SOME ASPECTS OF BIOCHEMICAL DIFFERENTIATION IN THE RAT VISCERAL YOLK SAC IN VIVO AND IN CULTURE

PHILIP C. ROWLANDS

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I dedicate this thesis

to my family

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ABSTRACT

By culturing 9.5 day rat egg cylinders as closed yolk sac vesicles, after surgical removal of the embryonic disc, it was shown that the cultured yolk sac retained the essential morphological characteristics of the *in vivo* yolk sac over an eight day culture period, and significant improvements were made in the original culture methodology, with regard to survival rate and serum requirement, by culturing in Medium 199-supplemented whole rat serum, and increasing oxygen tension over the last half of the culture period.

Yolk sacs cultured in this manner exhibited the same cellular morphology as *in vivo* yolk sacs removed after 17.5 days of gestation, but did not show the same degree of haemopoietic development or basement membrane synthesis. However, the cultured yolk sac endodermal cells appeared to be proliferative at 17.5 days, whereas 17.5 day *in vivo* yolk sacs did not have the same nuclear appearance.

The culture system was used to study aspects of carbohydrate metabolism and xenobiotic metabolising capability, and was used to study the expression and control of two genes - alphafetoprotein and insulin-like growth factor II, in rat visceral yolk sac.

Study of hexokinase, pyruvate kinase and succinate dehydrogenase activity in cultured and *in vivo* yolk sacs from 9.5 days to 17.5 days of gestation, indicated a rise in the level of aerobic tricarboxylic acid cycle catabolism

from 9.5 days to 13.5 days of gestation *in vivo* and in culture, as measured by an increase in succinate dehydrogenase activity. Concommitant with this rise in aerobic carbohydrate catabolism, there was a slight decrease in glycolytic activity as measured by hexokinase and pyruvate kinase activities *in vivo* and in culture. Activities of all three enzymes significantly decreased from 15.5 to 17.5 days *in vivo* ($p < 0.05$), but there was no observed decrease in carbohydrate catabolism in culture.

Expression of cytochrome P-450IA1 gene, measured by substrate-turnover enzyme assay and immunocytochemistry, was localised to the endodermal cells, and found to be inducible in *in vivo* yolk sacs from 15.5 to 18.5 days, but expressed as constitutive activity from 9.5 to 17.5 days in culture, postulated to be due to the absence of negative regulatory factors of either maternal or fetal derivation.

Using *in situ* hybridisation techniques to qualitatively and quantitatively determine AFP and IGF-II mRNA, expression of AFP mRNA was localised to endodermal cells, whilst IGF-II mRNA was expressed in both cell lineages.

Down-regulation of IGF-II mRNA was shown in neonatal liver by glucocorticoids, but these compounds did not suppress expression in the cultured yolk sac.

A thousand-fold decrease in detected AFP mRNA between 16.5 days and 21.5 days of gestation *in vivo* was not paralleled in culture, and preliminary data indicated that this was due to primary or secondary effects of a maternally-derived transcription factor.

CENTRAL AIM OF THE PROJECT

Although written in retrospect, this passage precedes the main body of the thesis, as a means to explain the rationale behind the wide divergence in both methodology and subject matter included in this thesis.

In the period 1982-1986, work in the Department of Anatomy, University of Leicester, had yielded an *in vitro* model of the rat visceral yolk sac - the 'giant' yolk sac (Dunton *et al.*, 1986). Experiments revealed the model's similarities to its counterpart *in utero*, with respect to structural morphology and uptake function.

In so far as this model represented a differentiating organ, cultured over a time period corresponding to a large window in gestation, the introduction of this model was a large step forward in *in vitro* methodology within developmental biology. The model offered better differentiation potential and retention of morphological integrity than methods such as fetal liver hepatocyte culture or micromass culture, whilst offering a larger gestational window in which to study, compared to whole embryo culture or limb bud culture.

In order to ascertain the usefulness of this culture model, the experiments described in this thesis were designed to characterise the biochemical and molecular differentiation patterns of the 'giant' yolk sac. By comparison to the differentiation patterns exhibited by the visceral yolk sac removed at corresponding gestational ages,

the ability of the 'giant' yolk sac to mimic normal yolk sac development could be assessed. Irrespective of the degree of similarity to the in vivo yolk sac, the 'giant' yolk sac could also be assessed as an in vitro model of a differentiating epithelial system.

CHAPTER ONE

Development of Structure and Function in the Rat Visceral Yolk Sac

1.1 EMBRYOLOGY OF THE RAT

In order to discuss the role of the visceral yolk sac in the development of the rat, one must first describe the origin of different embryonic and extra-embryonic cell lineages. This allows an understanding to develop, of the spatial arrangement of various structures in relation to each other, and also of the interaction of different lineages within a single structure.

The following brief description of embryological development is based on information from several reviews on myomorph rodent embryology (Huber, 1915; Boyd and Hamilton, 1952; Snell and Stevens, 1966; Theiler, 1972; Steven and Morriss, 1975). Many of these reviews concentrate on the embryological development of the mouse (Muridae mus), where gestational events are analogous to the rat (Muridae rattus), but chronologically advanced by thirty six hours due to a more rapid implantation (Butler and Juurlink, 1987).

Gestation in the rat is multiparous, and has a timespan of approximately 22 days, following fertilisation of the oocyte within the oviduct ampulla (Huber, 1915). Early embryological development can be split into four stages: formation of the blastocyst, implantation, onset of gastrulation, onset of organogenesis.

Formation of the blastocyst

The fertilised ovum, after successive cell divisions in the oviduct, enters the uterine horn at the late morula stage (8-12 cell stage). At this stage, a fluid-filled intercellular cavity appears at one pole of the ovoid morula, and this expands, pushing the inner cell mass to the opposite pole. The zona pellucida, which surrounds the newly formed blastocyst, then dissolves away (hatching), allowing a further increase in the rate of mitotic division (Huber, 1915).

A poorly understood mechanism of peristaltic contraction of the uterus, and cilia-mediated flow, allows distribution of the blastodermic vesicles (early blastocysts) along the uterine horns of the bicornate uterus, between days 4 and 5 after fertilisation (Snell and Stevens, 1966; Steven and Morriss, 1975).

Accompanying the increase in size of the blastocyst, changes occur in the uterine epithelium in close proximity to the blastocyst. The epithelial cells, which are usually flattened, become more cuboidal in nature, and the underlying mucosa becomes pitted (Huber, 1915). The initiation of physical contact with the blastocyst, which is thought to trigger these changes in the uterine wall, is called adhesion (Mossman, 1937).

Figure 1.1 Development of the rat embryo from blastocyst to headfold stage egg cylinder (modified from Beddington, 1985).

1.1a 6-day blastocyst - EB epiblast

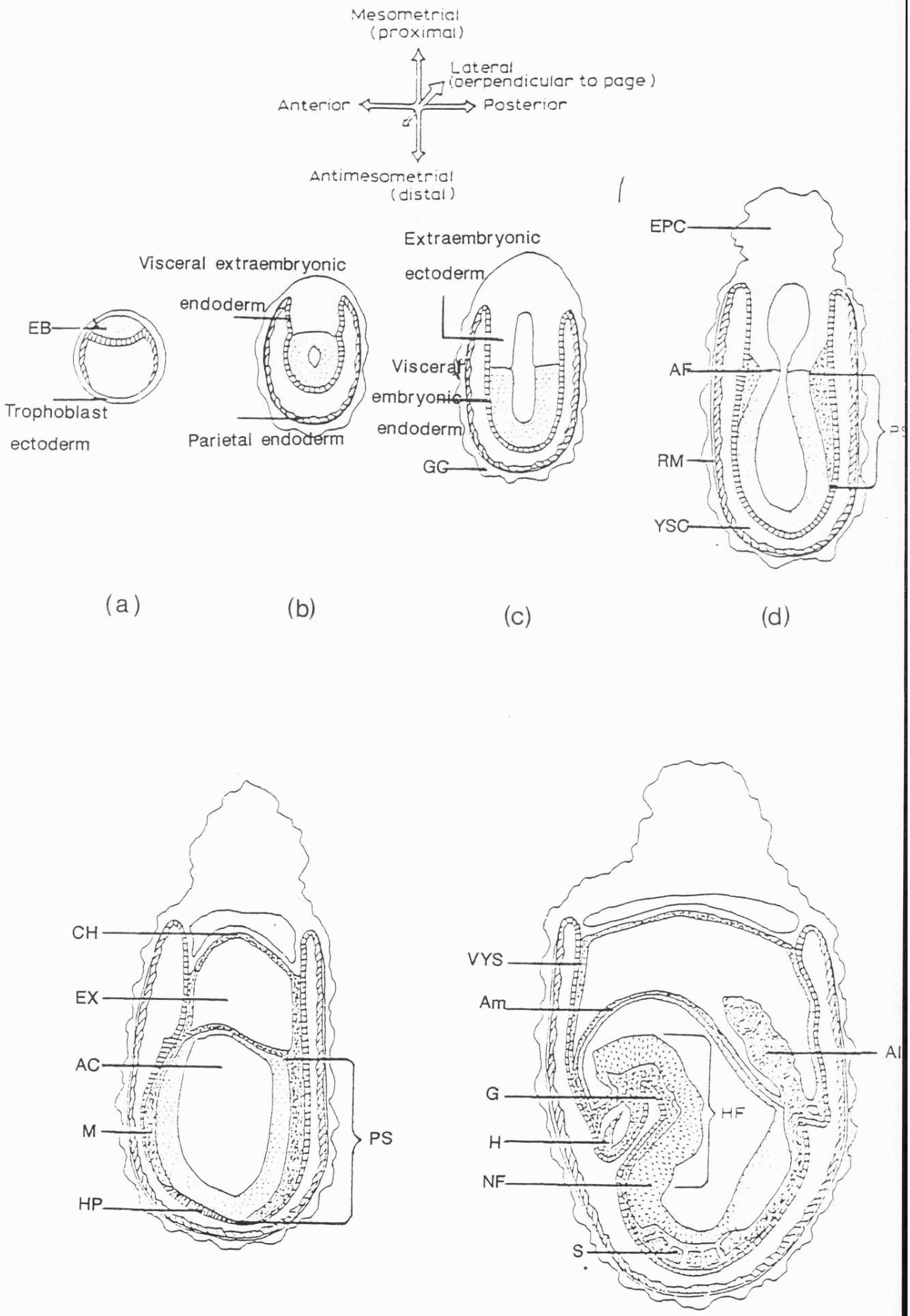
1.1b 7-day implanted blastocyst

1.1c 8-day egg cylinder - GC giant cells

1.1d 8.5 day egg cylinder - EPC ectoplacental core
AF amniotic fold
RM Reichert's membrane
YSC yolk sac cavity
PS primitive streak

1.1e 9 day three-chambered egg cylinder - CH chorion
EX exocoelom
AC amniotic cavity
M mesoderm
HP head process

1.1f 10 day headfold embryo - VYS visceral yolk sac
Am amnion
G gut
H heart
NF neural fold
S somite
HF headfold
Al allantois


Mesometrial
(proximal)
Lateral
(perpendicular to page)
Anterior
Posterior
Antimesometrial
(distal)
EB
Trophoblast
ectoderm
Visceral extraembryonic
endoderm
Parietal endoderm
Extraembryonic
ectoderm
Visceral
embryonic
endoderm
GG
EPC
AF
RM
YSC
(a)
(b)
(c)
(d)
CH
EX
AC
M
HP
PS
VYS
Am
G
H
NF
S
HF
(e)
(f)

Implantation

Thickening of the uterine mucosa on adhesion provides a groove or crypt in which the attached blastocyst sits. The permanent attachment and resulting deep penetration into the endometrium is termed implantation, and occurs on the 6th day of gestation in the rat (Huber, 1915). Implantation is interstitial and occurs on the antimesometrial side of the uterine horn (Mossman, 1937).

The uterine epithelium degenerates either as a result of contact with the fetal ectoderm, or by nutritive starvation caused by the thickening of the underlying mucosa (Mossman, 1937). The resulting cell death is marked by an increase in lipid and glycogen deposits in the uterine cavity.

As the uterine epithelium degenerates, the initiation of decidual formation is seen in the maternal tissues. The glandular tissue is displaced by an increase in size of the connective tissue cells. Irregular dilation of the underlying blood capillary network leads to the formation of sinuses (Amoroso, 1952).

The stages of development from late blastocyst to late headfold (days 6-10 of gestation) are diagramatically represented in Figs. 1.1a-1.1f.

Proliferation of the extra-embryonic ectoderm at the mesometrial pole of the blastocyst, covering the epiblast, pushes the epiblast downwards (Fig. 1.1b). The blastocyst becomes tubular, and the invagination within the tube causes the endoderm to surround the ectoderm. Hence the germinal

layers are said to be inverted, which is the reverse of the condition found in most chordates (Mossman, 1937). At this stage (approximately 7 days of gestation), the conceptus consists of: extra-embryonic and embryonic ectoderm, both having surfaces encroaching on the central pro-amniotic cavity; extra-embryonic and primary embryonic visceral endoderm, which is continuous with parietal endoderm, all impinging on the yolk sac cavity.

The mural trophoblast cells transform into primary giant cells (Fig. 1.1c), and form a distinct layer of cells between the yolk sac and the implantation cavity. These giant cells are phagocytic, and their role appears to be in enlarging the implantation cavity by destroying the decidual cells. The exclusive fetal origin of the giant cells has been challenged by a number of authors who postulate that some of the cells may be maternal in origin (discussed in Amoroso, 1952).

Adjacent to the mural trophoblast, the parietal endoderm starts to synthesise Reichert's membrane; this membrane, peculiar to rodents and insectivores, has been shown to be composed of parallel basement membrane-like layers of type IV collagen surrounded by a laminin-rich sheath (Inoue et al., 1983). The role of Reichert's membrane is unknown.

Ectoderm at the mesometrial pole of the blastocyst gradually thickens by proliferation to form the ectoplacental cone, which is the precursor of the fetal portion of the chorio-allantoic placenta. The proliferation of the cone, and the corresponding thickening of the crypt

walls at the mesometrial border of the implantation site, means that the blastocyst is completely enclosed in the uterine wall.

All these events occurring after implantation take place between 6 and 7.5 days of gestation in the rat (Steven and Morriss, 1975).

Finally, the extra-embryonic pole of the blastocyst elongates by proliferation of the ectodermal layer. Corresponding proliferation of the parietal endoderm is not seen, and therefore the cells are dispersed along Reichert's membrane by the expansion of the egg cylinder (Fig. 1.1d).

Onset of gastrulation

The term gastrulation is usually reserved to describe the anlage of the mesoderm from the primitive streak, and occurs on day 8 of gestation in the rat. The primitive streak arises in the posterior part of the epiblast, and the cells in this region lose their epithelial continuity, become densely packed (Fig. 1.1d), and eventually invaginate to form mesoderm. Mesoderm, emerging from the primitive streak, spreads anteriorly as two lateral sheets. At the anterior end of the primitive streak, the mesoderm condenses to form the head process.

Some of the primitive streak-derived mesoderm migrates into the extra-embryonic region. By the middle of gastrulation, this movement has resulted in the formation of the anterior and posterior amniotic folds (Fig. 1.1d) at the

junction between the embryonic and extra-embryonic regions. These folds fuse, forming the amnion on day 9 of gestation, and at the same time, a cavity develops in the mesoderm pushing both mesoderm and ectoderm into the pro-amniotic cavity. These two layers fuse to form the chorion, thus forming a three chambered egg cylinder, with ectodermally-lined epamniotic and amniotic cavities, and a mesodermally-lined exocoelomic cavity (Beddington, 1985).

Onset of organogenesis

Organogenesis begins some time before gastrulation is completed. Soon after formation of the headfold, the ectoderm, lying along the antero-posterior axis of the embryo, thickens to form the neural plate which subsequently rolls up to form the neural tube, possibly under the influence of the adjacent mesoderm/endoderm-derived notochord. This process is confused by the controversy over the origin of definitive endoderm (Beddington, 1983). The highly organised visceral embryonic endoderm of the late blastocyst (Fig. 1.1c) appears to dedifferentiate through gastrulation, and may be forfeited to make way for mesodermally-derived definitive embryonic endoderm. The fate of the original endoderm is unknown, although it may be incorporated into the visceral extra-embryonic endoderm of the yolk sac.

Beneath the headfold, the heart primordia emerge, fuse and begin rhythmical contraction, late on the 10th day of gestation.

Fig. 1.1f shows the rest of the embryonic mesoderm, subdivided into notochord extending under the neural plate, rows of segmental blocks of mesoderm (somites), and lateral plate mesoderm. Directly underneath the notochord is the gut, which assumes a tube-like configuration at anterior and posterior ends of the embryo, but remains open to the yolk sac cavity in the midgut region, until later in gestation.

The mesoderm at the posterior end of the embryo gives rise to the allantois, at 9.5 days of gestation, which grows in a mesometrial direction, through the exocoelom, to eventually fuse with the chorion at around 10.5 days of gestation. At the same time, the epamniotic cavity is obliterated by fusion of the chorion with the extra-embryonic ectoderm of the ectoplacental cone (Ellington, 1987). This allows the initiation of placental contact between the maternal blood lacunae, and the developing extra-embryonic sinuses (Welsh and Enders, 1987), although the complete chorio-allantoic placenta is not functional until day 11 of gestation.

Meanwhile, blood vessels appear in the lateral plate mesoderm, and by day 11 of gestation, an elaborate circulatory system is established between the embryo and its major nutritive organ at this stage, the visceral yolk sac. The structure and functions of the visceral yolk sac are discussed fully in section 1.2, but briefly, the visceral

yolk sac consists of extra-embryonic mesoderm surrounded by a layer of visceral extra-embryonic endoderm, surrounding the embryo and amnion, being separated from them by the exocoelomic cavity. Initially, the extra-embryonic endoderm is in contact with the yolk sac cavity, but with the subsequent breakdown of the decidua, and retraction of parietal endoderm and Reichert's membrane at around day 15 of gestation, the visceral yolk sac is exposed to the uterine lumen for the last third of gestation (Amoroso, 1952).

1.2 STRUCTURE AND FUNCTION OF THE RAT VISCERAL YOLK SAC

1.2.1. Structural morphology of the visceral yolk sac

The visceral yolk sac is an extra-embryonic structure with two epithelial surfaces. Between these two surfaces there are three distinct cell layers: endoderm, mesenchyme and mesothelium (Amoroso, 1952; Lambson, 1966; Gardner, 1983). The surface layer in contact with the yolk sac cavity is the visceral endoderm, and consists of simple columnar epithelial cells linked by numerous tight junctions, with a microvillus border, resting on a visceral basement membrane (Carpenter and Dishaw, 1979). Beneath this membrane is the central mesenchymal layer made up of fibroblast-like cells and vitelline capillaries in a connective tissue stroma (Clark et al., 1982). The mesenchymal layer is separated by a serosal basement membrane from the third layer which is the mesothelium. This relatively thin layer consists of simple squamous epithelial cells bordering the exocoelomic cavity (Clark et al., 1982).

Electron microscopic study of the visceral yolk sac reveals highly organised subcellular characteristics of all the cell layers. The endodermal cells have a basally situated nucleus, well developed rough endoplasmic reticulum and Golgi apparatus, and large mitochondria (Lambson, 1966). The apical cytoplasmic portion of the endodermal cells is characterised by numerous membrane-bound coated and uncoated

vesicles, lysosomal compartments and canaliculi (Jollie, 1984).

The mesenchymal cells also have a well-established rough endoplasmic reticulum and Golgi apparatus, and are held in a stroma of collagen fibrils incorporating numerous large and small vitelline capillaries with fenestrated endothelia and thin basal laminae (Jollie, 1986).

Since the visceral yolk sac is functional over a long period of gestation, the loading on various functional aspects would be expected to change, and this is reflected in a number of subcellular morphological changes in the yolk sac over the gestational period (Padykula and Richardson, 1963; Padykula et al., 1966; Lambson, 1966; Jollie, 1986).

Lipid droplets are observed in the endodermal cytoplasm from day 10, and remain in large numbers until days 15-16 (Diamant et al., 1980), when the phase of lipid storage is superseded by a phase of glycogen deposition until term. The glycogen is stored basally in the endodermal cells, and also in the mesenchymal cells and the endothelial cells of the vitelline circulation. Its storage form can either be in large α-rosette aggregates or small, fine β-particles (Chue, 1986; Dunton, 1988).

The apical microvillus border of the endodermal cells is present throughout gestation, with an extensive underlying canalicular system with multiple tubular endocytic vacuoles. This has been evident for a long time, but recently has been implicated in the process of membrane receptor recycling (Kugler and Miki, 1985). The length and thickness of the

microvilli appears to be variable, but the evidence for age-related change is mixed; Padykula et al. (1966) reported a decrease in length over gestational time, whereas Jollie (1986) found the opposite to be the case.

As the visceral yolk sac expands, the area immediately adjacent to the developing chorio-allantoic placenta develops an enlarged surface area due to increased invagination, resulting in the formation of 'villous' projections (Jollie, 1986). The core of these projections contain the main drainage capillaries and venules of the vitelline circulation.

1.2.2 Functions of the rat visceral yolk sac

As already alluded to in section 1.2.1, the visceral yolk sac of the rat performs a wide range of functional activities, which change in relative importance to the developing conceptus over the gestational period.

In order to provide a systematic account of these varied functions, they have been categorised under four simple headings: nutrition of the conceptus, transport of macromolecules, de novo protein synthesis, protection of the conceptus.

a) Nutrition of the conceptus

Meyer (1925), quoted in Amoroso (1952), described the nutritional material available to the embryo throughout gestation, as embryotroph, and this can be divided into two

types: the small energy-supplying nutrients obtained directly from the maternal blood stream (haemotroph), and the large digestible material obtained direct from the maternal blood stream or via the endometrial cells (histiotroph).

Histiotrophic nutrition can be defined as the intracellular breakdown of maternally supplied macromolecules by the extra-embryonic membranes, and the subsequent passage of the breakdown products to the conceptus (Beck, 1970). The histiotroph can be obtained from the digestion of endometrial cells, or in the form of macromolecules obtained from endometrial gland secretion or direct from the maternal blood stream, via extravasation (Amoroso, 1952).

Haemotrophic nutrition depends on a full chorio-allantoic placental connection between fetal and maternal circulation, and an adequate perfusion rate by the fetal circulation. The subsequent passage of solutes between the two circulations can occur by: simple diffusion across a chemical gradient, facilitated diffusion across a gradient, active transport against a gradient, or secondary flow in repsonse to the passage of another solute (Amoroso, 1952; Beck and Lloyd, 1977).

Because of the short time course covering organogenesis in the rat, the formation of the majority of the organ systems precedes the formation of the chorio-allantoic placenta, and therefore the role of histiotrophic nutrition in the rat is of ultimate importance.

Much work has been carried out on the rat visceral yolk sac, since it was proposed as the primary organ of histiotrophic nutrition (Everett, 1935). This role could only be proved by showing that the visceral yolk sac had the ability to take up macromolecules, to digest these macromolecules, and that the embryo was able to utilise the digested products.

In order to internalise macromolecules, a cell must be phagocytic or pinocytic. The characteristic microvillus border and well developed vacuolar system of the endodermal cells of the visceral yolk sac are indicative of an actively pinocytic cell type (Steinman et al., 1983). Pinocytosis (the interiorisation of extracellular fluid with solutes and particulate matter) may be fluid-phase (non-selective) or adsorptive (involving binding to the plasma membrane). In turn adsorptive pinocytosis can be split into two types: non-specific adsorptive pinocytosis or receptor-mediated endocytosis (Duncan and Pratten, 1985; Lloyd and Williams, 1984).

All three types of pinocytosis have been shown in rat visceral yolk sac in uptake studies, either in utero from the yolk sac cavity, or more often in vitro from the surrounding culture medium.

The initial stage of pinocytosis is marked by the invagination of the plasma membrane, and the formation of a small pinocytic vacuole, and this was observed in visceral yolk sac endoderm (Padykula et al., 1966; Jollie and Triche, 1971).

