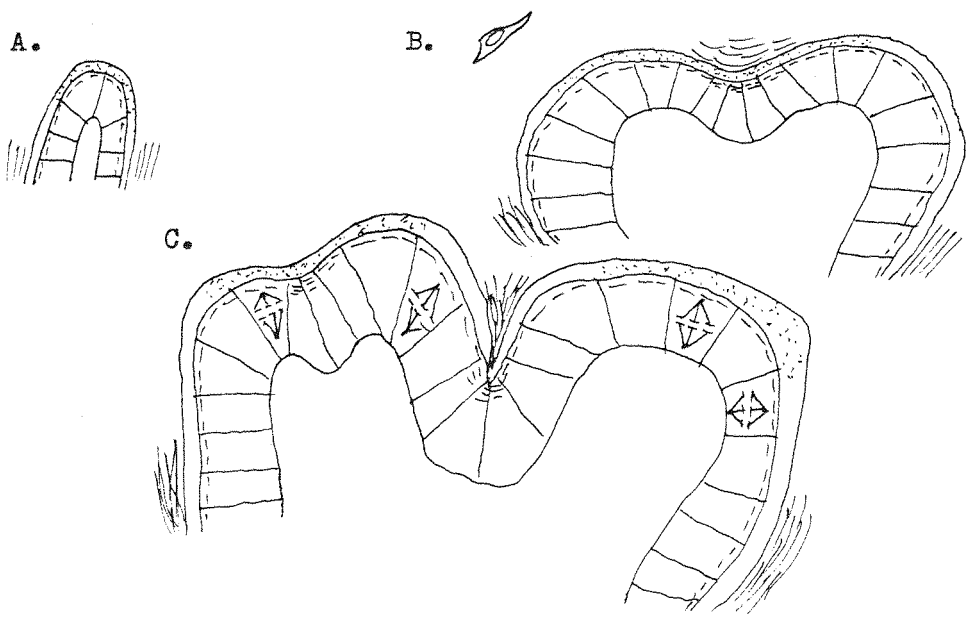
cytoskeleton. "The formation of protein networks in biological fluids enormously alters their fluid viscoelastic properties. It transforms a low viscosity, nearly Newtonian fluid into a viscoelastic gel"; Jen et. al., 1982; see also Pollard, 1976.) They looked at a simple cuboidal epithelium, with stable apical circumferential junctions attached to MF bundles. Contraction of these MF bundles will produce a "purse-string" effect. Active contraction is elicited by stretching of the bundles. Different "tubule" morphologies are produced by various starting conditions.

An in vitro model which seems to fit this model very well is Owaribe et. al.'s (1981) glycerinated model system of retinal pigmented epithelium. An in vivo system partially explained by the model is neurulation. Neural tube formation itself is well modelled, but regional modelling requires displacement of the whole neural plate caused by elongation of either the notochord or the overlying neural plate cells in the antero-posterior direction (Jacobson and Gordon, 1976). Thus, the neural plate as a whole seems to act more like a viscoelastic fluid, similar to nephric duct extension.

In vivo situations are normally complicated by a number of factors. For example, ECM can have a variety of effects on gene expression or the cytoskeleton (reviewed in Bissell et. al., 1982). Its role in gland formation has given rise to a simple model of branching morphogenesis (Fig. 17; Goldin, 1980; Bernfield et. al., 1973). Although not as rigourous as the mathematical or computer models, it accounts well for a variety of systems:- e.g. lung (Spooner and Wessells, 1970), salivary glands (Thompson and Spooner, 1982; Bernfield and Banerjee, 1982; Spooner, 1973), thyroid glands and pancreas

FIGURE 17.

Schematic Model depicting the Relationship between Cleft Formation and the Distribution of Extracellular Materials. (Taken from Bernfield et. al., 1973.)



A. A primary lobule. Newly synthesized Glycosaminoglycans (stippled areas) accumulates within the basal lamina at the distal end of the lobule. The basal laminae at the lateral aspects of the lobule are sites of low rates of new GAG accumulation and are associated with bundles of collagen fibres. Intra-cellular MF are present at the basal end of each cell.

B. Early cleft formation in a primary lobule. Contraction of MF bundles within cells at the top of the lobule causes the tissue to bulge inward, initiating the formation of secondary lobules. Tropocollagen derived in part from the mesenchymal cells begins fibrogenesis near the surface of the incipient cleft. This cleft would be sensitive to the action of cytochalasin.

C. Deepening of the cleft and growth of secondary lobules. Mitotic activity of cells within the secondary lobules and continued MF contractility cause the cleft to deepen. As secondary lobules grow, ECM originally at the surface of the primary lobule are incorporated into the cleft, where bundles of collagen fibres become organised and confer stability to the branch-point. This cleft would be insensitive to the action of cytochalasin. The basal laminae at the distal ends of the growing secondary lobules show the bulk of newly synthesized GAG, and with further growth undergo cleft formation.