Indigestible macromolecules have been used as markers of pinocytic activity in the yolk sac, either in morphological studies, using dyes (Beck et al., 1967; Beck and Lloyd, 1968), fluorescent antibodies (Wild, 1970) or ferritin (Lambson, 1966), or in kinetic studies using radiolabelled non-digestible molecules. These kinetic studies revealed differences in the rate of pinocytosis of ^{125}I-labelled bovine serum albumin and polyvinylpyrrolidine by the yolk sac in culture (Williams et al., 1975), suggesting a semi-selective fluid phase pinocytosis process in favour of bovine serum albumin (Agarval and Moore, 1979). These initial results were substantiated by Livesay and Williams (1979; 1982) who showed that denatured bovine serum albumin, insulin and lysozyme were taken up at different rates by the visceral yolk sac in culture, and that this selectivity was a result of differential binding affinities of the substrates for cationic and hydrophobic binding sites on the plasma membrane of the yolk sac. Other experiments revealed that molecules showing a high degree of hydrophobicity had an increased affinity for yolk sac pinocytosis (Kooistra and Williams, 1981).

Evidence for the energy-dependent nature of this pinocytic process was provided by numerous workers using low temperature or oxidative phosphorylation uncoupling agents (Duncan and Lloyd, 1978).

Yolk sac endoderm cells are rich in lysosomal enzymes such as acid phosphatases (Johnson and Spinuzzi, 1966; Beck et al., 1967), exopeptidases (Kugler and Huber, 1985) and

cathepsins (Beck and Lowy, 1982; Kugler and Beckenba, 1982), but evidence of the passage of ingested macromolecules through lysosomal degradadtion was required as an integral part of the proof of the importance of the visceral yolk sac in histiotrophic nutrition.

Beck et al. (1967) injected horseradish peroxidase into pregnant rats then performed a histochemical double stain on visceral yolk sac to determine the presence of acid phosphatase and horseradish peroxidase. The observed fusion of the two dyes was taken as evidence for the fusion of heterophagosomes (accumulations of pinocytic vesicles) and lysosomes, and the resultant hydrolysis of horseradish peroxidase.

Additional biochemical studies (Beck and Lloyd, 1968) showed that following maternal injection of horseradish peroxidase, short term accumulation of the compound in the visceral yolk sac was followed by a measured depletion, in yolk sacs removed after 24 hours. This depletion in horseradish peroxidase content was not parelleled by any detectable release of the parent compound.

Evidence supporting the role of the visceral yolk sac in supplying processed histiotroph to the developing embryo was supplied by Freeman et al. (1981) and Freeman and Lloyd (1983), who showed that amino acids released from maternally-injected radiolabelled bovine serum albumin and haemoglobin, were utilised by the embryo and visceral yolk sac to synthesise several proteins.

By interfering with normal yolk sac function in culture or *in utero*, using yolk sac antibodies (Cheewatrakoolpong and Leung, 1988; Freeman *et al.*, 1982) or by *in vitro* surgical removal of extra-embryonic membranes (Payne and Deuchar, 1972), the essential role of the yolk sac in providing histiotrophic nutrition to the embryo over the period of organogenesis can be observed. The fundamental role of yolk sac-mediated protein uptake and digestion can be shown by selectively blocking either pinocytosis using agents such as trypan blue (Kernis and Johnson, 1969) or exopeptidase digestion using agents such as leupeptin (Beck and Lowy, 1982).

b) Transport of macromolecules

Although the formation of the chorio-allantoic placenta at day 11 of gestation reduces the requirement for histiotrophic nutrition of the embryo via the visceral yolk sac, the yolk sac still retains the ability to adsorb certain macromolecules, that the chorio-allantoic placenta does not transport (Everett, 1935). The embryo/fetus often requires these molecules intact, therefore transport from the maternal exudate in the yolk sac cavity must be via receptor-mediated endocytosis. This process involves the pinocytosis of the macromolecule-receptor complex in a coated vesicle (Beck, 1980; King, 1982), and the passage of the complex through the cell, until the complex is

uncoupled, and the macromolecule exocytosed from the cell in an intact form (Goldstein et al., 1985).

In the rat, transmission of immunity is achieved by transfer of intact maternal immunoglobulin (IgG) to the fetus, via the visceral yolk sac and vitelline circulation (Brambell and Halliday, 1956).

IgG, epidermal growth factor, rat transferrin and insulin have all been shown to be transported intact across the visceral yolk sac (Huxham and Beck, 1981; Andrews, 1986; Beck et al., 1984; Weisbecker et al., 1983; Pratten et al., 1988).

c) Protein synthesis

There are a number of reports that indicate that the visceral yolk sac, as well as transporting proteins from the maternal circulation, can also synthesise proteins for its own use or specifically for embryonic use.

Alphafetoprotein is produced by the visceral yolk sac (Gitlin et al., 1967), and this synthesis is localised in the endodermal cells, from day 10 of gestation (Dziadek and Adamson, 1978). The synthesised protein could be shown to be present in embryonic ectoderm and mesoderm, both of which did not synthesise the protein. Janzen et al. (1982) showed the visceral yolk sac of the mouse produced alphafetoprotein and transferrin. The rate of synthesis of these proteins increased from day 9 to day 15 of gestation, then declined to term.

Experiments examining this function of de novo protein synthesis performed by the visceral yolk sac, are presented in Chapter 5 of this thesis.

Another important synthetic function of the visceral yolk sac is the specialised role of being the initiation site of haemopoiesis.

Study of the developing haemopoietic system of the chick embryo revealed that the yolk sac was the site where stem cells developed locally from precursor cells, and that subsequent sites of haemopoiesis were initiated by migration and colonisation of stem cells (Moore and Owen, 1965). However, avian yolk sac haemopoiesis is more extensive than mammalian yolk sac haemopoiesis, and involves the production of more differentiated cell types (Barker, 1968). The mammalian yolk sac is the first site of haemopoiesis in all species observed, and there is evidence that colony forming cells migrate from the yolk sac to colonise all other stem cell sites (Moore and Metcalf, 1970). These circulating pluripotent stem cells differentiate according to the micro-environment of the colonised tissue. This is the generally accepted view of stem cell colonisation, although there is some evidence from mouse chimera studies that the yolk sac and liver anlage have separate pluripotent stem cell populations (Le Douarin et al., 1982).

In the case of the visceral yolk sac, haemopoiesis takes place in the mesenchyme, where stem cells differentiate into primordial blood cells, and clump together to form blood islands, their characteristic red colour developing due to

haemocytoblast formation. Further differentiation then takes place with the central cells of the island giving rise to nucleated erythrocytes, while the peripheral cells flatten to form the capillary endothelium (Haar and Ackerman, 1971a and b). Evidence against mesoderm being the sole site of haemopoiesis was provided by studies of human yolk sac haemopoiesis (Takashina, 1987). The somewhat surprising findings of that study indicated that extra-embryonic endoderm was the site of stem cell differentiation to produce erythrocytes, and that mesenchyme is produced by the endoderm, to surround the blood islands, and form endothelial cells.

d) Protection of the conceptus

Because of the inversion of the germ layers that exist in the rat and mouse, the chorion does not surround the developing embryo, as it would in other mammals (Steven and Morriss, 1975), therefore the function of mechanical protection by the visceral yolk sac is of great importance in these species.

Fetal membranes protect the developing embryo or fetus against mechanical shock by maintaining a fluid environment in the surrounding cavities, to absorb the shock. Amniotic cells maintain the fluid balance and composition of amniotic fluid (Wild, 1965), but in vitro the amnion is unable to maintain its structural integrity, when the visceral yolk sac is removed (Payne and Deuchar, 1972).

The visceral yolk sac has been shown to be capable of ion transport (Kernis and Johnson, 1969) and Chan and Wong (1978) show that the Na^+ content of extra-embryonic coelomic fluid is greater than either fetal or maternal serum in late gestation. They postulate that the subsequent secondary influx of water through the yolk sac is the mechanism by which the extra-embryonic coelomic cavity is extended to encompass the growth of the fetus and increasing fetal movement. Chan and Wong (1978) showed that visceral yolk sac could actively transport sodium ions across a trans-epithelial potential gradient, and it was found that this transport process was at peak activity on day 18.5 of gestation, when the fluid sodium content was at its peak (Gibson and Ellory, 1984).

As well as mechanical protection, the visceral yolk sac is selectively tranporting macromolecules into the vitelline circulation, and is therefore in a position to protect the embryo against chemical insult.

Kochhar (1975) cultured embryos in medium containing 100µg/ml 6-diazo-5-oxonorleucine (an anti-glutamic agent). No decrease in DNA synthesis was observed with intact embryos, but if the visceral yolk sac was torn in a relatively avascular region, a 52% decrease in DNA synthesis was observed. This observed protective role of the visceral yolk sac <u>in vitro</u> is not paralleled <u>in vivo</u>, where this compound is a potent teratogen at lower doses, but this could be explained by passage of the chemical through the

chorio-allantoic placenta, or by a secondary effect brought about by an effect of the compound on maternal metabolism.

The visceral yolk sac has been shown to synthesise binding proteins for calcium (Delorme et al., 1983), and to take up and store a binding protein for vitamin D (Danan et al., 1985a). Therefore, the visceral yolk sac could regulate the bio-availability of these chemicals to the embryo.

The visceral yolk sac can also hydroxylate various compounds such as vitamin D and testosterone, and this metabolism is energy-dependent (Sheth et al., 1982; Danan et al., 1985b).

The potential of the visceral yolk sac to metabolise macromolecules is studied further in Chapter 4 of this thesis.

1.3 IN VITRO PROCEDURES IN MAMMALIAN DEVELOPMENTAL BIOLOGY

Because of the highly emotive nature of teratological disasters such as the thalidomide tragedy, society now demands that a pregnant woman should run no risk of unintentional exposure to a chemical with an embryotoxic potential. Thus pharmacological and environmental compounds must be proved to have no detrimental effect on normal embryonic development, and this requires the use of animal models to test for embryotoxicity (Beck, 1982a).

The study of mammalian developmental events *in utero* is complicated by a multitude of factors, including difficulty in determining the precise time in gestation, due to a lack of a precise fertilisation time, and difficulty in assessing interactions between maternal, embryonic and extra-embryonic systems. This leads to difficulty in making an accurate assessment of human risk from exposure (Bass and Neubert, 1980). Because of these difficulties, a number of *in vitro* procedures have been pioneered, which can be used both prospectively, as cheap and quick accessories to whole animal studies, to eliminate compounds with high embryotoxic potential, and retrospectively, to assess an *in utero* embryotoxic effect, in terms of species specificity and mechanism of toxicity. As a useful added advantage, these *in vitro* procedures can be utilised as research tools to elucidate mechanisms involved in normal mammalian development, thereby increasing the background data required

to make correct human risk assessments from animal model teratology studies.

The *in vitro* culture systems that have been successfully developed can be classified under the following categories (Neubert, 1982):

i) Isolated cell culture of cells of a set tissue type.
ii) Organ culture of a given anlage.
iii) Culture of whole pre-implantation embryos.
iv) Culture of whole post-implantation embryos.

1.3.1 *Isolated cell culture*

Cellular differentiation of liver, muscle, cartilage and other tissues has been extensively studied morphologically and biochemically, using primary cell cultures (Holtzer *et al.*, 1958; Nau *et al.*, 1977). These cells retain their cellular integrity for up to three days, but show no organotypic development when cultured on serum/nutrient media (Merker *et al.*, 1978).

Recent evidence indicates that synthetic culture media containing various support compounds can maintain primary fetal cell cultures over longer time periods, and that growth of liver cells in cords may indicate a certain degree of cell-cell communication and interaction (Strain, 1989). However, these developmental patterns bear little relationship to normal morphological development, and the normal primary cell cultures are usually used for investigating specific cellular questions. For example, Kremers *et al.*

(1981) used fetal hepatocyte culture to observe the specific pattern of cytochrome P-450 isoenzymes in a particular species, at a specific gestational age.

Experiments by Schacter (1970) using mass culture of chick embryonic limb cells, were developed by Ahrens et al. (1977) to develop the mammalian micromass culture. This culture technique involves the dispersion of blastema cells from embryonic limbs using trypsin. The dispersed cells are cultured in a single drop of culture medium, and after a couple of days, cartilagenous nodules have formed. In addition to observing the differentiation of cartilage, the method allows observation of cell aggregation and condensation, with the formation of cell contacts and specialised functions (Lewis et al., 1978). The method can also be used to observe the differentiation of lung cells (Merker et al., 1981), but both systems have the disadvantage of having no organotypic differentiation.

1.3.2 Organ culture

Organ development from the anlage can be studied in vitro by culture on a solid supporting filter (Trowell, 1954), or submerged in culture medium which is shaken or rotated (Barrach and Neubert, 1980). Many organ anlage can be cultured in this manner, for example, lung, lens, teeth, palatal shelves, bone, limb buds, kidney and pancreas (reviewed in Neubert, Barrach and Merker, 1980 and Neubert, 1982).

Taking limb bud culture as an example, this organ can be cultured on filters (Shepard and Bass, 1970; Beck and Gulamhusein, 1980) or submerged in culture medium in rotating bottles (Neubert et al., 1977). The extent of the differentiation of the cartilagenous anlagen depends on the developmental stage at which the cultures are initiated.

In general, the major disadvantage of organ culture for developmental study is the short time window in gestation available for study, but for researching specific induction or toxin-mediated effects, the organ culture system shows a marked improvement in organotypic development compared to cell culture techniques.

1.3.3 Whole pre-implantation embryo culture

Culture of mammalian embryos from one to two cell stage to blastocyst stage has been developed with pre-implantation stages of mice, rabbits and man (Biggers et al., 1971; Onuma et al., 1968; Steptoe and Edwards, 1978).

The culture methods often involve the non-physiological stimulus of superovulation (Hsu, 1980), where hormonal treatment leads to the number of ovulations being massively increased. However, the viability of the fertilised ova for implantation is low, and the changes initiated to produce this effect are poorly understood.

The culture technique has proved immensely valuable in the field of human embryology, allowing successful pregnancy in a large number of previously infertile women.

1.3.4 Whole post-implantation embryo culture

Any significant growth and development of post-implantation mammalian embryos can only be achieved with embryos surrounded by intact extra-embryonic membranes (Payne and Deuchar, 1972; New, 1978). This means that, of the common experimental animals, rats and mice are exclusively used for this type of culture.

All the early pioneering work on whole embryo culture was carried out in a watch glass apparatus, where a watch glass containing the embryos on or in a nutrient medium, was incubated in a closed Petri dish containing damp cotton wool to give constant humidity.

New and Stein (1964) cultured 9-10 day rat embryos on plasma clots, but New (1966) found that better growth could be obtained using rat serum.

The next major improvement in the culture technique was the culture of embryos in homologous sera in sealed, rotating tubes (New et al., 1973). This technique allowed more efficient oxygenation of the serum, and better nutritional exchange and removal of toxic by-products from around the developing embryo.

Studies by Steele and New (1974) showed that substantially improved development of rat embryos in culture was obtained by using immediately centrifuged whole rat serum, which was heat-inactivated to remove self-recognition factors.

Using this culture technique, rat embryos at early headfold (9.5 days of gestation) could be cultured over 48 hours, developing to the 30-somite stage of late organogenesis with comparable growth and development to the in utero situation (New et al., 1976).

However, due to the increasing demand for oxygen by cells in thickening internal organs, the lack of development of an embryonic/maternal chorio-allantoic placental exchange system restricts the culture system to this stage (New, 1978).

The culture system has similar advantages and disadvantages in mouse embryo culture (Sadler, 1979).

However, the development of a numerical scoring system for morphological development (Brown and Fabro, 1981) has allowed the culture system to be widely used for: teratogenic studies (Kao et al., 1981), study of potential teratogens in human or primate serum (Beck, 1982b; Klein et al., 1981), and assessment of mechanisms involved in normal organogenesis in the rat (Freeman and Lloyd, 1980).

1.4 IN VITRO CULTURE OF VISCERAL YOLK SAC

Sorokin and Padykula (1964) were the first to observe that fragments of yolk sac membrane, when cultured on solid medium in stoppered vessels, would survive and differentiate both biochemically and morphologically in a similar manner to normal yolk sac. This culture technique could be extended from 12.5 days of gestation, for up to two weeks.

Other shorter time-course culture techniques in fluid culture media allowed studies to be carried out on visceral yolk sac function.

Fridhandler and Zipper (1964) incubated sections of visceral yolk sac in Ringer phosphate buffer for one hour, and studied oxygen utilisation and protein and nucleic acid synthesis.

Williams et al. (1975) developed a culture technique where 17.5 day visceral yolk sac membranes were incubated for up to 8 hours in calf serum amd Medium 199, and the yolk sacs were gassed with 95% O_2/5% CO_2. This culture system allowed a large number of workers to establish the pinocytic role of the visceral yolk sac in the provision of macromolecules to the embryo, for example, Weisbecker et al. (1983).

The visceral yolk sac was studied at an earlier stage of gestation, by using the New method of whole embryo culture (New et al., 1973). Gupta et al. (1982) reported that the yolk sac developed in culture over 48 hours, in a similar

manner to the yolk sac *in utero*, with respect to morphological development and vacuolar size. Freeman and Lloyd (1983) used this technique to establish the role of the visceral yolk sac in providing histiotrophic nutrition to the embryo.

By extending the New culture method over eight days, Al-Alousi (1983) observed that the yolk sac continued to expand, but the embryo died and underwent autolysis. Rat chorio-allantoic placenta was known to survive *in utero* following induced embryonic cell death (Huggett and Pritchard, 1945), but this finding established that the yolk sac placenta could also grow, albeit in culture, without the influence of an intact embryo.

Dunton *et al*. (1986) developed this culture system, so that the embryonic pole of a 9.5 day egg cylinder was removed to leave the visceral yolk sac as an intact vesicle, which was then cultured in immediately centrifuged, heat-inactivated rat serum for up to 8 days, gassing every 24 hours with 20% O_2/5% CO_2. The so-called 'giant' yolk sac system was then used to study yolk sac function *in vitro* in terms of protein uptake and utilisation.

The 'giant' yolk sac culture system has the advantages of being organotypic, with the yolk sac developing as a closed vesicle with an external endodermal cell layer overlying a mesenchymal layer of cells, enclosing a closed fluid-filled cavity equivalent to the exocoelomic cavity. The prolonged nature of the culture period means that the yolk sac is available for study over its full range of functions during

gestation. The continuing growth and development of the yolk sac material means that sufficient tissue is generated for many assay procedures.

CHAPTER TWO

Methodology of Yolk Sac Culture

2.1 INTRODUCTION

At the start of this series of experiments, a model of visceral yolk sac development existed - the 'giant' yolk sac. Work in the Department of Anatomy, University of Leicester showed that this *in vitro* model had similar structural morphology to its counterpart *in utero*, after nine days in culture, the only major differences being that the *in vitro* tissue had a greatly increased endodermal vacuolar compartment, and that the mesenchyme was thicker and highly vascularised *in utero*. In terms of function, the 'giant' yolk sac had a similar pinocytic activity for polyvinylpyrrolidine as a corresponding yolk sac *in utero*, and showed similar enzyme activity patterns for acid phosphatase, hexokinase and malate dehydrogenase (Dunton *et al.*, 1986; Gale, 1986). The 'giant' yolk sac did not appear to deposit glycogen in the cytoplasm of its endodermal cells (Dunton *et al.*, 1986), but this situation could be rectified if excess glucose was added to the culture medium (Chue, 1986).

All these experiments used the 'giant' yolk sac culture method which was an extension of the New method of whole embryo culture (New, 1978). Thus the culture conditions established as optimal for whole embryo culture were used in the protracted culture of the 'giant' yolk sac.

The first series of experiments were therefore designed to optimise the culture conditions for yolk sac development, both in terms of scientific parameters, and also in terms of

cost efficiency. In the original 'giant' yolk sac culture technique, each yolk sac cultured for eight days required five millilitres of heat-inactivated rat serum. This means that for an average yield of ten yolk sacs from one mother, nine other rats would have to be sacrificed to provide sufficient serum. Growth of yolk sacs, cultured in different culture media and gas phase conditions, was assessed in terms of size, volume, DNA + protein content, and compared to control yolk sacs cultured by the original method.

The optimal culture conditions would then be used for the remaining experiments, in which histological examination using different stains would be used to examine the structural differences between yolk sacs in culture and in utero. This would not be a complete histological examination, but instead, would aim to provide additional morphological data with which to explain the biochemical and molecular findings of subsequent chapters.

2.2 METHODS

2.2.1 Animal Maintenance

Male and female SPF Wistar rats (Rattus norvegicus) were originally obtained from Charles River, UK, and housed, maintained and bred in the University of Leicester breeding colony.

Female rats were housed in cages in groups of five, while sexually mature male rats were housed individually in mating cages. Male rats were changed at three month intervals, whilst female rats were maintained in the mating stock over a weight range of 200-350g.

Environmental variability was minimised by maintaining a constant temperature (20^{o}C), humidity (60%), and light/dark cycle (12 hours). Food (rat and mouse No. 3 breeding diet - SDS) and water were available ad libitum.

2.2.2 Timed mating procedure

Male and female rats were paired in individual mating cages in the late afternoon. The presence of a sperm plug on the collecting tray under the cage the following morning was taken as an indication of successful fertilisation. Mating usually occurs two hours either side of midnight (Long and Evans, 1922), therefore the female was assumed to be 0.5 days into gestation at noon that day. Pregnant rats were

then tail-marked and kept in dated cages until used for experimentation.

2.2.3 Exsanguination and serum preparation

The pregnant female, at the appropriate age of gestation, or non-pregnant male or female serum donors, were anaesthetised with diethyl ether in a closed perspex box, and when anaesthetised, maintained under anaesthesia by placing a nose cone containing ether-soaked swabs over its head.

The ventral abdominal wall was soaked with 70% methanol to prevent contamination with fur, and a midline incision was made from pubis to xiphoid sternum. The relatively avascular linea alba was then bisected on either side of the incision to allow clear access to the abdominal cavity. The abdominal viscera were displaced to expose the descending aorta between the renal arteries and the bifurcation.

A 10ml syringe and 0.8mm sterile needle were used to draw the maximum quantity of blood from the aorta, after which the diaphragm of the animal was cut to ensure no recovery was possible. The blood was immediately centrifuged in a bench centrifuge at 3000rpm for 10 minutes. The tubes were allowed to stand for more than thirty minutes to ensure that a cell-free fibrin clot had formed, then the clots were squeezed with heat-sterilised forceps, and the tubes recentrifuged at 3000rpm for 5 minutes. The resulting clear supernatant (serum) was removed using a sterile needle and syringe, and pooled in 20ml aliquots containing 100 IU/ml

penicillin and 100µg/ml streptomycin (GIBCO, Paisley, UK). Serum was stored at -20°C. Prior to use, complement factors were denatured by heat-inactivating the serum at 56°C for 30 minutes, then equilibrating at 37°C.

2.2.4 Explantation of a 9.5 day rat conceptus

After exsanguination of a pregnant female rat at 9.5 days of gestation, the uterus was dissected free of the ovaries, vagina and associated fat.

The uterus was sectioned into individual segments containing one decidual mass (Fig. 2.1) and placed in autoclaved Hank's Balanced Salt Solution (Hank's BSS - see Appendix A), in a sterile Petri dish. The explantation procedure was then performed in a sterile laminar flow cabinet, under a dissecting microscope.

The muscular wall of the uterine segment was torn along the antimesometrial margin to expose the tip of the decidual mass, and this pear-shaped mass (Fig. 2.2) was teased free of the remaining uterine material, using two pairs of watchmakers' forceps. Because the conceptus lies in the narrower end of the decidua, a vertical incision was made in the broader base of the decidua, and by pulling obliquely on each side of this incision, the decidual mass was pulled in half, leaving the concpetus exposed on the surface of one of the halves (Fig. 2.3). The conceptus (Fig. 2.4) was removed from the decidual tissue and parietal endoderm by gently stroking under the conceptus with a closed pair of forceps.

Figure 2.1 Segment of 9.5 day pregnant rat uterus (x 7.4)

The decidual mass was exposed by ripping the antimesometrial wall of the uterus from a to b.

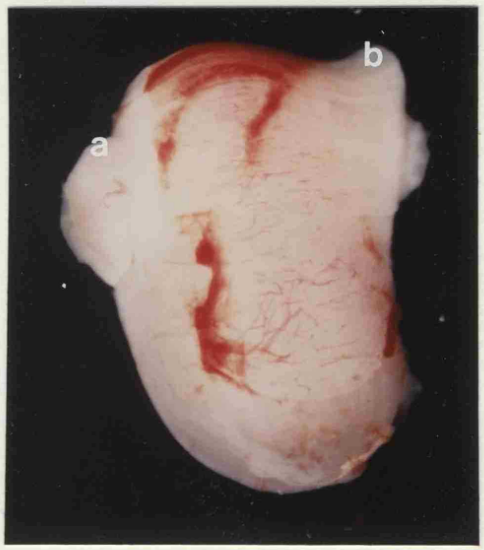
Figure 2.2 9.5 day decidual mass (x 9.3)

An incision was made at point (p), and ripped downwards to form two arms of the decidual mass, which could then be pulled in half.

Figure 2.3 9.5 day half decidual mass (x 11.9)

The egg cylinder (ec) was teased out of the decidual mass using closed watchmakers forceps.

2.1



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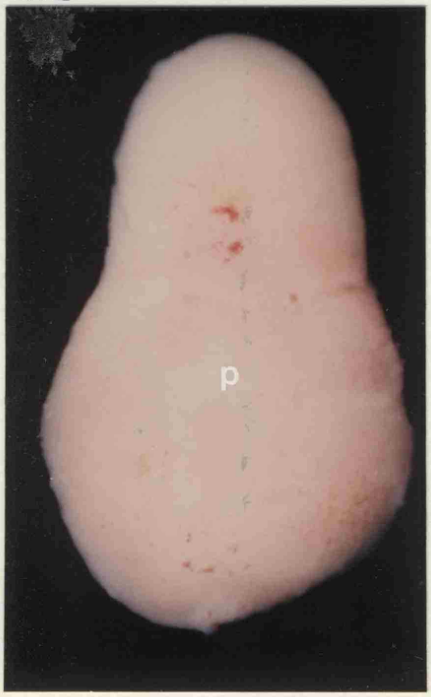

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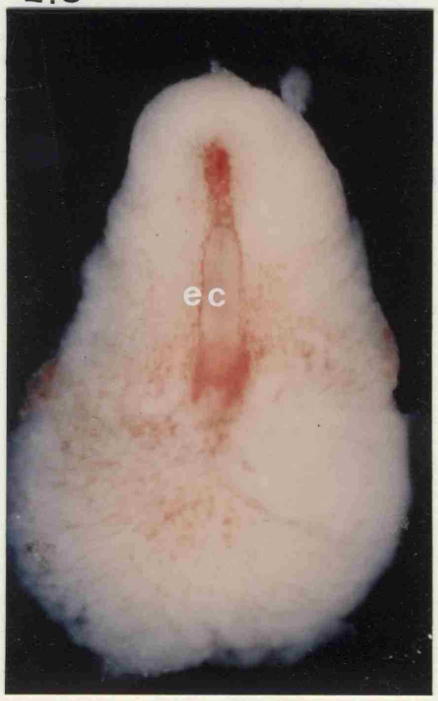

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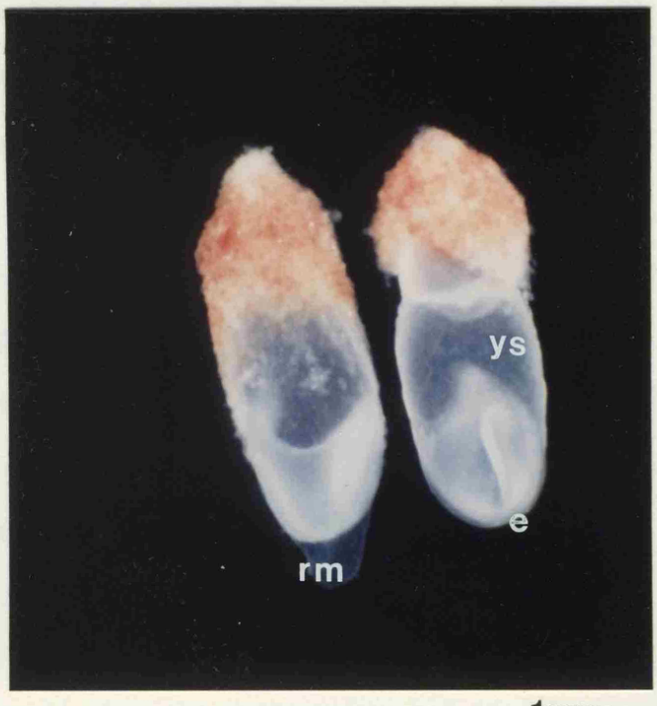
Figure 2.4 9.5 day egg cylinder (x 23.7)

The Reichert's membrane (rm) was removed to allow expansion of the egg cylinder. The embryonic disc (e) can be seen to be polar to the visceral yolk sac (ys).

Figure 2.5 Removal of 9.5 day embryo (x 37.1)

The polar embryonic disc was removed by single scalpel incision, ensuring that all embryonic material was removed. This left the yolk sac as a closed vesicle with ecto-placental cone (epc).

2.4



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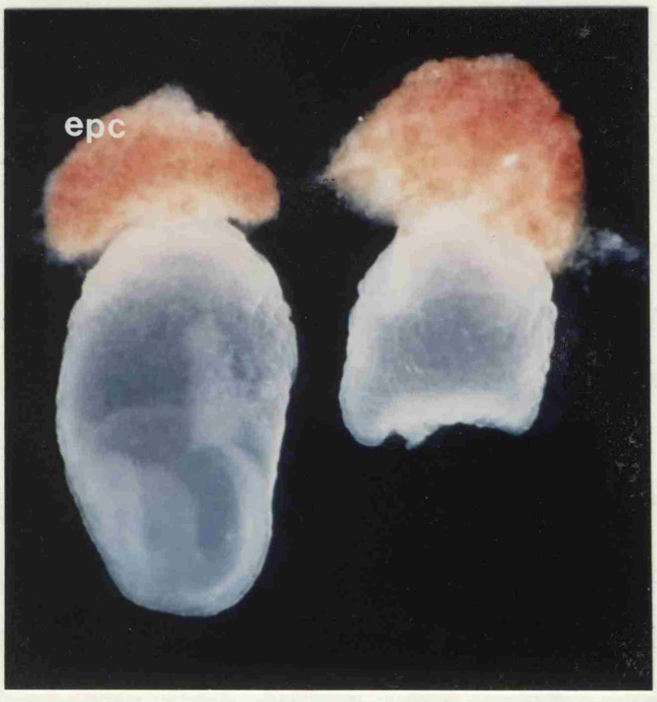
2.5



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The penultimate step of the explantation procedure was the removal of Reichert's membrane, by tearing it at the embryonic pole of the conceptus, and displacing it upwards to the ectoplacental cone, where it was torn off, taking care not to remove the ectoplacental cone (Fig. 2.4).

The final step was to remove the embryonic disc by a single incision with a size 15 sterile scalpel blade, making sure the angle of incision removed all the embryonic material including the developing allantoic bud. The single incision allowed the cut surfaces of the yolk sac to come into contact, and hence healing could be initiated (Dunton, 1988), and a closed vesicle formed (Fig. 2.5).

2.2.5 Original 'giant' yolk sac culture

This is the method first described by Dunton et al. (1986), modified from the original method of Al-Alousi (1983).

Following explantation, the 9.5 day yolk sac vesicles were cultured in 60ml sterile glass bottles, rotating horizontally in a roller incubator at 37^{o}C, as described by New (1973). Each vesicle was cultured in 1ml of immediately centrifuged, heat-inactivated whole rat serum, and a maximum of seven conceptuses were placed in each bottle. The serum was introduced to the bottles, prior to the addition of the yolk sacs, using a sterile syringe and bent needle, thus avoiding bubble formation, to which the yolk sacs could adhere. The yolk sacs were added to each bottle, and the

culture initially gassed with a mixture of 5% O_2; 5% CO_2; 90% N_2 for 2 minutes. Subsequent gassing was carried out every 24 hours with a mixture of 20% O_2; 5% CO_2; 75% N_2. The total culture period was usually 8 or 9 days.

After 2 days of culture the yolk sacs were transferred to bottles containing fresh serum, a maximum of three yolk sacs being placed in each bottle. The culture serum was changed every 2-3 days thereafter, used serum being carefully removed from the culture bottles using a syringe and bent needle, great care being taken not to damage the yolk sac membranes. Fresh serum was immediately placed in the bottles using a new syringe and bent needle, the flow of serum being aimed at the side of the bottle to create the least disturbance to the yolk sacs. Throughout the procedure the culture bottles were maintained in the horizontal position.

At the designated end of the culture period, the yolk sacs were harvested by a method which allowed them to remain as intact vesicles. The culture serum was removed using a syringe and bent needle. Using the same method for introduction and removal of fluid, the yolk sacs were gently washed with Hank's BSS at 37^{o}C. A 10ml volume of Hank's BSS was then added to the culture bottle, the bottle was stoppered, and then inverted under the surface of a glass container of 1% (w/v) NaCl. By opening the neck of the culture bottle, the yolk sacs sank into the 1% (w/v) NaCl, and were picked up on small disposable Petri dish lids.

2.2.6 Changing culture parameters

a) Serum concentration

Yolk sacs were cultured for 8 days from 9.5 days, gassing initially with 5% O_2; 5% CO_2; 90% N_2, and then every 24 hours with 20% O_2; 5% CO_2; 75% N_2.

Culture medium was changed at either day 2, 4 and 6 days, at day 2 and 5, or at day 4.

Having chosen the best serum changing regimen, the serum concentration was altered by diluting immediately centrifuged, heat-inactivated whole rat serum with either Hank's BSS or Medium 199 (GIBCO - see Appendix B). Dilutions of 0, 10, 25, 33, 50, 75 and 100% rat serum were prepared from the same serum sample, and used as culture media.

b) Oxygen tension

Selecting the serum concentration giving the best overall culture performance, the next set of experiments investigated the oxygen requirements of the yolk sac, by changing the gas mixtures used for reoxygenating the culture medium. The gas mixtures available were: 5% O_2; 5% CO_2; 90% N_2: 20% O_2; 5% CO_2; 75% N_2: 40% O_2; 5% CO_2; 55% N_2: and 95% O_2; 5% CO_2.

Six different gas regimens were employed, designated A, B, C, D, E and F.

Gas Regimen	Days of Culture								
	0	1	2	3	4	5	6	7	8
A	5% O_2	->	->	20% O_2	->	->	->	->	->
B	5% O_2	20% O_2	->	->	->	->	->	->	->
C	5% O_2	20% O_2	->	40% O_2	->	->	->	->	->
D	5% O_2	20% O_2	->	->	->	40% O_2	->	->	->
E	5% O_2	20% O_2	->	95% O_2	->	->	->	->	->
F	5% O_2	20% O_2	->	40% O_2	->	95% O_2	->	->	->

Each culture bottle was gassed for 1 minute every 24 hours, using a sterile Pasteur pipette, the tip being placed clear of the culture medium at the closed end of the bottle.

2.2.7 Assessment of yolk sac growth

All the yolk sacs from the serum concentration and oxygen tension experiments were harvested after 8 days in culture, and their growth assessed by the following series of tests:

a) Yolk sac diameter

The glass container of 0.1 (w/v) NaCl was placed under a dissecting microscope, and the diameter of the free-floating cultured yolk sac (Fig. 2.7) was measured using an eyepiece graticule.

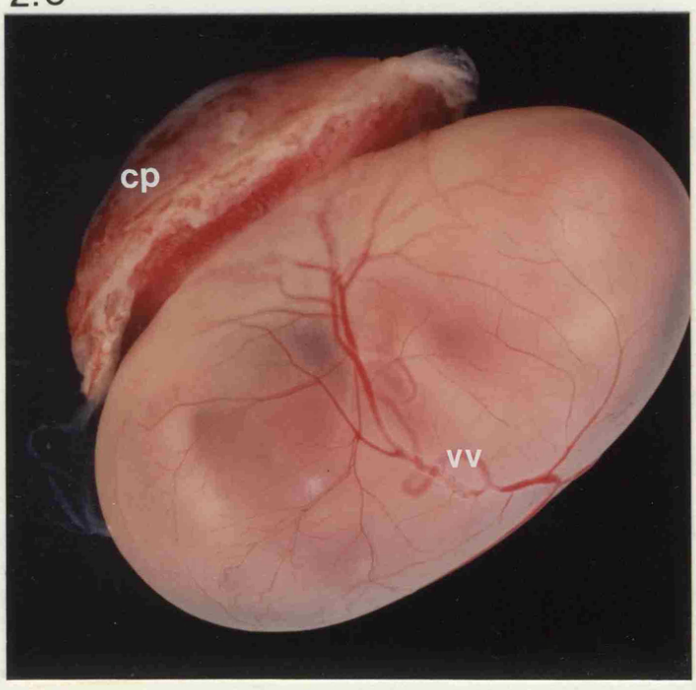
Figure 2.6 17.5 day rat conceptus (x 3.7)

The 17.5 day fetus is surrounded by the highly vascularised visceral yolk sac, with major vitelline vessels (vv) supplying the fetus. The other placental system - the chorio-allantoic placenta (cp) is also evident.

Figure 2.7 17.5 day cultured yolk sac (x 4.6)

The cultured yolk sac has developed from 9.5 days in serum-based culture medium in a rotating gassed bottle at 37^{o}C.

2.6



1cm

2.7

1cm

b) Extra-embryonic coelomic fluid volume

The yolk sac was removed from the container in a Petri dish lid and all the surrounding fluid removed by syringe. The yolk sac was then punctured with forceps, and the membrane removed and placed in a labelled tube on ice. The volume of the released fluid from the interior of the vesicle was then measured in a graduated 1ml syringe.

The yolk sac membranes were pooled in groups of six, 1ml of 0.1M phosphate buffered saline was added, and the yolk sacs were homogenised with a Potter-Elvejhem homogeniser, by six full strokes of a Teflon pestle, at $0^{o}C$.

c) Viability

The viability of yolk sacs cultured under different conditions was assessed as a simple percentage; the number of surviving yolk sacs after eight days as a percentage of the number of yolk sacs initially cultured at 9.5 days of gestation.

d) Protein content

The protein content of the yolk sacs was estimated by a modification of the method of Lowry et al. (1951).

0.1ml of yolk sac homogenate was mixed with 0.9ml of 1M NaOH, incubated at room temperature for 60 minutes, then neutralised with 0.145ml of 3M HCl. The resulting solution

was split into two duplicate 0.5ml aliquots, to which 2.5ml Folin A solution (see Appendix C) was added. After 20 minutes, the samples were mixed, and during mixing 0.25ml of Folin B solution (see Appendix C) was added. The samples were allowed to stand at room temperature for 45 minutes, then the absorbance was measured at 750nm on a LKB Ultra-spectrophotometer. The protein content of a sample was estimated by reading the absorbance of a series of standard bovine serum albumin solutions (0-1mg/ml), treated in the same manner.

e) DNA content

The DNA content of the yolk sac homogenates was estimated by the fluorimetric method described by Labarca and Paigen (1980).

0.1ml of yolk sac homogenate was further diluted by the addition of 9.9ml of 0.1M Na_2PO_4, 2.0M NaCl, 5mM EDTA. This diluted homogenate was split into two 0.5ml aliquots which were mixed with equal volumes of 2µg/ml bisbenzimidazole (Hoechst 33258 supplied by Aldrich Chemicals), and left to incubate at room temperature for 8 hours. Standard solutions of calf thymus DNA (0.10µg/ml) were treated in the same manner.

The fluorescence of the solutions was then read at λex = 358nm/λem = 460nm, with excitation and emission slit widths of 2.5nm, on a Perkin-Elmar LS5B spectrofluorimeter.

Four separate aliquots of 100%, 50% and 33% serum-cultured yolk sac homogenates were assayed for DNA content by the modification of the method of Burton (1955), described by Giles and Myers (1965).

2.2.8 Collection of in utero yolk sac material for comparative studies

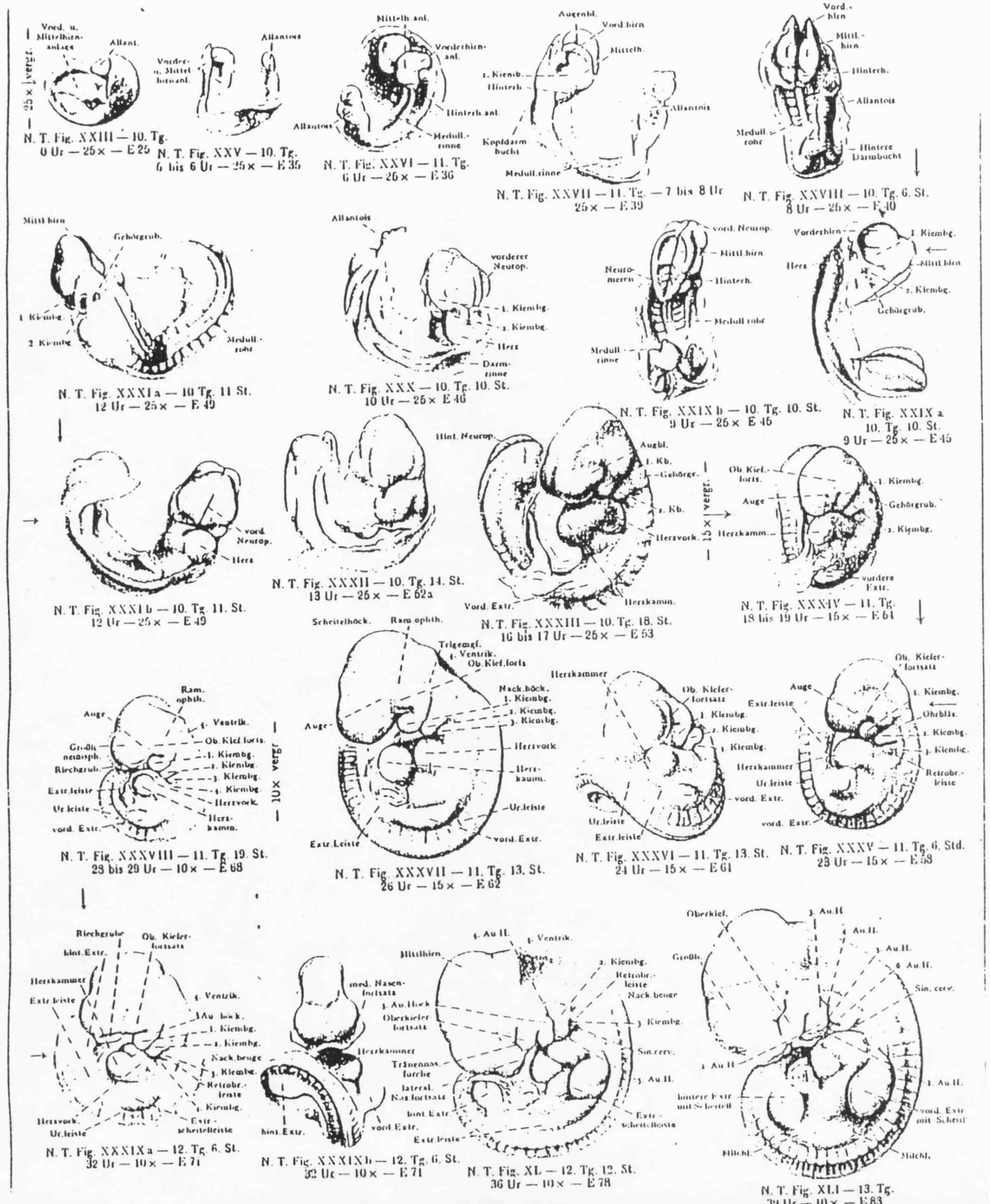
After defining the optimal culture conditions for growth of the visceral yolk sac in culture, yolk sacs from a comparable gestational age were collected to compare the cultured visceral yolk sac to its counterpart in utero.

The timed mated 17.5 day pregnant dam was sacrificed by the exsanguination method described in section 2.2.3, and the whole uterus removed, and placed in ice-cold Hank's BSS. The uterine wall was opened along the length of the anti-mesometrial border, and the exposed conceptuses were released by teasing the chorio-allantoic placenta away from the uterine mucosa. The conceptuses (Fig. 2.6) were transferred to fresh ice-cold Hank's BSS, where the chorio-allantoic placentae were removed using curved scissors. The resulting hole in the visceral yolk sac was enlarged, and the yolk sac inverted to release the fetus. The vitelline vessels and amnion were sectioned away from the visceral yolk sac, and discarded.

The fetal age was verified by checking topographical features against the diagrams of Keibel (1937; Fig. 2.8).

Figure 2.8 Drawings of the development of the rat from 10 days of gestation to 20 days of gestation, showing surface morphology development and crown-rump length (Keibel, 1937).

These drawings were used to accurately assess the gestational age of visceral yolk sac material removed in the last half of gestation.



N. T. Fig. XXIII — 10. Tg. 0 Ur — 25× — E 25

N. T. Fig. XXV — 10. Tg. 6 bis 6 Ur — 25× — E 35

N. T. Fig. XXVI — 11. Tg. 6 Ur — 26× — E 36

N. T. Fig. XXVII — 11. Tg. — 7 bis 8 Ur 25× — E 39

N. T. Fig. XXVIII — 10. Tg. 6. St. 8 Ur — 26× — E 40

N. T. Fig. XXXI a — 10 Tg. 11 St. 12 Ur — 25× — E 49

N. T. Fig. XXX — 10. Tg. 10. St. 10 Ur — 25× E 46

N. T. Fig. XXIX b — 10. Tg. 10. St. 9 Ur — 25× E 46

N. T. Fig. XXIX a 10. Tg. 10. St. 9 Ur — 25× — E 45

N. T. Fig. XXXI b — 10. Tg. 11. St. 12 Ur — 25× — E 49

N. T. Fig. XXXII — 10. Tg. 14. St. 13 Ur — 25× — E 62a

N. T. Fig. XXXIII — 10. Tg. 18. St. 16 bis 17 Ur — 26× — E 53

N. T. Fig. XXXIV — 11. Tg. 18 bis 19 Ur — 15× — E 64

N. T. Fig. XXXVIII — 11. Tg. 19. St. 23 bis 29 Ur — 10× — E 68

N. T. Fig. XXXVII — 11. Tg. 13. St. 26 Ur — 15× — E 62

N. T. Fig. XXXVI — 11. Tg. 13. St. 24 Ur — 15× — E 61

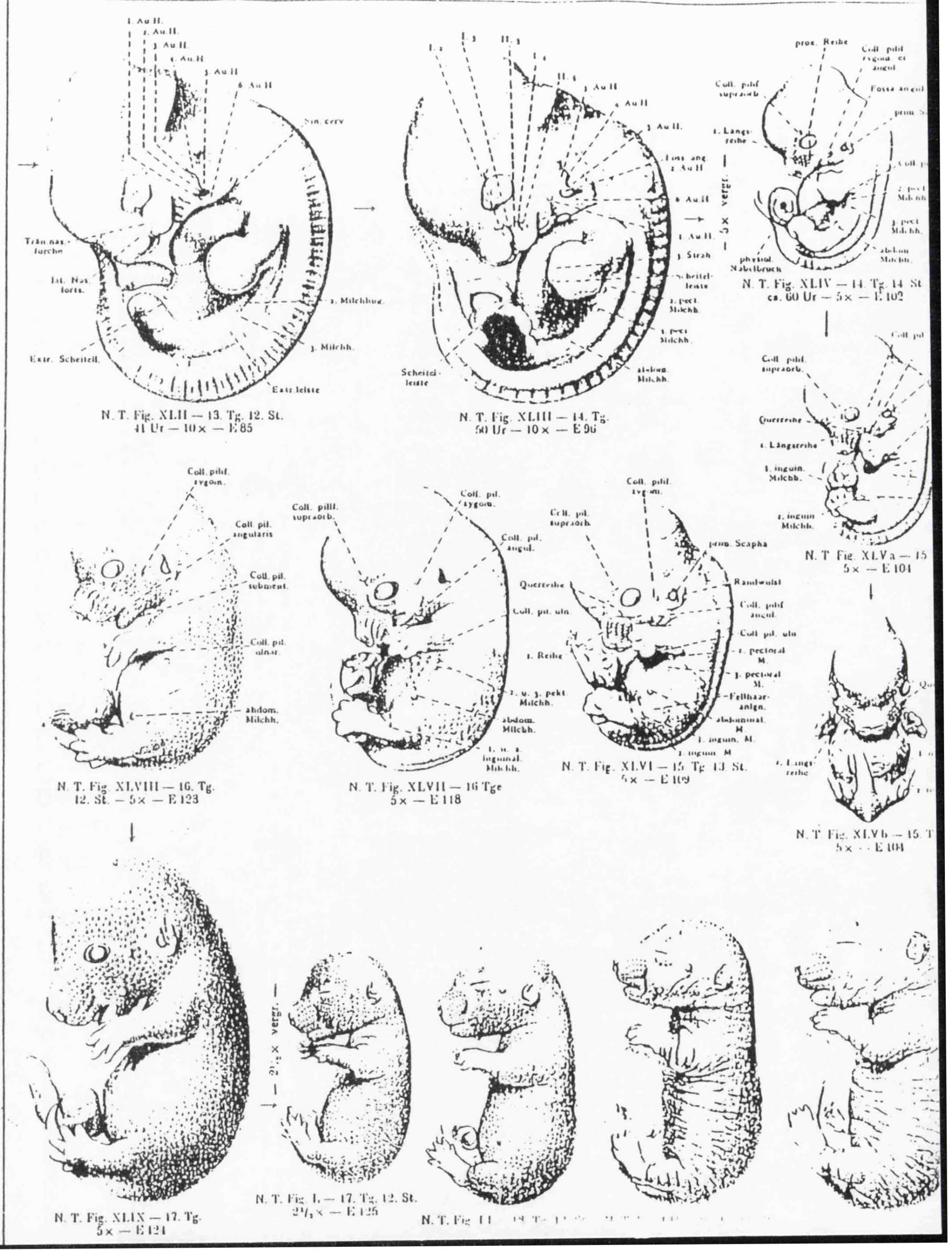
N. T. Fig. XXXV — 11. Tg. 6. Std. 23 Ur — 15× — E 58

N. T. Fig. XXXIX a — 12. Tg. 6. St. 32 Ur — 10× — E 71

N. T. Fig. XXXIX b — 12. Tg. 6. St. 32 Ur — 10× — E 71

N. T. Fig. XL — 12. Tg. 12. St. 36 Ur — 10× — E 78

N. T. Fig. XLI — 13. Tg. 39 Ur — 10× — E 83



N. T. Fig. XLII — 13. Tg. 12. St. 41 Ur — 10× — E 85

N. T. Fig. XLIII — 14. Tg. 50 Ur — 10× — E 96

N. T. Fig. XLIV — 14. Tg. 14. St. ca. 60 Ur — 5× — E 102

N. T. Fig. XLV a — 15 5× — E 104

N. T. Fig. XLVIII — 16. Tg. 12. St. — 5× — E 123

N. T. Fig. XLVII — 16 Tge 5× — E 118

N. T. Fig. XLVI — 15. Tg. 13. St. 5× — E 109

N. T. Fig. XLV b — 15. T 5× — E 104

N. T. Fig. XLIX — 17. Tg. 5× — E 124

N. T. Fig. L — 17. Tg. 12. St. $2^1/_2$× — E 125

N. T. Fig. LI — 19. Tg. …

2.2.9 Histological comparison of cultured and in vivo yolk sacs

Freshly removed 17.5 day visceral yolk sac, and cultured yolk sac harvested after 8 days in culture, were placed in 4% (w/v) paraformaldehyde at rtp for 24 hours. The fixed material was then washed in distilled water, and sequentially dehydrated in graded alcohols, before passing through two changes of xylene for a day to clear. The yolk sac material was then embedded in paraffin wax (Monoject) at 62^{o}C under vacuum.