(reviewed in Goldin, 1980). However, all these systems involve folding of an epithelial sheet. The early development of the mammary gland (Ceriani *et. al.*, 1970) and all three types of kidney involve lumen formation in an accumulation of cells; *in vitro* modelled by the collagen "sandwich" systems. The two types of system are similar, however, in that mesenchyme also usually has an inductive effect and ECM changes are involved (e.g. see the Introduction).

To return to the XTC cells; no specific reason for their failure to show organised morphogenesis can be given. In the case of the mesonephric explants, one knows that the cells normally develop into tubules, thus their failure to do so must be due to some insufficiency in the experimental conditions. On the other hand, the exact origin of the XTC cells is unknown. Although derived from adult kidney, there is no guarantee that they derived from tubular epithelium. Even if they were, there is no knowing whether their genetic equipment would enable them to reform tubules *in vitro*; this is disregarding any genetic changes that may have occurred during culturing (remember they are a heteroploid, aneuploid, permanent cell line). Added to all that, the failure of the mesonephric explants puts doubt on the ability of the "inductors" to induce tubules (including nephric duct, as it degenerates *in vitro*). Thus, the part of this project examining induction gave little useful information. This means that the project consists of 3 sections:- Cytoskeletons; Description of early kidney development in *Xenopus*; Behaviour of the XTC cells. The connecting link seems to be at a more formal level. The cytoskeletal work gives some basis for a viscoelastic model of a cell. This leads on to the formal models of morphogenesis, of which a

simple *in vitro* example is the overgrowth formation of the XTC cells. However, such simple models ignore the subtleties of intracellular structure (obvious even in whole-mount TEM work on the cytoskeleton) and extracellular environment. The latter is illustrated by the descriptive work on kidney development. The largest gap, however, concerns the genetic control of differentiation. This is well illustrated by the failure to induce organised morphogenesis in the XTC cells.

On a general level, simple sets of cellular properties and interactions are involved in a wide variety of systems. Simple changes in the initial parameters of such systems ("bifurcation") can often lead to drastic morphological changes and are obviously important in evolution (discussed in Oster and Alberch, 1982; Maderson, 1975; Alberch, 1980; Gould, 1977).

To summarise, the relation between "cytoarchitecture", ECM, and morphogenesis is a complex network, presumably under the final control of the genes. This project attempted to investigate this both *in vivo* and *in vitro*. The XTC cell line failed to show any organised morphogenesis. This is probably linked to their lack of any ultrastructural polarization and low ECM production. These cells did, however, provide some useful information on the cytoskeleton. Finally, the simple form of morphogenesis shown by the XTC cells can be explained by referring to the properties these cells do show in culture. More complex morphogenesis was studied by looking at kidney development *in vivo*. Here, too, it seems that a few simple cell properties can interact to produce organised structures. The "bifurcation" idea can be brought in to suggest how the 3 kidneys evolved. But, again, there is very little information as to the genetic control of such processes.

APPENDIX A

MEDIA USED IN CELL CULTURING.

a). L-15 Medium. (Flow Labs.)

Without Glutamine. Figures in mg/L.

L-Alanine . . . 225.0

L-Arginine . . . 500.0

L-Asparagine H_2O . . . 250.0

L-Cysteine . . . 120.0

Glycine . . . 200.0

L-Histidine . . . 250.0

L-Isoleucine . . . 125.0

L-Leucine . . . 125.0

L-Lysine HCl . . . 93.70

L-Methionine . . . 75.0

L-Phenylalanine . . . 125.0

L-Serine . . . 200.0

L-Threonine . . . 300.0

L-Tryptophan . . . 20.0

L-Tyrosine . . . 300.0

L-Valine . . . 100.0

D-Calcium pantothenate . . . 1.0

Choline chloride . . . 1.0

Folic acid . . . 1.0

i-Inositol . . . 2.0

Nicotinamide . . . 1.0

Pyridoxine HCl . . . 1.0

Riboflavin phosphate, sodium salt . . . 0.1

Thiamin monophosphate $2H_2O$ . . . 1.0

$CaCl_2 \cdot 2H_2O$ . . . 185.5

KCl . . . 400.0

KH_2PO_4 . . . 60.0

$MgSO_4 \cdot 7H_2O$ . . . 400.0

NaCl . . . 8000.0

NaH_2PO_4 . . . 190.0

D-Galactose . . . 900.0

Sodium phenol red . . . 10.0

Sodium pyruvate . . . 550.0

b). XTC Cell Culture Medium.

70% Liebovitz L-15 (Flow Labs.)

8% Foetal Calf Serum (Gibco or Flow Labs.)

2mM Glutamine (Sterilised by Milliporing)

22% Distilled Water (Sterilised by Autoclaving)

25μg Fungizone (Squib; Flow Labs.)

0.006g Pennicillin-G

0.01g Streptomycin sulphate

c). Modified Steinberg's Solution. (Sterilised by autoclaving; Freed and Mezger-Freed, 1970.)