5µm sections were cut on a Leitz microtome, and taken up on clean glass slides. Sections were rehydrated by clearing in xylene and passing through graded alcohols to water, and were then stained using one of three staining techniques, following protocols described by Cook (1974):

a) Haematoxylin and eosin

Sections were dipped in Harris's haematoxylin for 1 minute, rinsed in distilled water, dipped in acid alcohol for 1 minute, then blued in running tap water. The sections were then quickly rinsed in eosin for 5 seconds, dehydrated through graded alcohols to xylene, then coverslipped with DPX mountant (BDH).

b) Periodic acid - Schiff's

Sections were placed in 1% (w/v) periodic acid for 20 minutes, rinsed with distilled water, then stained with Schiff's reagent (BDH) for 15 minutes. The sections were rinsed again with distilled water, and counter-stained for 1 minute with Harris's haematoxylin (see haematoxylin and eosin staining). The sections were then dehydrated, cleaned and coverslipped as described previously.

c) Picro-Mallory's trichrome

Sections were stained in 1% alcian green 2GX in acetic acid for 3 minutes, washed, then stained with iron haematoxylin for 30 minutes and blued in tap water. A second 3 minute stain with alcian green was followed with 0.2% orange G in alcoholic picric acid for 7 minutes. The sections were differentiated in water for 5 seconds, then treated in Ponceau-acid fuchsin (0.5% Ponceau 2R/0.5% acid fuchsin in 1% acetic acid) for 2 minutes. After rinsing with 2% acetic acid and differentiating with 'red' differentiator, the sections were stained with filtered 2% aniline blue for 10 minutes, rinsed with 2% acetic acid, and differentiated in 'blue' differentiator. After a final rinse in 2% acetic acid, the sections were dehydrated, cleaned and coverslipped. 'Red' and 'blue' differentiator solutions were made up from stock phosphotungstic/picric acid solution.

2.3 RESULTS

2.3.1 DNA content

Measurement of yolk sac DNA content using the method of Labarca and Paigen (1980) proved unsuccessful, due to large variations in content within experimental groups, and non-correlation of DNA content when compared to a single measurement, using the Giles and Myers (1965) modification of the diphenylamine reaction described by Burton (1955).

The DNA content of yolk sacs cultured in 100%, 50% and 33% serum are shown in Table 2.1, expressed in terms of µg DNA/mg protein.

Although the fluorescence procedure, when applied to replicate aliquots, gave a maximum variability in DNA content of 1.2%, when the procedure was applied to yolk sac samples cultured under the same conditions, the variability ranged from 12% to 83%.

For duplicate aliquots of cultured yolk sac homogenate, cultured in 100% rat serum, the DNA content varied by up to 40µg, when results from the Labarca and Paigen assay were compared to the diphenylamine reaction assay.

2.3.2 Serum concentration

Changing serum only once during the eight day culture period caused significant decreases in all parameters measured (protein content, diameter, exocoelomic fluid

Table 2.1

DNA content of cultured yolk sacs grown in different concentrations of whole rat serum

% Serum	DNA content (mg DNA/mg protein)		
	I	II	III
100	46.72	46.37	82.17
100	70.36	70.24	74.25
100	52.67	52.47	80.64
100	77.40	77.30	77.62
50	90.47	90.49	86.38
50	62.36	62.39	82.46
50	48.71	48.48	74.65
50	84.74	85.01	79.81
33	52.41	52.95	40.14
33	22.47	21.94	36.24
33	74.13	74.06	39.46
33	12.16	12.18	35.44

I and II represent duplicate DNA contents measured by the fluorimetric method of Labarca and Paigen (1980). III represents DNA content measured by the method of Giles and Myers (1965). Protein content was estimated by the method of Lowry et al. (1959).

Immediately centrifuged, heat-inactivated whole rat serum was diluted with Medium 199.

Table 2.2

The effect of number of serum changes on growth parameters of the cultured yolk sac

No. of Serum Changes	Growth Parameters of Cultured Yolk Sacs after 8 days			
	Protein Content (mg)	Diameter (mm)	Exocoelomic Fluid Volume (ml)	Viability (%)
3	2.04 ± 0.11	16.2 ± 1.1	0.51 ± 0.02	82.7 ± 1.2
2	2.01 ± 0.07	16.4 ± 1.3	0.53 ± 0.04	88.2 ± 0.9
1	1.44 ± 0.14	10.1 ± 1.7	0.39 ± 0.04	63.4 ± 1.4

Serum was changed at 4 days (1 change), 2 and 5 days (2 changes) or 2, 4 and 6 days (3 changes).

Estimation of growth parameters was made on 24 observations for each experimental group.

volume and viability) when compared to changing the serum after 2 days, 4 days and 6 days or 2 days and 5 days (Table 2.2). Results in this table also show a significant mean increase in viability of 5.5 ± 1.3%, if the serum was only changed twice ($p<0.05$).

Therefore, using two changes of serum in the protocol, the effect of changing serum concentration was investigated.

Firstly, pH and osmolality of the culture media were measured, on ten separate occasions, using a standardised Phillips PW 9409 pH meter and a calibrated Camlab C11 osmometer.

Serum concentration (%)	pH range	Osmolality range
100	7.2 - 8.1	280 - 365
75	7.2 - 8.0	290 - 355
50	7.3 - 7.6	285 - 360
33	7.3 - 7.6	280 - 340
25	7.3 - 7.6	295 - 315
10	7.3 - 7.7	280 - 325
0	7.5 - 7.7	290 - 320

There were no detectable changes in the range of pH or osmolality results as serum concentration was decreased, but a trend of narrowing of the range was observed as more Medium 199 was introduced.

Changes in yolk sac diameter, protein content, exocoelomic fluid volume and viability were plotted against

serum concentration in the culture medium diluted with Medium 199 (Fig. 2.9).

Growth increased in all parameters measured. as serum concentration increased, with the number of yolk sacs being:

% rat serum	No. of yolk sacs
0	12
25	30
33	58
50	84
100	112

Comparing the results with 50% serum to 100% control yolk sacs, there was no significant difference in any of the parameters. When the results with 33% serum and 100% serum were compaerd, yolk sac diameter, protein content and exocoelomic fluid volume were all significantly different ($p < 0.005$) using Student's 't' test. There was no significant decrease in viability of the cultured yolk sacs until no serum was present in the medium, when no yolk sacs survived the culture period.

2.3.3 Oxygen tension

Using 50% serum diluted with Medium 199 as the culture medium, and maintaining one minute gassing per day, six different gassing regimens were used, designated A-F, and

the resulting changes in yolk sac growth are shown in Fig. 2.10.

Yolk sacs cultured by the original method, using an initial gas mixture of 5% O_2, followed by subsequent gassing with 20% O_2, (B), had a mean diameter of 16.34mm, a protein content of 2.15mg, an internal fluid volume of 0.54ml, and a viability of 85% (n = 63).

Increasing the oxygen concentration to 40% O_2 after 72 hours (C) and 120 hours (D) in culture, had no significant effect on yolk sac diameter, protein content or exocoelomic fluid volume after 8 days in culture (n = 46 and 40 respectively). However, by increasing the oxygen concentration after 72 hours, there was a significant rise in viability to 93 ± 3% ($p < 0.05$).

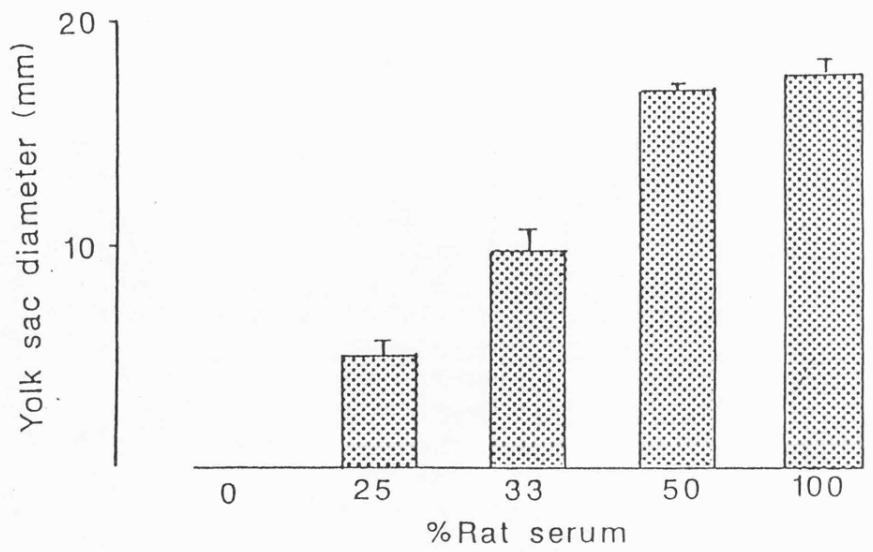
Continuing to gas with low oxygen concentration mixture for 72 hours (A) resulted in decreased growth in all parameters except viability.

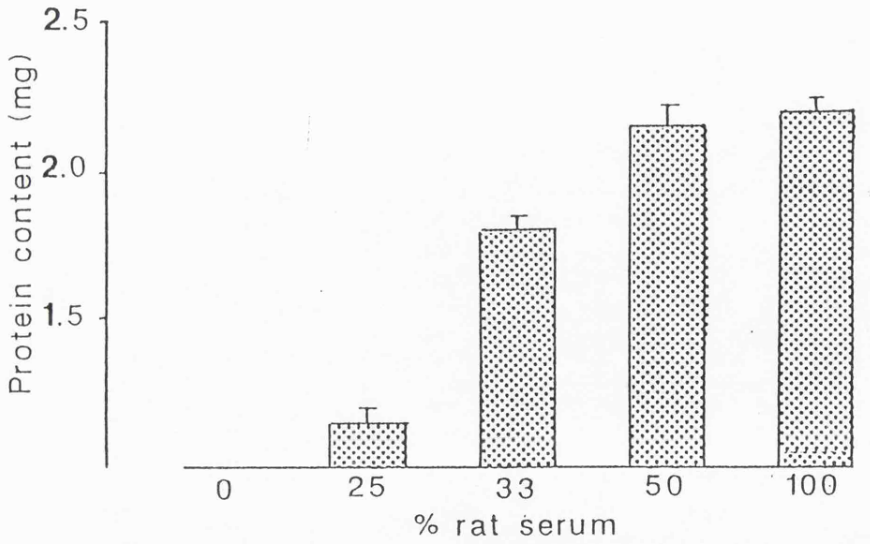
Increasing the oxygen concentration to 95% O_2 after 72 hours (E) and 120 hours (F) caused a large shrinkage in the yolk sac vesicle, and no subsequent recovery over the 8 day culture period. This event was delayed by 18-24 hours after a single gassing with 95% O_2.

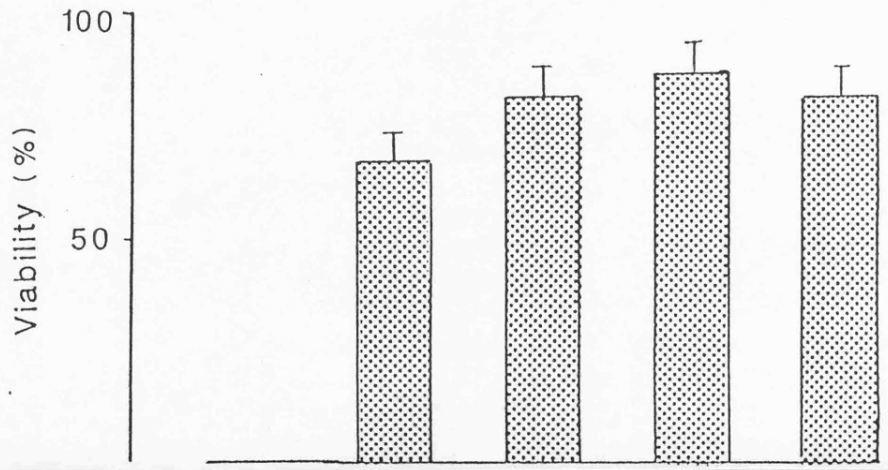
On five separate occasions, the culture media were removed two hours after gassing with the different mixtures, and assessed for pH and oxygen tension.

Figure 2.9 Parameters of 17.5 day cultured yolk sac growth versus serum concentration in the culture medium

Immediately centrifuged, heat-inactivated rat serum was diluted in Medium 199, and culture medium was changed after 2 and 5 days in culture. Yolk sacs were maintained in roller culture at 37°C, gassed every 24 hours with $O_2/CO_2/N_2$ mixture, and harvested after 8 days in culture.


Yolk sac diameter (mm)
20
10
0
25
33
50
100
%Rat serum


Protein content (mg)
2.5
2.0
1.5
0
25
33
50
100
% rat serum


Viability (%)
100
50

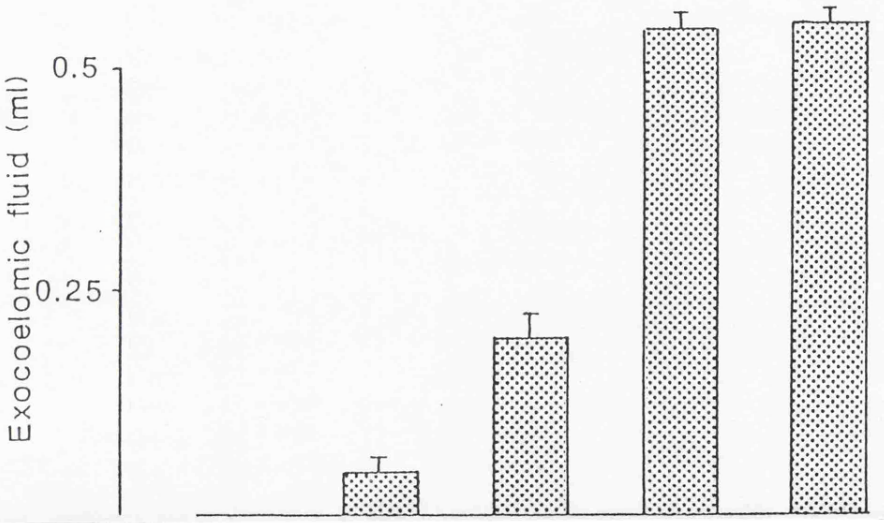
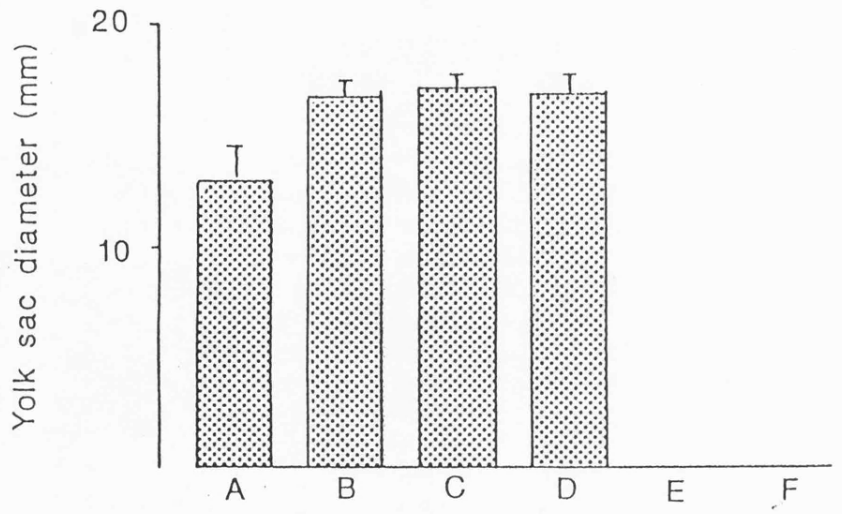

Exocoelomic fluid (ml)
0.5
0.25

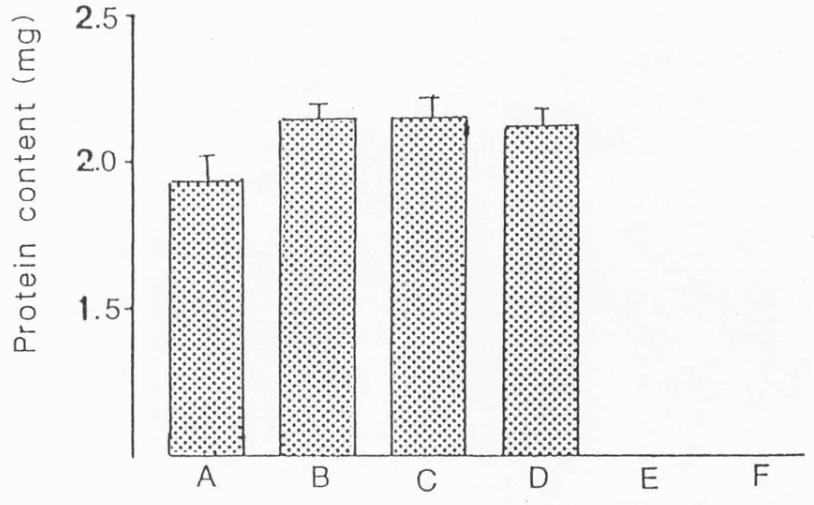
Figure 2.10 Parameters of 17.5 day cultured yolk sac growth versus different oxygen tension

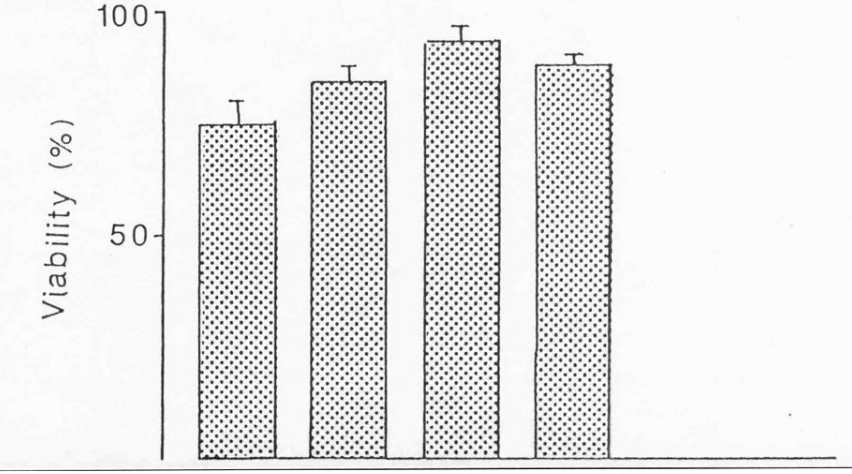
Yolk sacs were maintained in roller culture at 37°C and gassed every 24 hours. Culture medium was changed after 2 and 5 days.

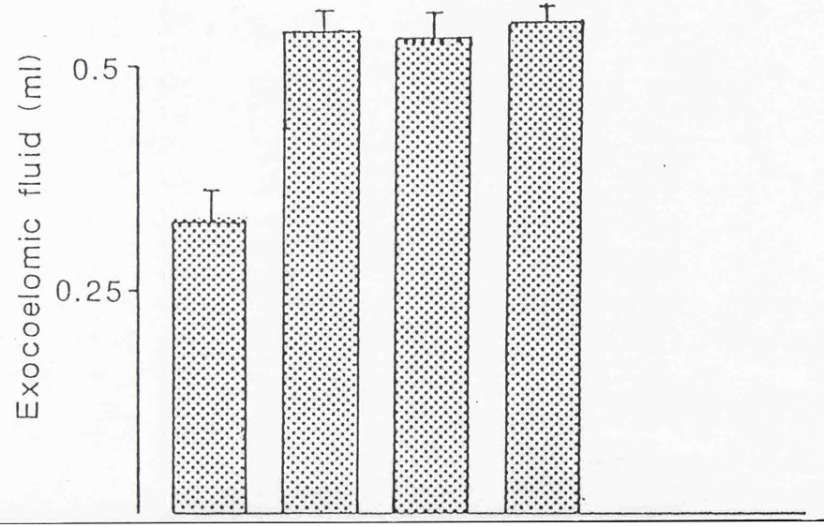
A-F represent different oxygen concentrations in the gas medium.

A represents an elongation of 5% O_2 gassing from 24 to 72 hours.
B represents control gassing - 5% O_2 for 24 hours followed by 20% O_2.
C represents an increase to 40% O_2 after 72 hours.
D represents an increase to 40% O_2 after 120 hours.
E represents an increase to 95% O_2 after 72 hours.
F represents an increase to 95% O_2 after 120 hours.


Yolk sac diameter (mm)
20
10
A
B
C
D
E
F


Protein content (mg)
2.5
2.0
1.5
A
B
C
D
E
F


Viability (%)
100
50


Exocoelomic fluid (ml)
0.5
0.25

% O_2	pH	pO_2 (mm Hg)
5	7.43 ± 0.07	52 ± 3
20	7.42 ± 0.06	140 ± 6
40	7.46 ± 0.04	190 ± 4
95	7.56 ± 0.08	703 ± 7

pH was measured using a standardised Phillips PW 9409 pH meter, whilst pO_2 was measured using a Yellow Springs Instruments 5301 oxygen electrode.

2.3.4 Histological assessment

Figs. 2.11a-c show sections of 17.5 day in vivo yolk sac stained with haematoxylin and eosin. In Fig. 2.11b, the plane of section passes through the 'rugose' area of the visceral yolk sac adjacent to the chorio-allantoic placenta. Larger capillaries, lined with endothelium, are visible in the core of the villous projections. Small capillaries are visible in the mesenchyme in Fig. 2.11c, and these vessels can be seen to contain nucleated erythrocytes. In both high power magnifications, variation in the degree of vacuolation of the endodermal cells can be seen.

In comparison, there is a lower degree of vascularisation apparent in Figs. 2.12a-c. These are sections of 17.5 day cultured yolk sac stained with haematoxylin and eosin. The

nucleoli of the endoderm cell nuclei are far more apparent than in Fig. 2.11.

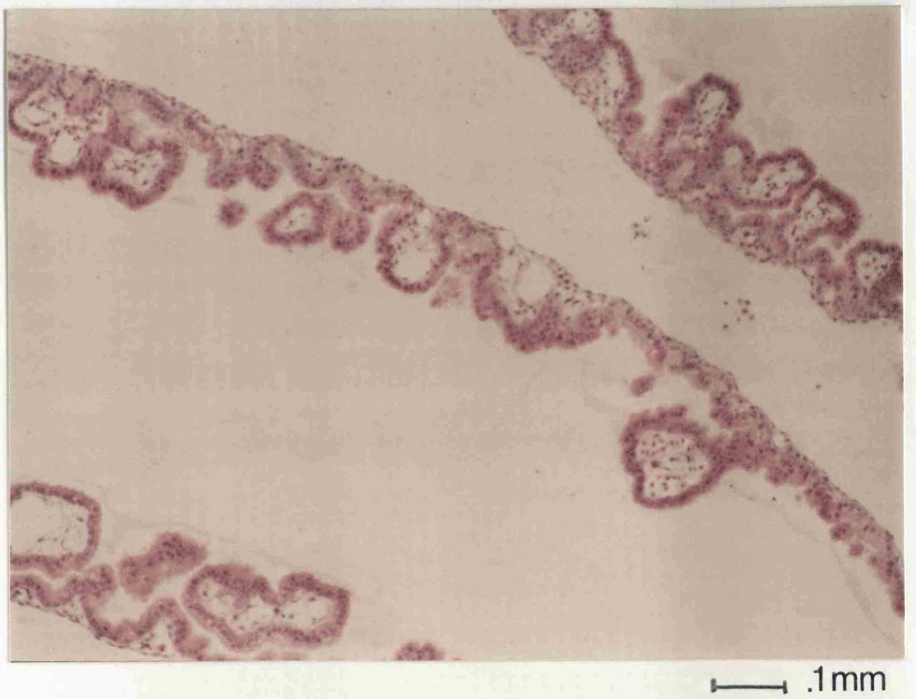
Fig. 2.13a is a section of 17.5 day in vivo yolk sac, and Fig. 2.13b is a section of 17.5 day cultured yolk sac. Both sections are stained by the periodic acid-Schiff's reaction, and counterstained with haematoxylin. In Fig. 2.13b the PAS positivity is confined to the apical cytoplasm of the endodermal cells which are intensely pink. The PAS positivity in Fig. 2.13a is also in the apical cytoplasm of the endodermal cells, but also in the mesothelium.

Figs. 2.14 and 2.15 are stained with the Picro-Mallory trichrome stain. 17.5 day in vivo yolk sac (Fig. 2.14a and Fig. 2.15a) endodermal cells have a pale green acid mucin-positive apical cytoplasm, and numerous red staining vacuoles. The mesothelium has a broad band of pale blue collagen underlying the cells, and this connective tissue band can also be seen in a thinner form underlying the endothelium of the large and small blood vessels. The 17.5 day cultured yolk sac (Fig. 2.14b and Fig. 2.15b) has fewer red vacuole-containing endodermal cells, but in those cells that do contain them, they are more abundant. There is no evidence of any collagen deposition under the mesothelium, or around the capillaries. Blood cells resembling late normoblasts can be seen in a number of capillaries in Fig. 2.15b.

Figure 2.11 5μm paraffin sections of 17.5 day *in vivo* yolk sac stained with haematoxylin and eosin x 95 (a) or x 238 (b and c).

The visceral yolk sac consists of endoderm (e) and mesoderm (m) cells with numerous blood capillaries (c) in the mesenchyme.

a



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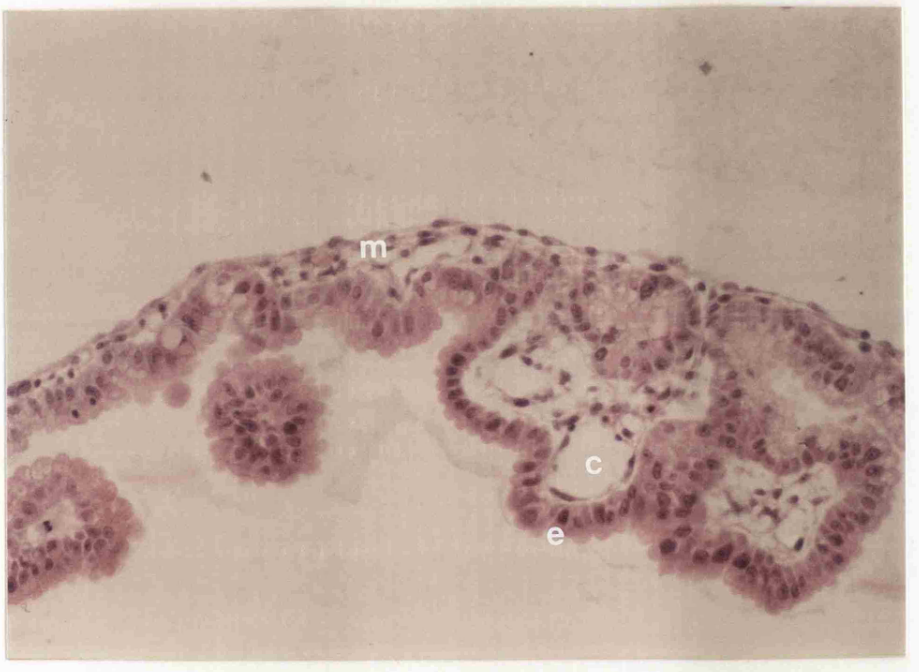
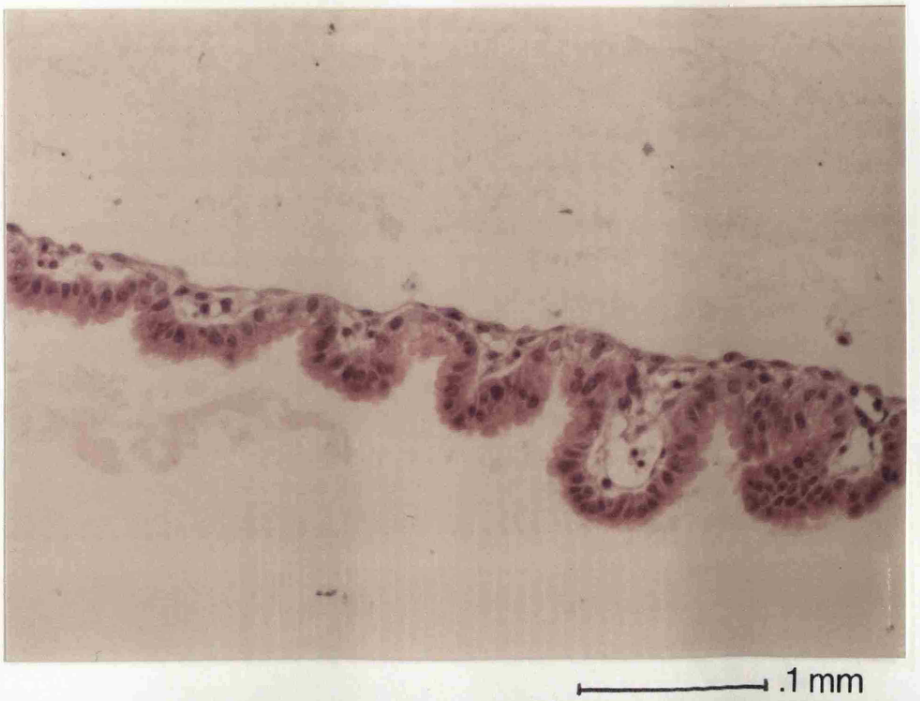
b



c

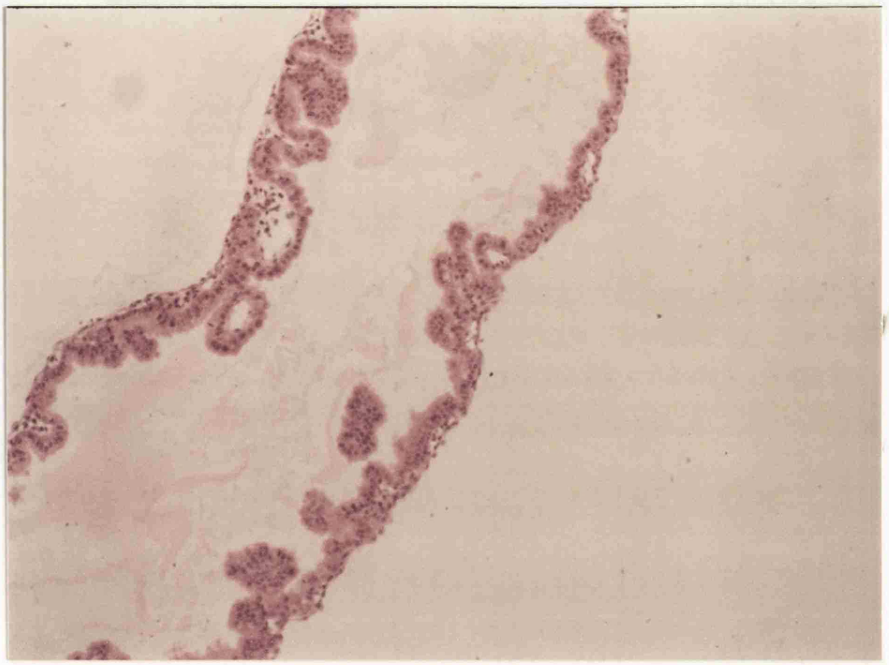


.1 mm

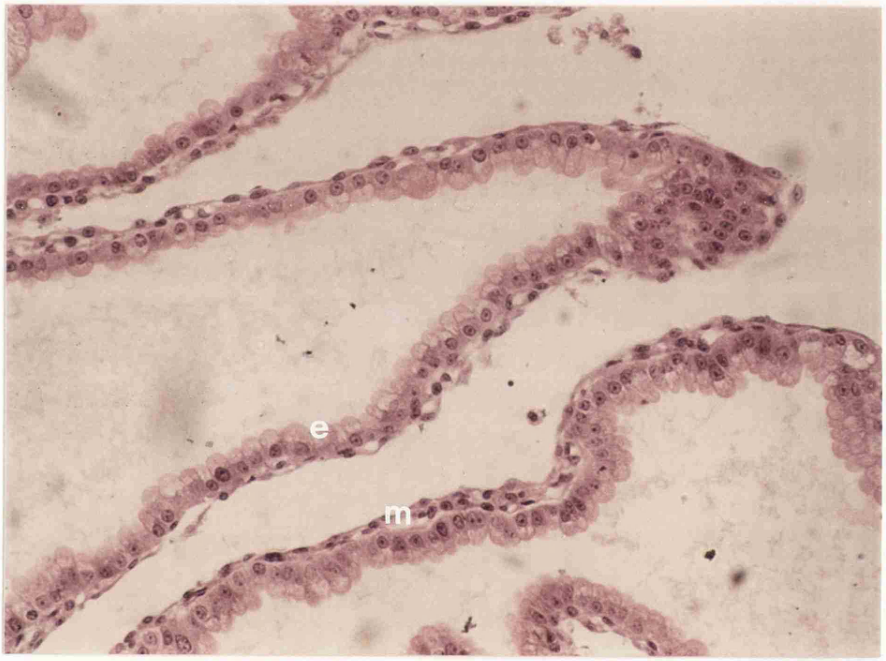
Figure 2.12 5μm paraffin sections of 17.5 day cultured yolk sac stained with haematoxylin and eosin x 95 (a) or x 238 (b and c).

The yolk sac in culture can be seen to maintain the same differentiation between endoderm (e) and mesoderm (m), but there are fewer capillaries in the mesenchyme.

a



b



c

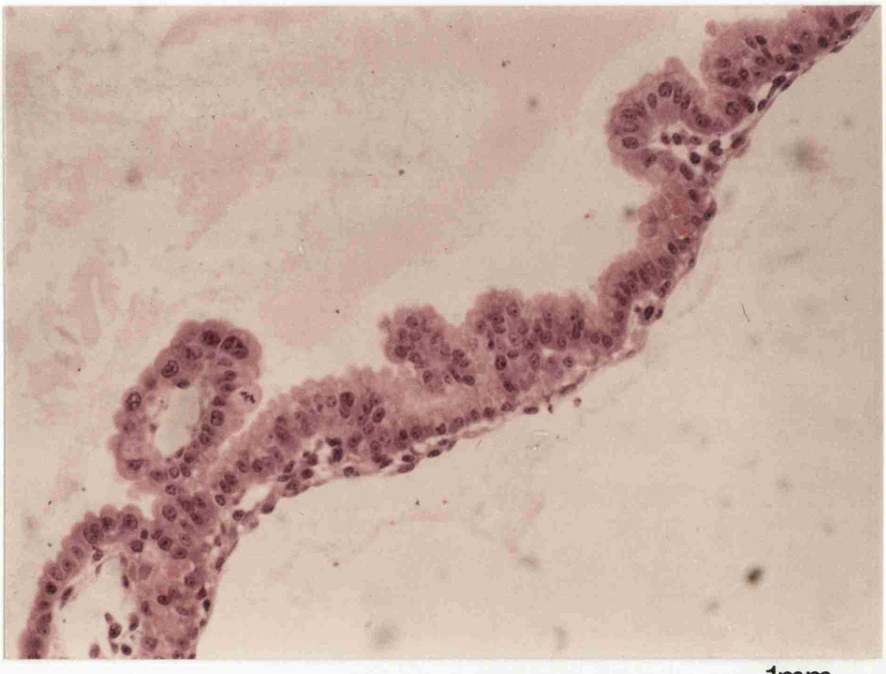
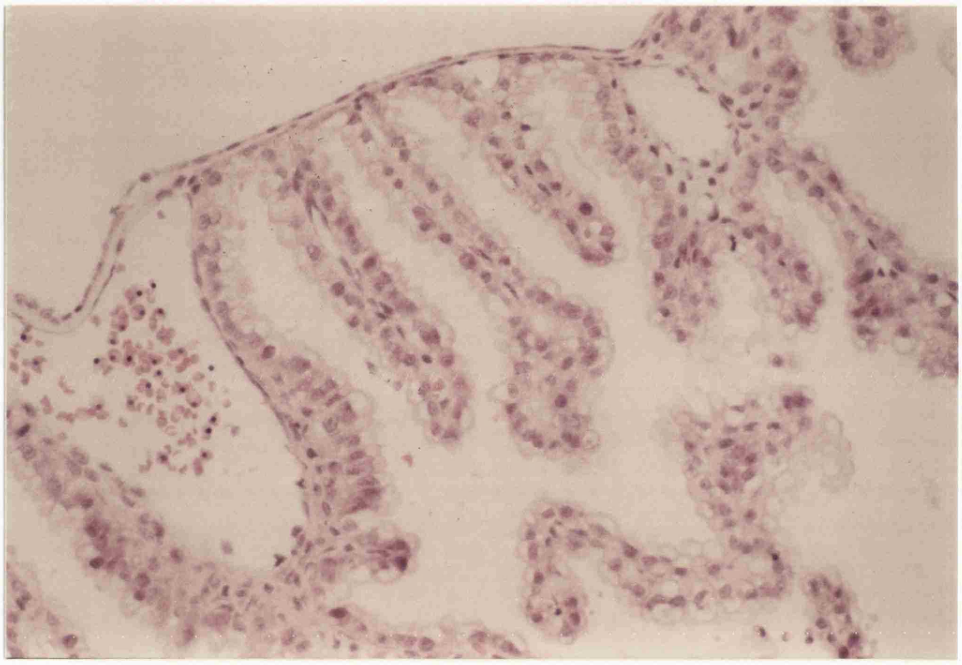


Figure 2.13 5μm paraffin sections of *in vivo* and cultured 17.5 day yolk sac stained with Periodic acid-Schiff reagent (x 238)

PAS positive staining is indicated by pale pink staining, counterstained in the nuclei by Harris' haematoxylin. *In vivo* yolk sac (a) shows PAS positivity in the mesothelium and the apical cytoplasm of the endoderm cells. Cultured yolk sac (b) show intense PAS staining in some but not all endodermal cells.

a



b

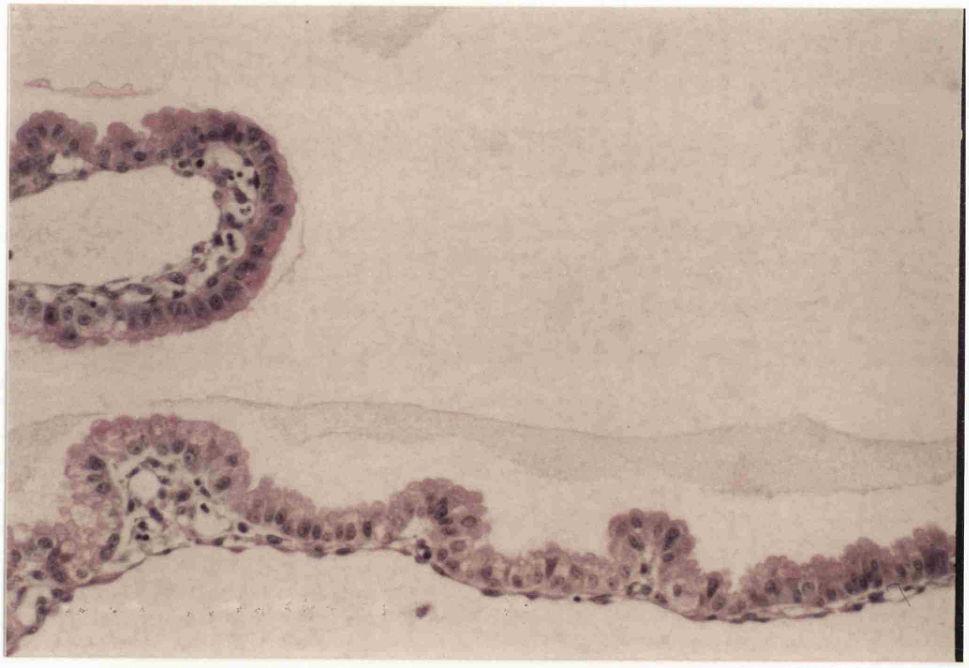


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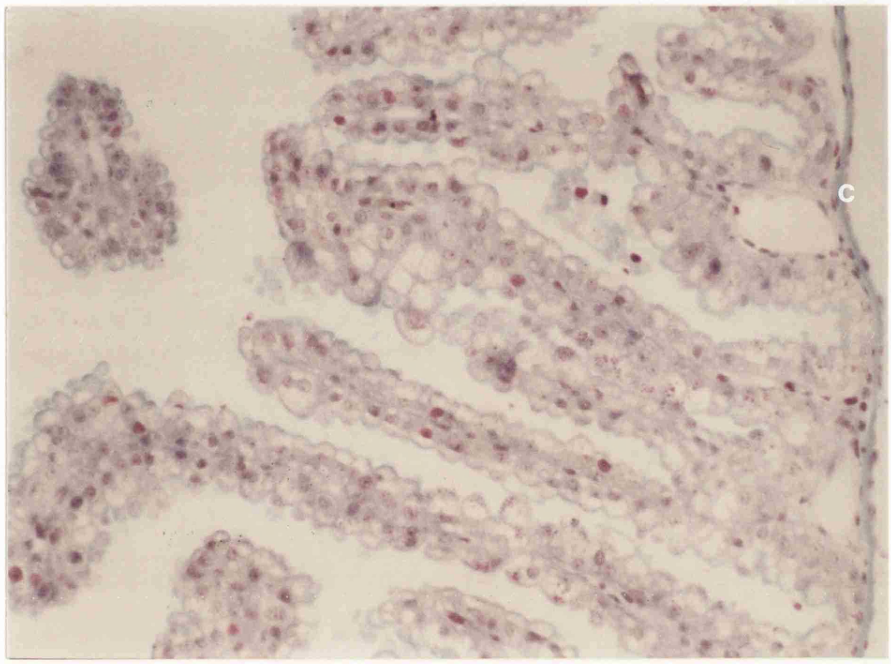
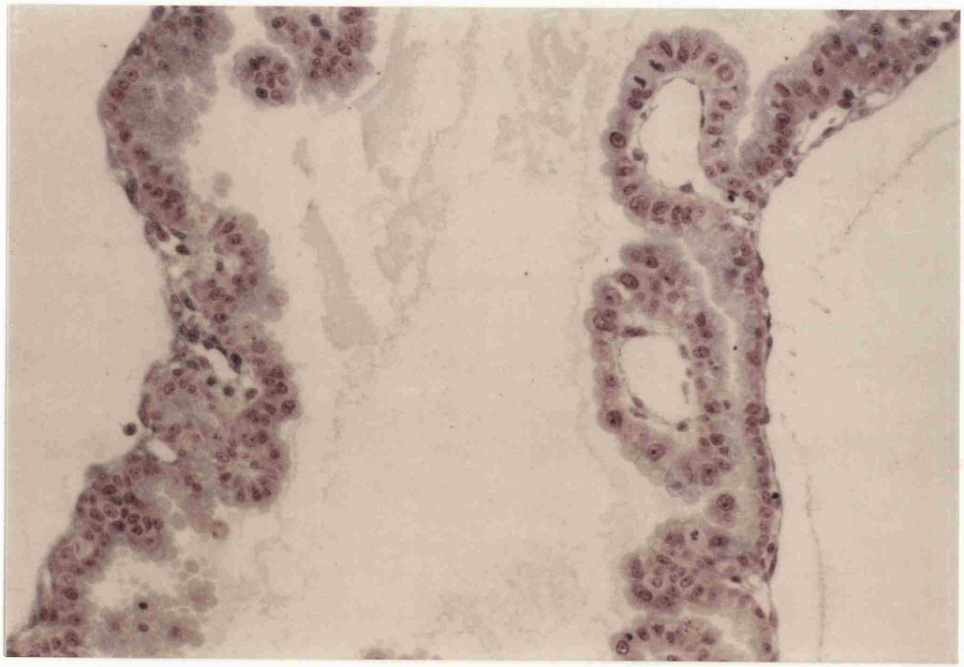
Figure 2.14 5μm paraffin sections of 17.5 day in vivo and cultured yolk sac stained with Picro-Mallory trichrome (x 238)

In vivo yolk sac (a) shows a pale blue collagen (c) band between the mesenchyme and the mesothelium. This is not seen in cultured yolk sac (b).

a



b



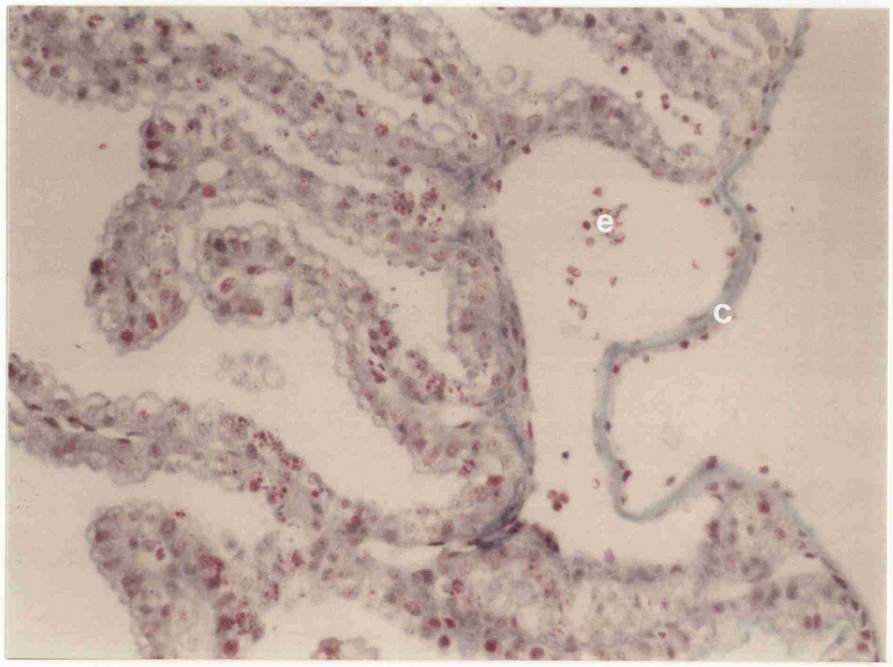
.1mm

Figure 2.15 5μm paraffin sections of 17.5 day *in vivo* and cultured yolk sac stained with Picro-Mallory trichrome (x 238)

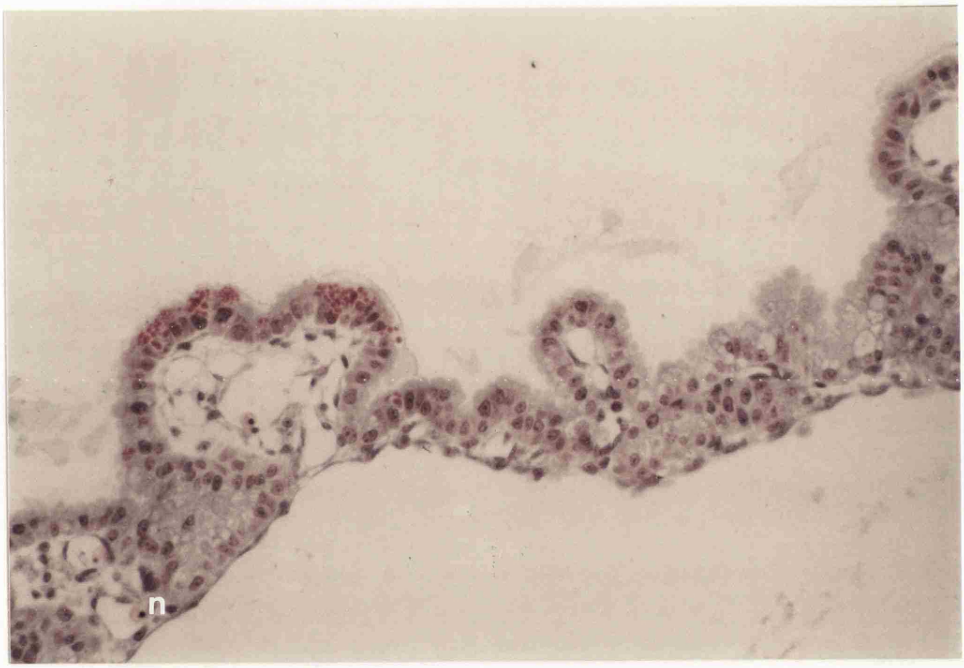
The blue band of collagen (c) is seen in *in vivo* yolk sac (a) but not in cultured yolk sac (b). This collagen is also observed around the blood vessels in the *in vivo* yolk sac.