17% NaCl - 20ml

0.5% KCl - 10ml

1.0N HCl - 4ml

Glass-distilled water - 966ml

Tris(hydroxymethyl)aminomethane - 691mg

d). Trypsin Solutions. (pH = 7.8)

0.05% Trypsin solution for XTC cells:-

17% NaCl - 10ml

0.5% KCl - 5ml

Tris(hydroxymethyl)aminomethane - 0.28g

Ethylenediaminetetra-acetic acid (EDTA) - 1.0g

Made up to 1 litre and sterilised by autoclaving before adding trypsin.

0.1% Trypsin solution for salmon cells (Wolf and Quimby, 1969):-

Distilled water - 960ml

NaCl - 8.0g

KH_2PO_4 - 0.2g

KCl - 0.2g

$NaHPO_4$ - 1.15g

Disodium EDTA - 0.2g

Autoclave before adding trypsin.

e). Gurdon's Modified Barth's (Gurdon, 1968)

x10

NaCl - 51.4g

KCl - 0.75g

$NaHCO_3$ - 2.02g

Tris(hydroxymethyl)aminomethane - 2.42g

$Ca(NO_3)_2 \cdot 4H_2O$ - 0.78g

$CaCl_2 \cdot 2H_2O$ - 0.6g

$MgSO_4 \cdot 7H_2O$ - 0.79g

The first 4 chemicals are dissolved and pH adjusted to 7.6 with conc. HCl. Make volume upto 500ml.

Dissolve the last 3 chemicals in 500ml distilled water.

Both solutions are sterilised seperately by autoclaving. Then they are mixed to give 1L of x10 GMB.

f). Hank's Balanced Salt Solution. (Flow Labs.)

Without Calcium or Magnesium, with added HEPES

mg/L

KCl - 400.0

NaCl - 8000.0

Na_2HPO_4 - 60.0

Glucose - 1000.0

Sodium phenol red - 17.0

KH_2PO_4 - 60.0

HEPES - 2mM

g). Minimum Essential Medium Eagle (Modified). (Flow Labs.) With Hank's salts. Without Glutamine or bicarbonate, plus 10mM HEPES.

mg/L.

L-Arginine HCl - 126.4

L-Cystine disodium salt - 28.42

L-Histidine - 41.9

L-Isoleucine - 52.5

L-Leucine - 52.5

L-Lysine HCl - 73.06

L-Methionine - 14.9

L-Phenylalanine - 33.02

L-Threonine - 47.64

L-Tryptophan - 10.2

L-Tyrosine - 36.22

L-Valine - 46.9

Glucose - 1000.0

Sodium phenol red - 17.0

D-Ca pantothenate - 1.0

Choline chloride - 1.0

Folic acid - 1.0

i-Inositol - 2.0

Nicotinamide - 1.0

Pyridoxal HCl - 1.0

Riboflavin - 0.1

Thiamin HCl - 1.0

$CaCl_2.2H_2O$ - 185.5

KCl - 400.0

KH_2PO_4 - 60.0

$MgSO_4.7H_2O$ - 200.0

NaCl - 8000.0

Na_2HPO_4 - 47.5

h). Salmon Cell Culture Medium.

90% MEM (Flow Labs.)

8% Foetal Calf Serum (Gibco or Flow Labs.)

2mM Glutamine (sterilised by milliporing)

2% Distilled water (containing the antibiotics; sterilised by milliporing)

0.006g Pennicillin-G

0.01g Streptomycin sulphate

25µg Fingizone (Squib; Flow Labs.)

APPENDIX B

SOLUTIONS FOR ELECTRON MICROSCOPY.

a). 0.1M Phosphate Buffer (Humason, 1972).

7.008g Na_2HPO_4

7.8g $NaH_2PO_4 \cdot 2H_2O$

500ml distilled water

b). 0.1M Cacodylate Buffer (Humason, 1972).

4.28g Sodium cacodylate.

200ml distilled water.

c). Karnovsky's Fixative.

2.5% Glutaraldehyde

2.0% Paraformaldehyde

2mM $CaCl_2$

0.1M Cacodylate buffer

(Heated to c.60'C in a fume cupboard to dissolve the paraformaldehyde.)

d). Araldite Resin (TAAB).

0.4g Dibutyl Phthalate

5.0g Epoxy Resin (Araldite CY212)

5.0g Dodecenylsuccinic anhydride hardner 964 (DDSA)

0.25g 2,4,6-tri(dimethylaminomethyl)phenol (DMP-30)

APPENDIX C

SOLUTIONS FOR LIGHT MICROSCOPY.

(Humason, 1972.)

a). Bouin's Fixative.

Picric acid, saturated aqueous - 75.0ml

Formalin - 25.0ml

Glacial acetic acid - 5.0ml

b). Buffered Formalin.

10% Formalin - 1L

$NaH_2PO_4.H_2O$ - 4.0g

Na_2HPO_4 - 6.5g

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