The large venule in the *in vivo* yolk sac contains fetal erythrocytes (e), whilst the small capillaries seen in the cultured yolk sac appear to contain late normoblast-like cells (n).

a



b



.1mm

2.4 DISCUSSION

Because of the high variability in DNA content within culture groups, it was deemed impossible to make any comparison between culture techniques, using the DNA content as measured by the method of Labarca and Paigen (1980). This finding was emphasised when comparison of the contents was made between the fluorimetric technique and the time-consuming spectrophotometric method of Burton (1955). In all cases there was variance between the two DNA estimations. Considering the variation between DNA contents of yolk sacs within the same culture group, estimated by the fluorimetric technique, the reproducibility between duplicate samples of the same yolk sac homogenate was remarkably high. It therefore seems likely that the main problem in the fluorimetric assay stemmed from an inability of the Hoechst 33258 compound to intercalate with the yolk sac DNA in the same manner, in each yolk sac homogenate. The presence of 2M sodium chloride should have ensured that all chromatin protein was dissociated from the DNA (Labarca and Paigen, 1980). The concentration of EDTA used in this assay was far in excess of that usually required to effectively block DNAase activity (Maniatis et al., 1982) and the fluorescent adduct of H33258 and DNA is reported to be stable for up to 16 hours (Labarca and Paigen, 1980). Therefore, the only apparent reason for the lack of success of the assay is that the rate of penetration of H33258 into the yolk sac homogenate varied between samples over the eight hour

incubation. The homogenisation procedure used in this series of assays was more extensive than that recommended in the original reference, but the procedure may have benefited from the use of sonication to provide a finer and more homogenous suspension of cellular material. The Burton DNA assay was not employed as a substitute, since a rapid screen of growth tests was needed for comparison of parameter changes in the culture system, and the lengthy procedure for nucleic acid extraction required for each series of yolk sacs, made the technique unusable.

The reproducibility of results recorded by the Lowry protein assay and the viability ratio meant that the DNA content assay was deleted from the screen.

When the percentage of heat-inactivated rat serum in the culture medium was changed, there was no significant difference in any of the growth parameters measured, until serum concentration fell below 50%. This is in broad agreement with other published accounts of whole embryo culture in medium containing less serum. 10% rat serum in human serum will support embryonic growth and development from 9.5 to 11.5 days in culture, whilst 30% serum in balanced salt solution will also support growth and development (Gupta and Beck, 1983; Lear et al., 1983; Al-Alousi, 1983). Rat serum contains essential growth and differentiation factors, some of which are common between species and can be found in fetal calf serum and human placental cord serum (Hsu, 1980), and others which are species specific and account for the lack of development of

rat embryos in unsupplemented human serum (Lear et al., 1983). Serum also contains essential nutrients and vitamins, such as glucose, pantothenic acid, riboflavin, inositol and folic acid (Cockcroft, 1979), but these constituents are available in the same concentrations in Medium 199. In effect, there was less variation in 50% serum compared to 100% serum, because of the added buffering capacity and fixed salt concentration of the synthetic medium. This may have accounted for the decrease in variation of measured pH and pO_2.

When the number of medium changes was decreased from three to two, a significant increase in viability was observed, probably reflecting the decreased mechanical handling of the developing vesicles. A further decrease to one medium change resulted in significant decreases in all growth parameters, and was probably attributable to excess build up of waste products in the medium, such as lactate, creatinine and urea (Sanyal, 1980).

Initiating the culture with 5% oxygen assumes the major catabolic pathway in the yolk sac to be anaerobic glycolysis (see Chapter 3), and follows previous work with whole embryo culture, where low oxygen concentrations at this stage, improve growth and development (New et al., 1976). Sanyal and Wiebke (1979) showed that extending low oxygen gassing past 10.5 days was detrimental to embryo culture, and this effect was observed to be true for yolk sac culture, probably reflecting an energy metabolism change over to aerobic metabolism.

The finding that high oxygen concentrations were toxic in the culture system was less expected. Many short term organ culture techniques routinely use 95% O_2 as the gassing medium, for example it is used to gas yolk sacs cultured for up to six hours at 17.5 days, in the study of protein uptake (Dunton et al., 1986; Livesey and Williams, 1979). The mechanism by which 95% O_2 causes a delayed toxicity in the cultured yolk sac can only be speculated on, but it may cause physico-chemical changes in permeability of the membrane, or it may have a specific inhibitory action on a life-critical function of the yolk sac. Although oxygen is a powerful oxidising agent, it is unpredictably stable in biological systems due to spin restriction in its outer orbital. However, reaction with a single electron donor such as a transition metal, can lead to formation of superoxide and hydroxyl radicals, which are extremely reactive, and can damage macromolecules, including DNA (Halliwell and Gutteridge, 1985). Subsequent to this oxygen toxicity finding, Miki et al. (1988) reported specific high oxygen toxicity in visceral yolk sac targeted at protein uptake. They reported a significant decrease in horseradish peroxidase uptake in 11.5 day yolk sacs in culture, 24 hours after being gassed with 95% O_2. The loss of uptake of essential macromolecules from the serum medium may have contributed to the loss of integrity of the vesicle structure.

The findings from the histological study were that:

i) Cultured yolk sacs still retain intact capillary-like structures which contain erythrocyte precursors, although the haemopoietic system is not as advanced as that seen *in vivo*, and large blood vessels do not form. The capillary structures were also seen in cultured yolk sacs processed for electron microscopy (Decatris, 1988). The lack of development of the vitelline vessels in culture is probably due to the lack of haemodynamic factors from the development of the embryonic heart primordium.

ii) *in vivo* yolk sacs deposit collagen in a dense connective tissue band between the mesenchyme and the mesothelium, and this connective tissue invades the mesenchyme to surround the major blood capillaries. Cultured yolk sacs exhibited no detectable collagen deposits. Clark *et al*. (1982) showed that this collagen in the visceral yolk sac stroma was rat type I collagen, and was synthesised as pro-collagen. They speculated that the synthetic cell type was a fibroblast-like cell in the mesenchyme. The cultured yolk sac may not contain this cell type, may not synthesise the necessary transcription factor required to activate the collagen synthesis, or may not be capable of transporting hydroxyproline (this amino acid is present in Medium 199 at a concentration of 10mg/l).

iii) The H & E and trichrome stains both reveal differences in the nuclear structure of cultured and *in vivo* 17.5 day yolk sacs. The prominent nucleoli observed in

cultured yolk sac sections are characteristic of an active, protein-manufacturing cell type (Junqueira and Carneiro, 1980). This finding suggests the possibility that the normal metabolic shut-down observed in visceral yolk sac at around 16.5 days (Padykula, 1958; Johnson and Spinuzzi, 1966) may not occur in yolk sac cultured in the absence of a developing embryo. This finding is partially supported by the continued differentiation of rat visceral yolk sac for as long as 28 days in culture, reported by Lu et al. (1984).

The standard of PAS-staining was variable between in vivo and cultured yolk sacs, with some staining being potentiated, when washed in acid alcohol after haematoxylin counterstaining. This meant that it was difficult to draw comparative conclusions between the two situations.

2.5 MODIFIED YOLK SAC CULTURE TECHNIQUE

After assessment of the results of changing certain parameters of the original 'giant' yolk sac culture technique, the following procedure was chosen for culture of yolk sacs in the remaining experiments described in this thesis.

After explanting 9.5 day egg cylinders from the pregnant dam, and preparing yolk sac vesicles, as described in section 2.2.4, six vesicles were transferred into 4ml of culture medium in a sterile 60ml glass culture bottle. The culture medium consisted of 2ml of immediately centrifuged, heat-inactivated whole rat serum, and 2ml of Medium 199 (GIBCO). Both stock solutions contained 100 IU/ml penicillin and 100µg/ml streptomycin (GIBCO).

The culture bottles were gassed for 1 minute with a gas mixture of 5% O_2; 5% CO_2; 90% N_2, stoppered, and placed on a roller (30-40rpm) in a 37°C incubator. The bottles were subsequently gassed every 24 hours for 1 minute with 20% O_2; 5% CO_2; 75% N_2 for three days, and 40% O_2; 5% CO_2; 55% N_2 for the remaining days of culture.

After 48 hours and 120 hours of culture, the yolk sacs were removed from the culture medium using the wide base of a sterile Pasteur pipette, and transferred to fresh medium in sterile culture bottles.

When comparing groups of yolk sacs in a culture experiment, the same number of yolk sacs were cultivated in each bottle. Therefore, if one yolk sac died in the course

of the experiment, a yolk sac was removed from the corresponding bottles of each of the experimental groups.

2.6 CULTURE NOMENCLATURE

The original term 'giant' yolk sac was not used in the experimental chapters of this thesis for two main reasons:

a) The word 'giant' already occurs in the field of placentation on two separate occasions, in association with trophoblastic giant cells. Firstly, there are two separate giant cell invasions during the process of egg cylinder formation (Amoroso, 1952 - see Chapter 1.1). The role of these cells appears to be in phagocytosing decidual material to enlarge the implantation cavity and allow subsequent embryonic development. Secondly, in prolonged culture of visceral yolk sac material, proliferation of poorly differentiated cells, frequently associated with trophoblastic giant cells, was observed (Lu et al., 1984).

b) In the experimental chapters of this thesis, when comparing cultured yolk sacs to yolk sacs taken from the mother at a similar age, the cultured yolk sac is always smaller than its counterpart. Therefore, in these cases, the 'giant' yolk sac appears to be a misnomer.

Hence, yolk sacs grown in culture were referred to as CULTURED YOLK SACS, and yolk sacs removed from a pregnant animal at comparable gestational ages were referred to as IN VIVO YOLK SACS.

Strictly speaking, once the yolk sac vesicle is prepared for culture at 9.5 days of gestation, it has been removed from the normal gestational process, and can only be referred to in terms of culture age. For example, a yolk sac

explanted at 9.5 days, and cultured for 3 days, is a 3-day cultured yolk sac, and not a yolk sac 12.5 days into gestation.

However, for ease of comparison between cultured and *in vivo* yolk sacs, the equivalent gestational age was always used in both cases:

Day of Culture	Age of Cultured Yolk Sac or *in vivo* Yolk Sac (equivalent gestational age)
0	9.5
1	10.5
2	11.5
3	12.5
4	13.5
5	14.5
6	15.5
7	16.5
8	17.5

CHAPTER THREE

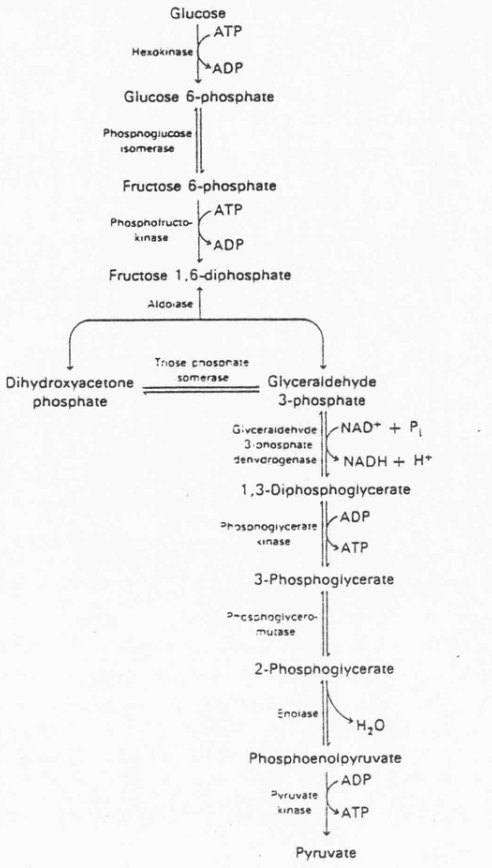
Aspects of Carbohydrate Metabolism in the Rat Visceral Yolk Sac

Figure 3.1 Glycolytic and tricarboxylic acid cycle catabolism of carbohydrates

The glycolytic pathway (a) of glucose metabolism yields two molecules of pyruvate and a net gain of two molecules of ATP.

The tricarboxylic acid cycle (b) starts with the condensation of acetyl CoA with oxaloacetate to give citrate. The following cycle of oxidation and decarboxylation reactions produces a variety of energy-yielding molecules - GTP, NADH and $FADH_2$.

(a)

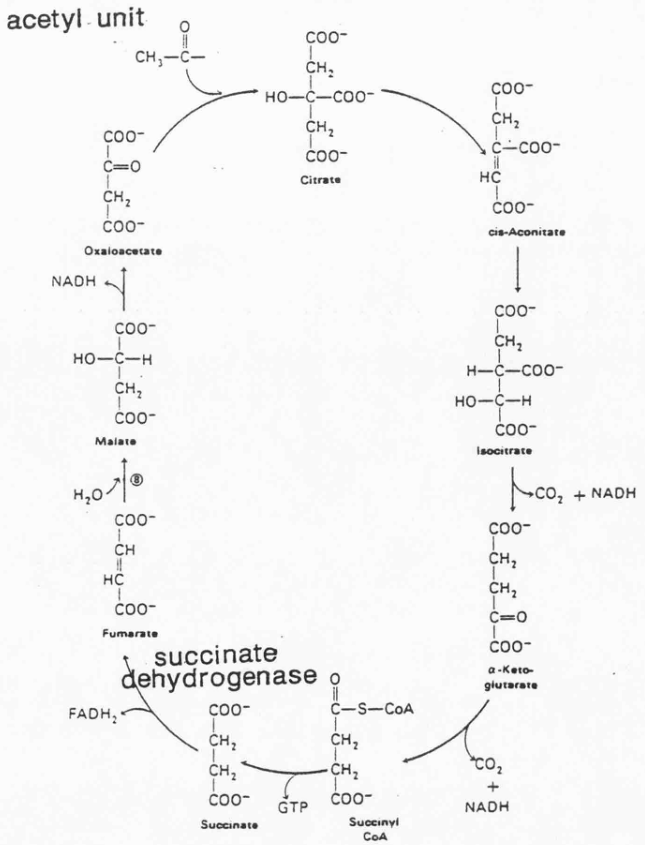


CYTOSOL

MITOCHONDR

Pyruvate + NAD$^+$ + CoA ⟶ acetyl CoA + CO_2 + NADH

(b)



3.1 INTRODUCTION

3.1.1 Oxidative carbohydrate catabolism in mammals

Mammals extract energy from ingested foodstuffs by converting the nutrient material into macromolecular precursors, ATP and NADPH. ATP is an energy-rich molecule which can be used to drive energy-dependent processes, whilst NADPH provides reducing power in the biosynthesis of cell components from more oxidised precursors. Proteins, fats and carbohydrates are digested to produce amino acids, fatty acids and glucose respectively, and in a normal balanced diet situation fatty acids and amino acids are used to create cell components, whilst glucose is utilised as the preferred substrate for energy production (Stryer, 1981).

The flux of glucose within a cell is regulated by the maintenance of a store of inactivated glucose (glycogen), from which it can draw glucose when supply of digested carbohydrate is low. Glycogen is synthesised from glucose by glycogen synthetase and broken down to release glucose by glycogen phosphorylase. The two enzymes are coordinately controlled by phosphorylation reactions, such that when one enzyme is activated, the other is inactivated; these phosphorylation reactions are triggered by changes in cyclic AMP initiated by glucagon or adrenaline (Jungermann and Katz, 1986).

Glucose is catabolised by a series of reactions in the cytosol, making up the process of glycolysis (Fig. 3.1a),

yielding two molecules of pyruvate and two molecules of ATP. Under anaerobic conditions, pyruvate is converted to lactate, regenerating the NAD^+ utilised in the oxidation of glyceraldehyde-3-phosphate. In aerobic conditions, pyruvate is actively transported into the mitochondria (Ottaway and Mowbray, 1977), where it is oxidatively decarboxylated by the pyruvate dehydrogenase complex to form acetyl coenzyme A (acetyl CoA). The acetyl CoA then condenses with oxalo-acetate to form citrate, and is then completely oxidised to CO_2 by a series of reactions known as the tricarboxylic acid cycle (Fig. 3.1b), with the release of three NADH molecules, one $FADH_2$ molecule and one molecule of GTP per cycle. The electron acceptors (NAD^+ and FAD) are regenerated when NADH and $FADH_2$ transfer their electrons through a chain of asymmetrically-orientated transmembrane complexes (ubiquinone, cytochrome b, cytochrome c_1, cytochrome c and cytochromes a/a_3) to molecular oxygen. This electron transfer causes protons to be pumped out of the membrane creating a proton gradient. The reverse flow of protons back to the mitochondrial matrix is facilitated by coupling factor 1, and generates ATP. NADH yields three ATP molecules whilst $FADH_2$ enters the respiratory chain at a later stage and yields two ATP molecules (Boyer et al., 1977).

In conditions of low glucose availability, fatty acids can be oxidised via acetyl CoA and certain amino acids can be oxidised via pyruvate and α-ketoglutarate (Garland et al., 1969; Felig, 1975).

The majority of reactions in glycolysis and the tricarboxylic acid cycle are reversible, but some reactions, due to their negative free energy changes, are physiologically irreversible, and are therefore key enzymes in the regulation of carbohydrate catabolism. In glycolysis three enzyme reactions are irreversible - hexokinase, pyruvate kinase and phosphofructokinase. Of these enzymes, phosphofructokinase is the most important control element in glycolysis, and it is inhibited by high levels of ATP and citrate, and is activated by AMP. This results in activation of phosphofructokinase when requirements for energy and essential cell components are high (Newsholme and Start, 1973). Hexokinase activity is inhibited by its product - glucose-6-phosphate, however this product is also the initial compound in a series of reactions leading to the formation of NADPH and ribose-5-phosphate - a precursor of nucleic acids. This series of reactions termed the pentose phosphate shunt is rate-limited by glucose-6-phosphate dehydrogenase (Newsholme and Start, 1973). Depending on the relative requirements for NADPH and nucleic acids, the six carbon fragment of glucose-6-phosphate is either incorporated into ribulose (5-carbon) or recycled into the glycolytic pathway in the form of glyceraldehyde-3-phosphate. Pyruvate kinase activity is allosterically inhibited by ATP, resulting in high energy-inhibition of further oxidative carbohydrate catabolism.

The tricarboxylic acid cycle can be controlled at a number of sites, including citrate synthetase, isocitrate

dehydrogenase and α-ketoglutarate dehydrogenase, as well as pyruvate dehydrogenase (Stryer, 1981). The funneling of two-carbon fragments into the cycle and the rate of the cycle are reduced when a cell has a high level of ATP, and this control is usually achieved by a variety of complementary inhibitive mechanisms, mediated directly by ATP, or indirectly by product inhibition or covalent phosphorylation.

3.1.2 Development of carbohydrate metabolism in rodents

The first three cell divisions of the fertilised ovum are pyruvate-dependent (Wales, 1975), and it is postulated that a large cytosolic pool of citrate inhibits glycolysis in these early embryos, whilst tricarboxylic acid cycle enzymes are active (Barbehenn et al., 1974). Early cleavage stage embryos possess a specific facilitated glucose-transport system (Wales and Brinster, 1968), however glucose only supports cell division after the 8-cell stage, and even then, only a small proportion of that glucose is oxidised to CO_2, the remainder being stored as glycogen (Clough, 1985).

Implantation marks two major changes for the embryo and its environment. Firstly, the embryo ceases to be free within the uterine lumen, and instead, it becomes surrounded by decidual tissue. Secondly, cleavage divisions of the blastocyst are succeeded by growth (Snow, 1977), and therefore there is a greatly increased demand for protein and nucleic acids. New (1978) showed that post-implantation embryos required glucose as the major energy source, and

that *in vitro*, low oxygen tension produced the best growth profile. At this stage, in rodents, glycolytic conversion of glucose to lactate accounts for about 98% of metabolised glucose, whilst the remaining 2% was metabolised via the pentose phosphate shunt to yield nucleic acid precursors (Clough and Whittingham, 1983). After implantation, the activities of a number of glycolytic enzymes are 10-50 fold higher than in pre-implantation embryos, whilst mitochondrial energy systems activities are negligible (Clough, 1985). Around 10.5-11.5 days of gestation, tricarboxylic acid cycle activity starts to rise, coinciding with organogenesis and a consequential rise in energy-dependent pathways (Shepard *et al.*, 1970). This also coincides with haemopoietic development in the rat, and the rise in tricarboxylic cycle activity may be in response to rising oxygen tension.

Since the yolk sac culture system spans from 9.5 to 17.5 days of gestation, by measuring specific activities of glycolytic and tricarboxylic acid cycle enzymes, a method is available to assess biochemical differentiation of the yolk sac in culture.

Glycogen deposition continues in many fetal tissues until parturition, when hepatic glycogen stores are rapidly depleted (Walker, 1971). However, no apparent role for glycogen storage in yolk sac or chorio-allantoic placenta has been elucidated (Harkness *et al.*, 1985). Since a comparative study of energy metabolism is being made between cultured and *in vivo* yolk sac, an attempt to control any

differences brought about by glucose flux was made by inducing glycogen storage in culture, using increased glucose concentrations in the culture medium (Chue, 1986; Decatris, 1988).

3.1.3 Choice of enzymes to study carbohydrate metabolism in the visceral yolk sac

In order to assess carbohydrate catabolism in *in vivo* and cultured yolk sac, three enzymes were chosen for study - hexokinase, pyruvate kinase and succinate dehydrogenase.

Hexokinase, the first enzyme in glycolysis, catalyses the irreversible 6-phosphorylation of glucose:

$$\text{Glucose} + \text{ATP.Mg}^{++} \rightarrow \text{Glucose-6-phosphate} + \text{ADP} + \text{H}^{+} + \text{Mg}^{++}$$

Hexokinase (EC.2.7.1.1) can be isolated into four iso-enzymes with different k_m for glucose and different electrophoretic mobilities (Purich *et al.*, 1973). Types I, II and III have low k_m for glucose and are inhibited by glucose-6-phosphate, whereas Type IV (glucokinase) is a liver-specific enzyme with high k_m for glucose and a refractivity to product inhibition (Balinsky *et al.*, 1975).

Hexokinase activity has been assessed in visceral yolk sac, both *in vivo* and in culture (Walsh, 1985; Gale, 1986), therefore the activities detected in this study can be compared to previous results. By comparing hexokinase activity to the activity of another irreversible glycolytic

enzyme - pyruvate kinase, the potential exists to differentiate between simple glycolytic activity and pentose phosphate shunt activity.

Pyruvate kinase (EC.2.7.1.40) catalyses the final step of glycolysis:

$$\text{Phosphoenol pyruvate} + \text{ADP} + H^{+} + Mg^{++} \rightarrow \text{Pyruvate} + \text{ATP}.Mg^{++}$$

This enzyme also has four isoenzymes - types L, R, M_1 and M_2, named on their tissue specificity to liver (L), erythrocytes (R), muscle (M_1) and fetal tissues (M_2). These isoenzymes have different substrate k_m values and differing responses to activation by fructose-1,6-diphosphate (Engstrom et al., 1987).

Tricarboxylic acid cycle reactions take place in the mitochondria and this can lead to difficulty in measuring specific activities, without substantial purification of the enzymes. To avoid the requirement of a large mass of yolk sac tissue, succinate dehydrogenation was chosen as the assay for tricarboxylic acid cycle activity. Succinate dehydrogenase (EC.1.3.99.1) is the only enzyme of the tricarboxylic acid cycle which is bound to the inner membrane of mitochondria (Hatefi and Stiggall, 1976) and therefore by semi-purification of the membrane bound Complex II of the inner mitochondrial membrane electron transport chain, isolation of succinate dehydrogenase activity could be assessed. Previous reports of succinate dehydrogenase activity in in vivo rat visceral yolk sac revealed

increasing activity from 11.5 to 16.5 days followed by a sharp decline in activity. Therefore assay of this activity in in vivo and cultured yolk sac would have the potential of indicating continued yolk sac differentiation throughout the culture period.

3.2 METHODS

3.2.1 Collection of yolk sac material

9.5 day rat conceptuses were explanted, and yolk sac vesicles prepared as described in Chapter 2.2.4.

Yolk sacs were cultured from 9.5 to 17.5 days as described in Chapter 2.5, with the exception that from day 14.5 the serum-based medium contained an additional 2.5mg/ml D-glucose supplement. Cultured yolk sacs were harvested at 11.5, 13.5, 15.5 and 17.5 days, and a matched series of *in vivo* yolk sacs were collected from pregnant rats at the corresponding gestational ages, as described in Chapter 2.2.8. Additionally, yolk sac vesicles prepared from 9.5 day egg cylinders were collected, and used as a 9.5 day *in vivo* and cultured yolk sac source.

The yolk sac material was stored at -20°C, before being pooled and homogenised on ice, in nine volumes of 0.25M sucrose/0.1M Tris-HCl buffer, pH 7.5, using a Potter-Elvejhem homogeniser and Teflon pestle. Homogenates were differentially centrifuged for 10 minutes at 9,000rpm in a Sorvall-OTD RC-5B ultracentrifuge at 4°C. The nuclear pellet was discarded, and a mitochondrial pellet obtained from the supernatant by recentrifuging at 12,000rpm for 20 minutes at 4°C.

The supernatant (cytosolic fraction) from the mitochondrial preparation was diluted 1:40 with 50mM Tris-HCl,

pH 7.5, then frozen at -20°C until assayed for hexokinase and pyruvate kinase activity.

The pellet was resuspended in half a volume of homogenisation buffer, supplemented with 0.5mM sodium succinate, and 1mM calcium chloride was added to a final concentration of 10μM. The resulting mitochondrial suspension was stored at -20°C, until all samples had been homogenised. Complex II was then prepared from the mitochondrial suspension by a modification of the method of Hatefi and Stiggall (1978).

The suspension was thawed, then maintained at 0-4°C for all the subsequent steps, unless otherwise stated. The suspension was diluted by a factor of two by the addition of 0.25M sucrose/1M potassium phosphate, pH 7.4, then incubated at 38°C for 30 minutes to dissolve 0.5ml of 20% (w/v) potassium cholate, pH 8.0 and 1.6g/10ml ammonium sulphate. After cooling on ice, the mixture was centrifuged for 30 minutes at 30,000rpm, in a cooled (4°C) Sorvall-OTD 65 ultracentrifuge, then the supernatant was retreated with ammonium sulphate and centrifuged as before. The precipitate was washed in 0.25M sucrose, resuspended in the same solution, then dialysed against 0.25M sucrose/10mM potassium phosphate, pH 7.4, for 5 hours, with two buffer changes. The dialysed material was then frozen overnight at -20°C. After thawing on ice, the solution was diluted in two volumes of 10% (w/v) potassium deoxycholate, pH adjusted to 7.3 with 1M HCl, then mixed with half a volume of 95% ethanol at 0°C. The mixture was centrifuged for 30 minutes at 30,000rpm in a Sorvall-OTD 65 ultracentrifuge at 0°C, then the supernatant

was decanted, mixed with an equal volume of cold cyclohexane, and recentrifuged at 30,000rpm for 45 minutes. The resulting pellet was resuspended in the original volume of 0.25M sucrose, and diluted in two volumes of 10% (w/v) potassium deoxycholate and one volume of neutral, saturated ammonium sulphate. After cooling on ice for 30 minutes, the suspension was centrifuged for 30 minutes at 30,000rpm in a Sorval-OTD 65 ultracentrifuge. The supernatant was pipetted onto a 30cm Sephadex G25 column, equilibrated and eluted with 20mM potassium phosphate, then the eluted Complex II suspension was centrifuged for 2 hours at 40,000rpm in the Sorvall-OTD 65 ultracentrifuge. The Complex II pellet was resuspended in 2ml of 20mM potassium phosphate, then frozen at -20^{o}C until assayed for succinate dehydrogenase activity.

3.2.2 Hexokinase assay

Hexokinase activity of 9.5 to 17.5 day cultured and *in vivo* yolk sac cytosolic fractions was assayed by the method of Easterby and Qadri (1982). Assay buffer contained 500µM D-glucose, 30mM $MgCl_2$ and 30mM Tris-HCl, pH 7.5. 10µl yolk sac cytosol, 100 nmoles NADP and 0.2 units of glucose-6-phosphate dehydrogenase were diluted to a reaction volume of 1.0ml with assay buffer, and incubated at 30^{o}C in the temperature-regulated carousel of an LKB 5040 Ultraspectrophotometer, until no increase in absorbance at 340nm was observed. The hexokinase reaction was then initiated by the addition of 10 µmoles ATP, pH 7.5. A standard curve of

absorbance of 0-5 µmoles NADPH was constructed, and specific activities of hexokinase were calculated using protein values obtained by a modification of the method of Lowry et al. (1951) (see Chapter 2.2.7).

3.2.3 Pyruvate kinase assay

Pyruvate kinase activity of 9.5 to 17.5 day cultured and in vivo yolk sac cytosolic fractions was assayed by a modification of the method described by Imamura and Tanaka (1982). 10µl yolk sac cytosol was incubated for 2 minutes at 37°C, with 50mM Tris-HCl, pH 7.5, containing 0.1mM fluoroacetate. At the same time, a 1.0ml cuvette containing assay mixture (50mM Tris-HCl, pH 7.5,; 0.1M KCl; 5mM $MgSO_4$; 2mM ADP; 2mM phosphoenolpyruvate; 0.5mM fructose-1,6-diphosphate;0.18mM NADH and 8 units lactate dehydrogenase) was incubated at 37°C in the LKB 5040 ultraspectrophotometer. The pyruvate kinase reaction was initiated by the addition of 10µl of fluoroacetate-treated yolk sac cytosol, and the resulting absorbance decrease at 340nm recorded. A standard curve of absorbance of 0-25 µmoles NADH was constructed, and specific activities of pyruvate kinase were calculated using protein values obtained by a modification of the method of Lowry et al. (1951) (see Chapter 2.2.7).

3.2.4 Succinate dehydrogenase assay

Succinate dehydrogenase activity of Complex II fractions of yolk sac homogenates, from 9.5 to 17.5 day cultured and *in vivo* yolk sac material, was assayed using the method of Hatefi and Stiggall (1978). 1.0ml of assay buffer containing 50mM potassium phosphate, pH 7.4; 20mM sodium succinate; 100µM EDTA; and 0.1% (w/v) bovine serum albumin, was incubated at 37°C in the LKB 5040 ultraspectrophotometer for 2 minutes, then 75µM 2,6-dichloroindophenol (DCIP) and 1.6mM phenazine methosulphate were added, mixed, and the reaction was immediately initiated by the addition of 2µl of Complex II, pre-incubated for 2 minutes at 37°C in 10µl of 0.1M 2-thenoyltrifluoroacetone.

The decrease in absorbance at 600nm was recorded, and specific activities of succinate dehydrogenase were calculated using protein values obtained by the method previously described (Chapter 2.2.7), and an extinction equivalent of DCIP that indicated that 1 absorbance unit was equivalent to 21 µmoles DCIP (Hatefi and Stiggall, 1978).

3.3 RESULTS

3.3.1 Hexokinase activity

Fig. 3.2 shows the pattern of hexokinase activity in *in vivo* and cultured yolk sac from 9.5 to 17.5 days. Both *in vivo* and in culture there was a trend of decreasing activity from 9.5 to 17.5 days. In *in vivo* yolk sac, there appeared to be two phases of decreasing activity; from 9.5 to 11.5 days, there was a significant decrease in specific activity from 194 nmoles NADPH/min/mg to 169 nmoles/min/mg ($p< 0.05$), whilst from 15.5 to 17.5 days, there was another significant decrease from 165 to 131 nmoles/min/mg ($p< 0.005$). In culture, although there was the same trend of decreasing activity, there was no significance in the differences between adjacent culture ages.

Lineweaver-Burke analysis of the hexokinase activity in 15.5 day cultured and *in vivo* yolk sac cytosol revealed essentially identical kinetic profiles for the two enzyme sources (Fig. 3.3). The apparent k_m values using glucose as substrate were 75μM and 78μM for cultured and *in vivo* yolk sac respectively.

3.3.2 Pyruvate kinase activity

Fig. 3.4 shows the pattern of pyruvate kinase activity in *in vivo* and cultured yolk sac from 9.5 to 17.5 days. From 9.5 to 15.5 days, there was no significant difference

between activities *in vivo* or in culture at any age. However, at 17.5 days, whilst activity in culture remained constant at 11.9 µmoles NADH oxidised/min/mg, the activity *in vivo* fell to 9.1 µmoles NADH oxidised/min/mg, which represents a 28% decrease in activity from 15.5 to 17.5 days *in vivo* ($p<0.005$).

Kinetic analysis of pyruvate kinase activity in 15.5 day *in vivo* and cultured yolk sac cytosols revealed apparent k_m values for phosphoenolpyruvate as substrate, of 326µM and 404µM for cultured and *in vivo* yolk sac respectively (Fig. 3.5).

In a single experiment using 15.5 day *in vivo* and cultured yolk sac cytosol, increasing fructose-1,6-diphosphate from 0.5mM to 5mM had no significant effect on reaction rate, whilst leaving the compound out of the assay mixture reduced measured pyruvate kinase activity by 21% in cultured yolk sac and 32% in *in vivo* yolk sac.

3.3.3 Succinate dehydrogenase activity

Fig. 3.6 shows the pattern of succinate dehydrogenase activity in cultured and *in vivo* yolk sac from 9.5 to 17.5 days. No activity was detected in 9.5 day yolk sacs, then activity rose in both cultured and *in vivo* yolk sacs from 11.5 to 15.5 days, reaching values of 148.5 nmoles/min/mg (*in vivo*) and 125.5 nmoles/min/mg (cultured) at 15.5 days. From 15.5 to 17.5 days, there was a small increase in detected activity in cultured yolk sac, but a significant

50% decrease in succinate dehydrogenase activity in in vivo yolk sac ($p < 0.005$).

In a single experiment, using 15.5 day in vivo and cultured yolk sac, pre-incubation of Complex II preparations in 1mM dithiothreitol for 3 minutes caused complete loss of detectable activity in either preparation.

Figure 3.2 Hexokinase activity in cultured and *in vivo* yolk sac cytosol

Cytosolic fractions of cultured and *in vivo* yolk sacs from 9.5 to 17.5 days were prepared by differential centrifugation.

Hexokinase activity was assessed by coupling the reaction to glucose-6-phosphate dehydrogenase, and measuring the increase in absorbance of the generated NADPH, at 340nm.

The values are means ± s.e.m. of ten estimations.

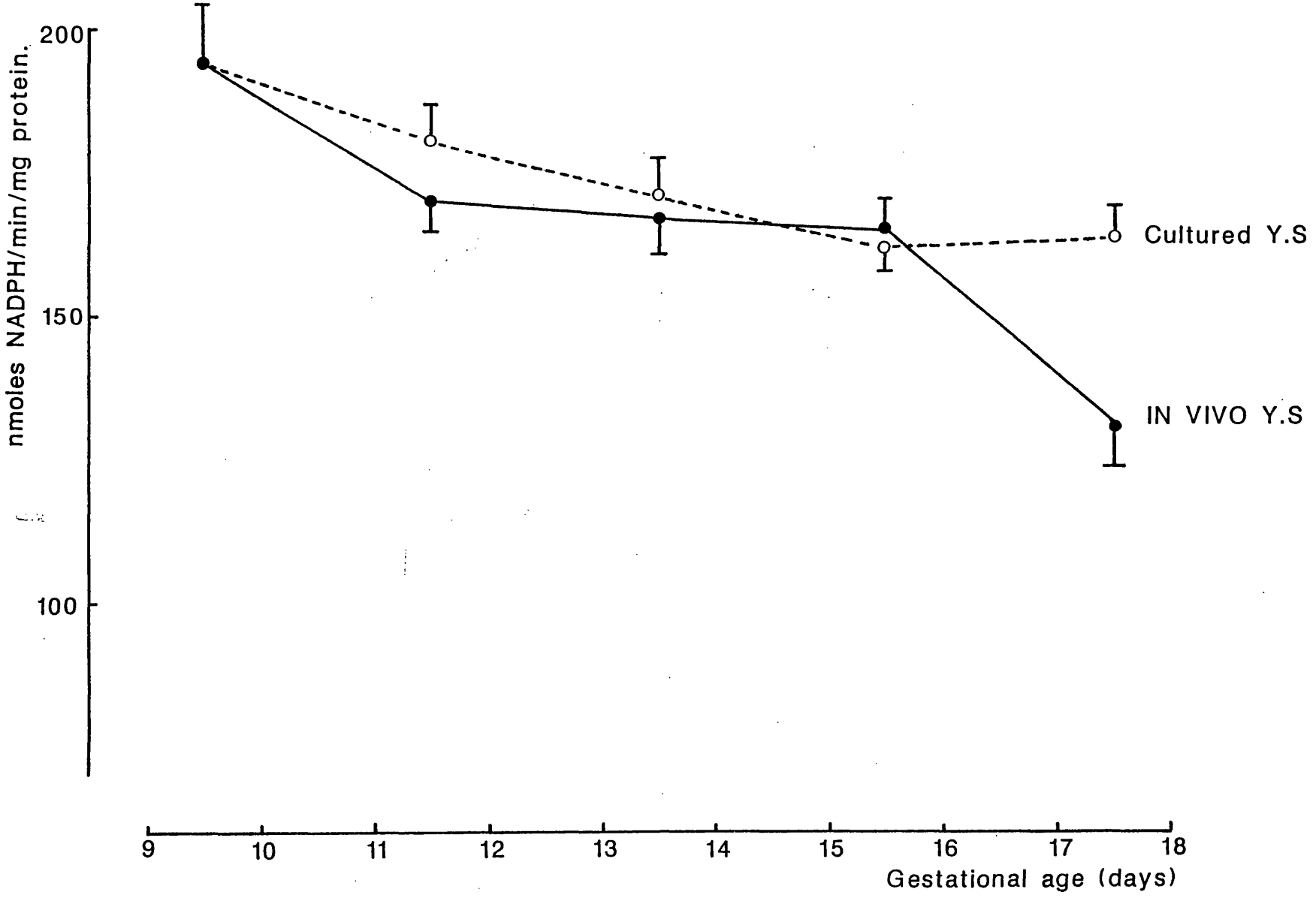

200
150
100
nmoles NADPH/min/mg protein.
Cultured Y.S
IN VIVO Y.S
9
10
11
12
13
14
15
16
17
18
Gestational age (days)

Figure 3.3 Plot of the reciprocal of glucose concentration against the reciprocal of initial reaction rate for hexokinase activity in cytosolic fractions of 15.5 day cultured and *in vivo* yolk sacs

Hexokinase activity was assessed by coupling the reaction to glucose-6-phosphate dehydrogenase, and measuring the increase in absorbance of the generated NADPH, at 340nm. From the y-axis intercepts, the estimated k_m values, using glucose at 30°C were:

	k_m (μM)
15.5 day *in vivo* yolk sac	78.3
15.5 day cultured yolk sac	74.7

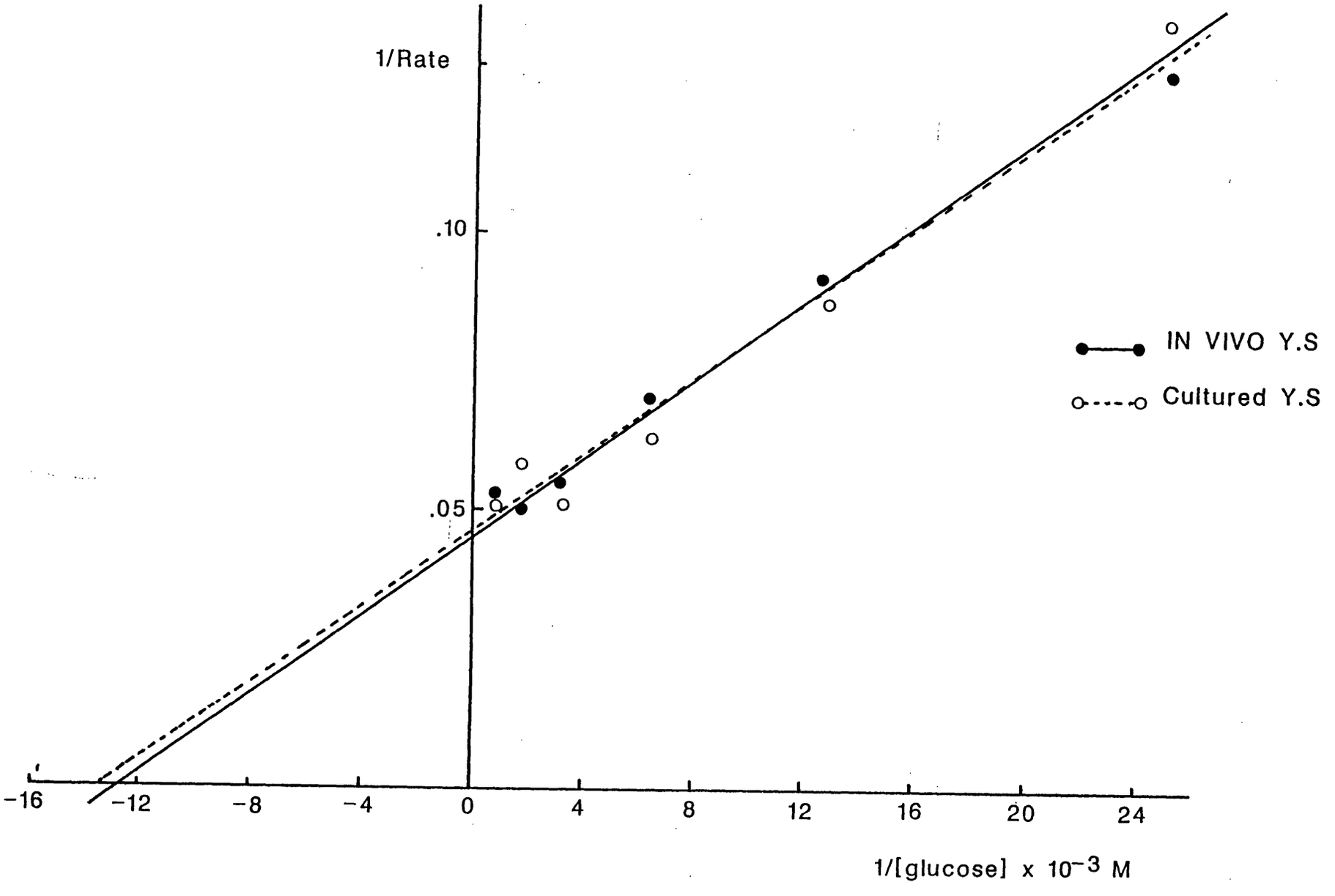

1/Rate
.10
.05
IN VIVO Y.S
Cultured Y.S
-16
-12
-8
-4
0
4
8
12
16
20
24
1/[glucose] x 10⁻³ M

Figure 3.4 Pyruvate kinase activity in cultured and *in vivo* yolk sac cytosol

Cytosolic fractions of cultured and *in vivo* yolk sacs from 9.5 to 17.5 days were prepared by differential centrifugation.

Pyruvate kinase activity was assessed by coupling the reaction to lactate dehydrogenase, and measuring the decrease in absorbance at 340nm, due to the utilisation of NADH.

The values are means ± s.e.m. of ten estimates.

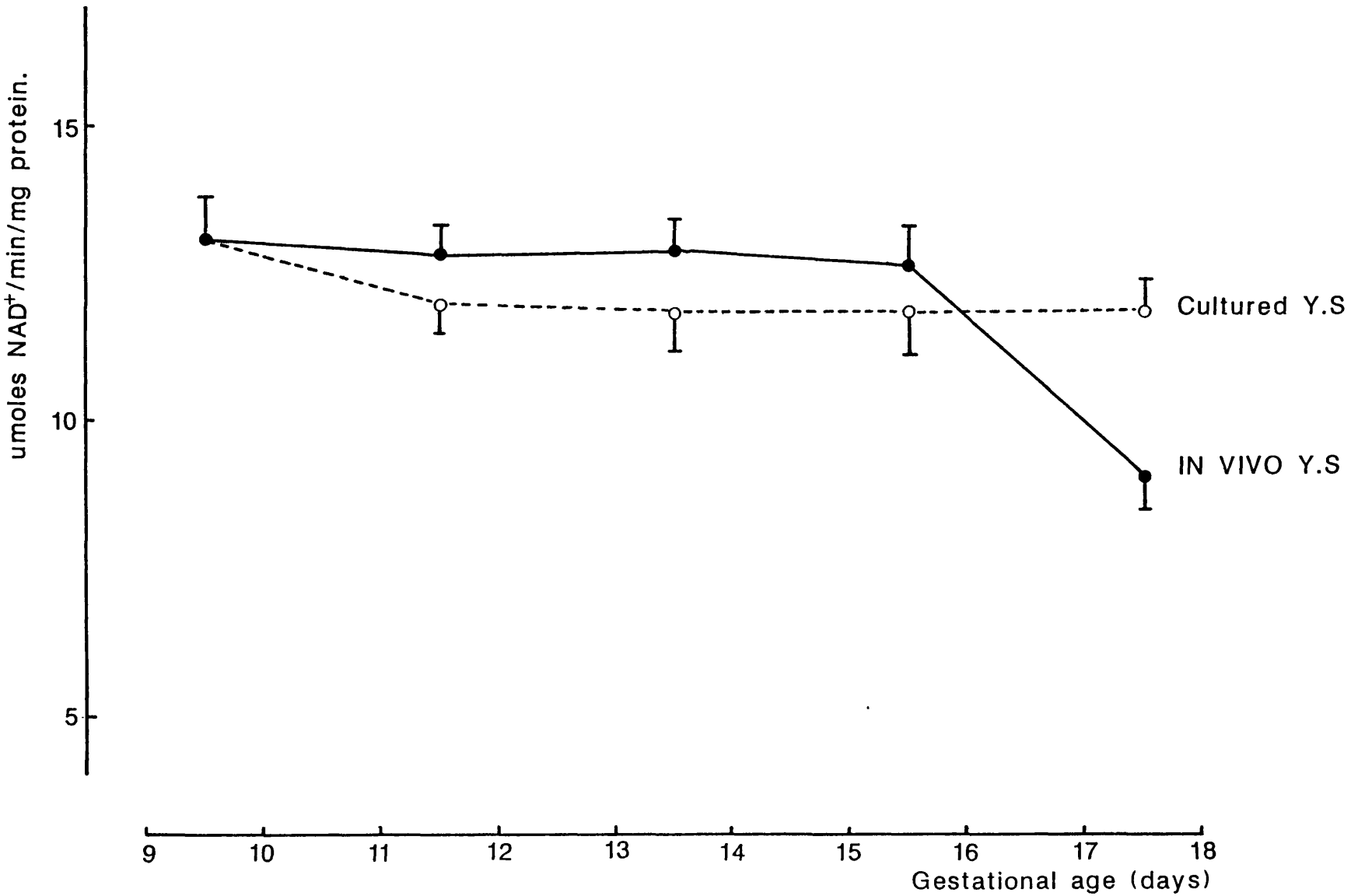

umoles NAD+/min/mg protein.
15
10
5
Cultured Y.S
IN VIVO Y.S
9
10
11
12
13
14
15
16
17
18
Gestational age (days)

Figure 3.5 Plot of the reciprocal of phosphoenol pyruvate against the reciprocal of initial reaction rate for pyruvate kinase activity in cytosolic fractions of 15.5 day cultured and *in vivo* yolk sacs

Pyruvate kinase activity was assessed by coupling the reaction to lactate dehydrogenase, and measuring the decrease in absorbance at 340nm, due to the utilisation of NADH.

From the y-axis intercept, the estimated k_m values using phosphoenol pyruvate at 37°C were:

	k_m (μM)
15.5 day *in vivo* yolk sac	326
15.5 day cultured yolk sac	404

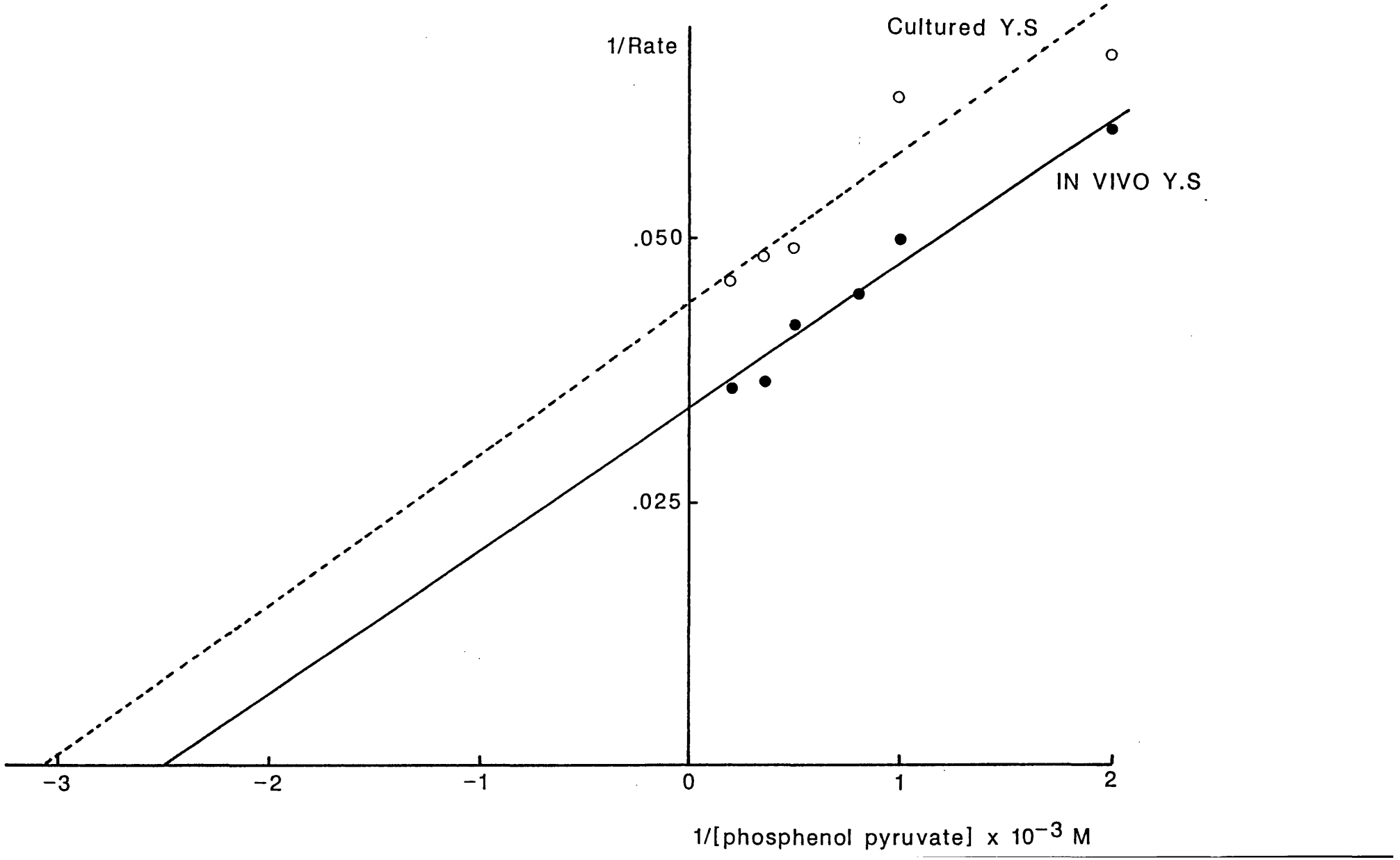
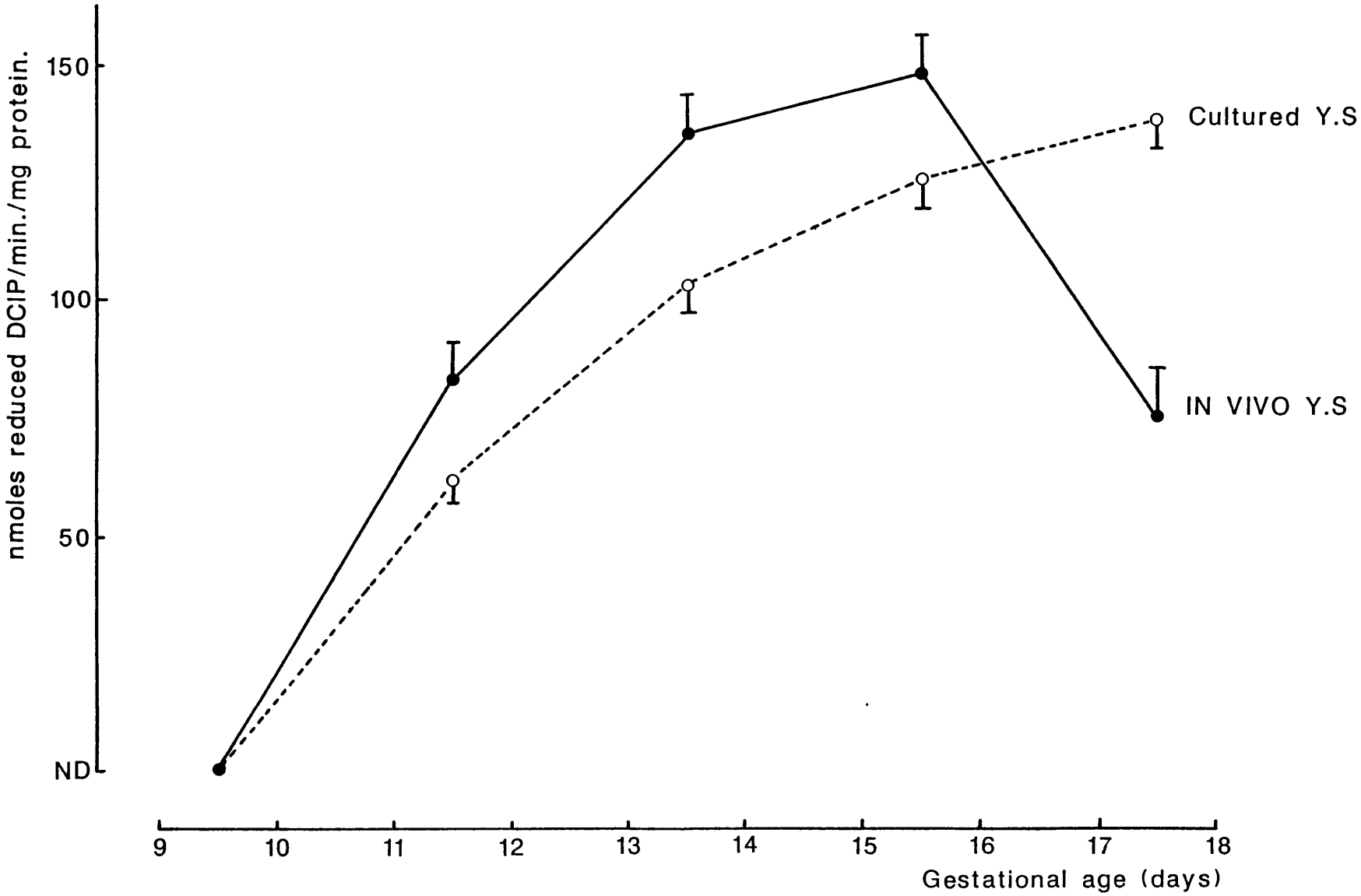

Cultured Y.S
1/Rate
IN VIVO Y.S
.050
.025
-3
-2
-1
0
1
2
1/[phosphenol pyruvate] x 10^{-3} M

Figure 3.6 Succinate dehydrogenase activity in Complex II fractions of cultured and *in vivo* yolk sacs

Crude mitochondrial fraction of cultured and *in vivo* yolk sacs from 9.5 to 17.5 days were prepared by differential centrifugation. Complex II fractions were then isolated by ammonium sulphate fractionation and solvent extraction, followed by deoxycholate-ammonium sulphate extraction.

Succinate dehydrogenase activity was assessed by measuring the reduction in absorbance at 600nm, of dichloro-indophenol (DCIP), mediated by phenazine methosulphate, in the presence of 2-thenoyltrifluoroacetone.


nmoles reduced DCIP/min./mg protein.
150
100
50
ND
Cultured Y.S
IN VIVO Y.S
9
10
11
12
13
14
15
16
17
18
Gestational age (days)

3.4 DISCUSSION

A major problem of assessing actual levels of enzyme activity in the glycolytic or tricarboxylic acid cycles is the inter-relatedness of many of the enzymes. One must be sure that the measured activity in the chosen enzyme assay is attributable to a single enzyme, and not to a net result of competing enzyme actions. The ideal solution to this problem is to use purified enzyme preparations, but this approach suffers from two major disadvantages when studying samples of relatively low protein content. Firstly, each step of the purification process results in a small loss in enzyme activity which can accumulate to a considerable cumulative loss (cf. Boyer and Krebs, 1987). Secondly, because of the nature of purification whereby a small constituent of a large mass of protein is isolated, large quantities of tissue are required to give an appreciable yield of purified product.

In the assay of succinate dehydrogenase, the enzyme preparation was semi-purified by isolation of Complex II from the crude mitochondrial pellet. Spectrophotometric assays using mitochondrial or nuclear fractions are extremely difficult, because of the variable turbidity of the assay mixture in the cuvette (Harris and Bashford, 1987). The densely packed nature of the mitochondrial pellet meant that relatively small numbers of yolk sacs were required to produce sufficient protein material to make Complex II extraction feasible. Complex II is a membrane-

bound segment of the respiratory chain that catalyses the transfer of reducing equivalents from succinate to ubiquinone. Succinate dehydrogenase constitutes about 50% of the complex protein weight, and is the only enzyme of the tricarboxylic acid cycle to be bound to the inner membrane of the mitochondria (Hatefi and Stiggall, 1976). Complex II has two major enzyme activities - flavin-linked succinate dehydrogenase and succinate-ubiquinone reductase (Hatefi and Stiggall, 1978). Incorporating 2-thenoyltrifluoroacetone into the assay mixture assured that all ubiquinone-mediated reduction of the dye (dichloroindophenol) was inhibited (Ziegler and Rieske, 1967), and therefore the only reduction of dichloroindophenol was mediated by succinate dehydrogenase-mediated electron transfer to the dye-phenazine methosulphate complex. Bovine serum albumin was present in the assay mixture to facilitate the interaction between the enzyme, dye and electron acceptor.

The relatively low protein content of cytosolic preparations meant that purification of pyruvate kinase or hexokinase enzymes would have required large numbers of yolk sacs both *in vivo* and in culture. Therefore activities were measured in cytosol, and corresponding precautions were taken to attempt to ensure the specificity of the measured activity.

The hexokinase reaction was linked to excess glucose-6-phosphate dehydrogenase, thus preventing the accumulation of glucose-6-phosphate, which is a known inhibitor of hexokinase (Purich *et al.*, 1973). By coupling the hexokinase

reaction to the rate-limiting step in the pentose phosphate shunt, cytosolic NADPH formation could be followed as the end-point of the overall reaction. The other major site of NADPH synthesis is the mitochondrial transfer of acetyl CoA by exchange of pyruvate and citrate (Utter, 1969), whilst the major sites of NADPH utilisation in mammalian systems are cytochrome P-450-mediated reactions and cholesterol biosynthesis (Walsh, 1979). In the yolk sac, the majority of NADPH-dependent reactions take place in the mitochondria (Sheth et al., 1982), therefore preparation of cytosolic fractions for the hexokinase assay remove the potential for interference by these reactions. A pre-incubation step was incorporated into the hexokinase assay, so as to remove all endogenous glucose-6-phosphate from the enzyme preparation prior to assay.

The pyruvate kinase activity in the cytosolic fractions was assessed by coupling the reaction to NADH-dependent lactate dehydrogenase. Utilisation of NADH as the end-point indicator in this assay introduces a number of possible interfering reactions, since NADH is produced or utilised by many reactions in carbohydrate metabolism, as well as being the principal molecule oxidised by the respiratory chain to generate ATP (Stryer, 1981). It was therefore essential to ensure that the cytosolic fraction was essentially mitochondria-free, and therefore the differential centrifugation step to pellet the mitochondria was extended, both in terms of centrifugal force and spin time, on the literature values for mitochondrial preparation (Racker and Stoeckenius,

1974). An additional step to block any contaminant tricarboxylic acid cycle activity was incorporated by pre-incubation with fluoroacetate. Fluoroacetate is irreversibly converted into fluorocitrate by citrate synthetase, and the product potently inhibits aconitase by fluoro-chelating the active site ferrous iron (Gribble, 1973). By blocking aconitase which catalyses the first two steps of the tricarboxylic acid cycle, no further carbohydrate catabolism can take place through this pathway. However, this does not preclude further cycle activity utilising amino acids or fatty acids.

The presence of fructose-1,6-diphosphate in the assay mixture maximally activates pyruvate kinase activity, especially L and M_2 isoenzymes (Imamura and Tanaka, 1982), but its presence may stimulate the utilisation of NAD^+ in the glycolytic conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate.

A limited degree of cross-interference between hexokinase and pyruvate kinase assays may also have been introduced by assaying at 340nm in both assays. However, since both lactate dehydrogenase or glucose-6-phosphate dehydrogenase were added in excess in either assay, the cross-interference should have been negligible.

The assays of glycolytic and tricarboxylic acid cycle enzyme activities in visceral yolk sac all indicated a small decrease in specific activity of glycolysis between 9.5 and

11.5 days, concommitant with switching on of expression of tricarboxylic acid cycle activity.

These findings parallel similar reports of a changeover from anaerobic glycolysis to oxygen-dependent tricarboxylic acid cycle metabolism, in post-implantation rat embryos at this stage of gestation (Tanimura and Shepard, 1970; Shepard et al., 1970). The fall in glucose utilisation between 9.5 and 11.5 days detected in the early reports is more closely paralleled by the measured decrease in hexokinase activity in this study, compared to the findings with the pyruvate kinase assay. This could be explained by a large decrease in pentose phosphate shunt activity in the yolk sac, between these stages of gestation, in agreement with the findings of Clough and Whittingham (1983), who reported such a decrease in post-implantation mouse embryos. A decreased requirement for ribose moieties in nucleic acid synthesis is a likely reason for this decrease in shunt activity, and this would mean a reduced requirement for the product of hexokinase activity - glucose-6-phosphate. Since Clough and Whittingham (1983) reported fairly constant production of lactate, whilst this decrease in shunt activity was measured, one can assume that pyruvate kinase activity was relatively constant, and this may explain the fairly constant levels of pyruvate kinase activity detected in the yolk sac between 9.5 and 11.5 days.

Vollborth (1985) reported decreased glycolytic activity in rat visceral yolk sac from 11.5 to 19.5 days of gestation, measuring aldolase, enolase and hexokinase

activities. In this study, enzyme activities of hexokinase and pyruvate kinase appeared to plateau between 11.5 and 15.5 days, before falling sharply *in vivo*. Gale (1986) also showed a sharp decline in hexokinase activity from 11.5 to 17.5 days in the 'giant' yolk sac model. It is difficult to compare the actual values in the studies, because the earlier results are semi-quantitiative with respect to absorbance values, however, the presence of higher concentrations of glucose in the culture medium may explain why the cultured yolk sac activities remained at a plateau, since uptake of glucose into the cells is likely to have been increased (Jungermann, 1986).

The pattern of expression of succinate dehydrogenase rising to a peak of activity at 15.5 days then decreasing *in vivo*, closely parallels the findings of Walsh (1985) measuring isocitrate, malate and succinate dehydrogenases. Very low succinate dehydrogenase activities were detected in the study of Walsh (1985), probably due to the inaccessibility of the enzyme in a crude tissue homogenate.

Kinetic studies of hexokinase and pyruvate kinase activity in the *in vivo* and cultured yolk sacs revealed isoenzyme patterns in close agreement with the published literature findings. There was very close correlation between the kinetic data of 15.5 day *in vivo* and cultured yolk sac cytosol with respect to hexokinase activity. The k_m values for glucose of 78μM and 75μM are indications of the presence of low k_m hexokinase isoenzymes I, II and III, whose k_m values vary from 16-140μM (Purich *et al.*, 1973;

Balinsky et al., 1975). At high concentrations of glucose (1.2mM), there was no significant change in the kinetics of hexokinase activity, indicating the absence of high k_m glucokinase (Purich et al., 1973). This finding is in agreement with the literature reports of the exclusive appearance of glucokinase in post-natal liver (Walker, 1971).

The apparent k_m values for phosphoenol pyruvate of 404μM and 326μM for 15.5 day in vivo and cultured yolk sac respectively, are in agreement with the published range of k_m values for mammalian pyruvate kinase types L or M_2 (Mildvan and Cohn, 1966; Imamura and Tanaka, 1982; Engstrom et al., 1987), and exclude the presence of types R or M_1. Both types L and M_2 are activated by fructose-1,6-diphosphate, which is in agreement with the findings of this study.

Further resolution of the isoenzyme configuration of visceral yolk sac for hexokinase or pyruvate kinase would require bulk collection of tissue and full purification and electrophoretic identification (Purich et al., 1973; Imamura and Tanaka, 1982).

The decreasing activity of carbohydrate catabolising enzymes in the in vivo yolk sacs between 15.5 and 17.5 days of gestation is probably indicative of a general switch down in metabolic activity of the yolk sac as parturition approaches. Johnson and Spinuzzi (1966) reported similar decreases in activities of alkaline phosphatase, adenosine

triphosphatase, succinate dehydrogenase and non-specific esterases, whilst Padykula (1958) demonstrated histochemically a decrease in succinate dehydrogenase enzyme at 16.5 days of gestation. The mechanism by which the visceral yolk sac metabolic activity is down-regulated is as yet unknown, but a role for fetal or maternally-derived factors is indicated by the apparent absence of down-regulation in the cultured yolk sac. The continued metabolic activity of the cultured yolk sac at 17.5 days may relate to the histological findings in Chapter 2, where the endodermal nuclear appearance of 17.5 day cultured yolk sac indicated the presence of actively synthesising cells. These findings may also explain the prolonged growth of rat visceral yolk sac material in culture for up to 28 days, in the absence of fetal or maternal influence (Lu et al., 1984).

CHAPTER FOUR

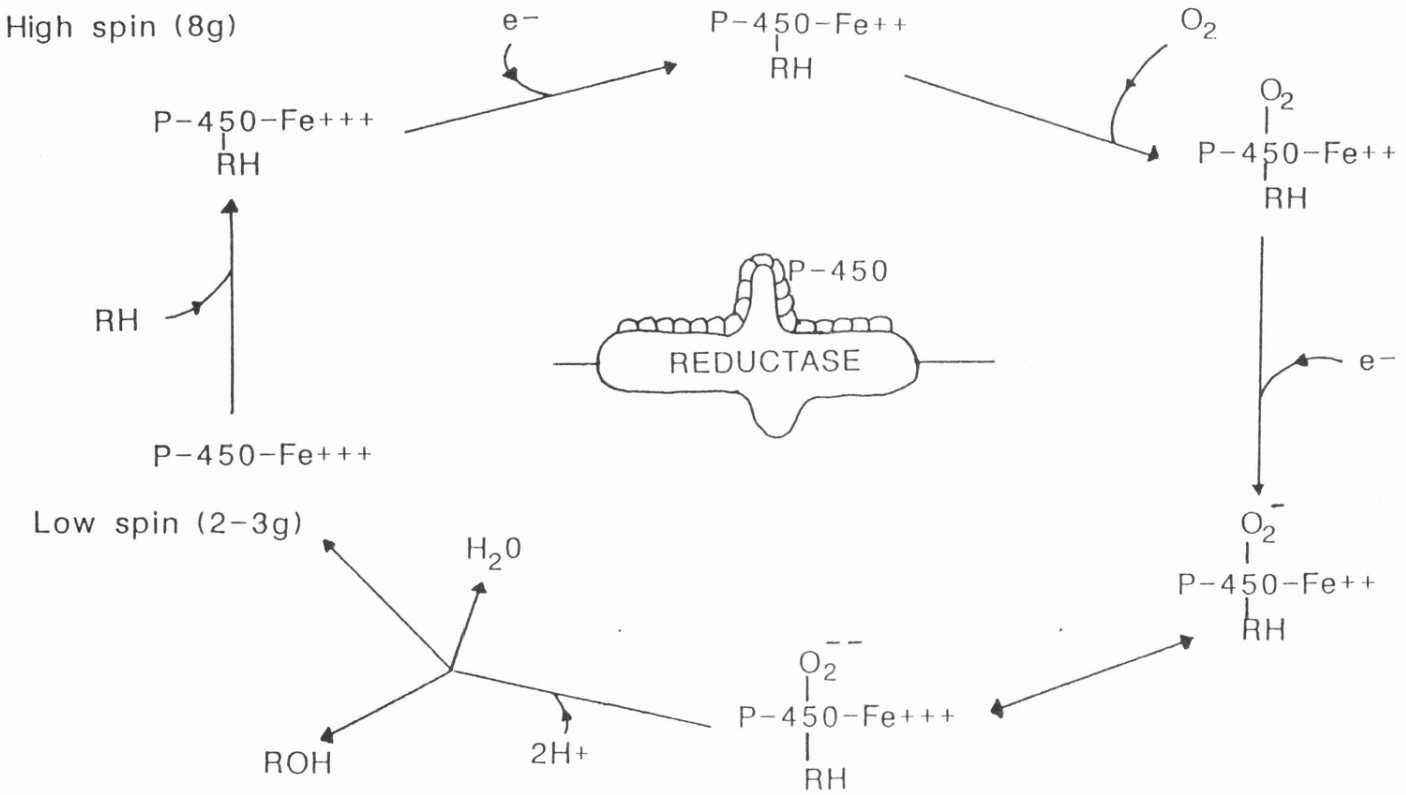
Xenobiotic Metabolising Capability of the Rat Visceral Yolk Sac

Figure 4.1 Electron transfer of iron haem in cytochrome P-450

To enable cytochrome P-450 to act as a mixed function oxidase by binding molecular oxygen, and splitting the molecule into two atoms, one donated to the substrate (R) and one to water, the iron haem of P-450 cycles between ferrous and ferric states, accepting electrons from its adjacent reductase molecule.

In order to bind the substrate whilst in its ferric state, the iron atom promotes electrons in its outer orbitals, into a high spin energy state.

CYTOCHROME P-450



4.1 INTRODUCTION

4.1.1 Cytochrome P-450

Since the original discovery of cytochrome P-450 in hepatic microsomes (Garfinkel, 1958), a large number of investigations have led to the concept that this carbon monoxide-binding pigment represents a large family of b-type cytochromes, that may be found in a wide variety of eukaryotic and prokaryotic cells, in both animal and plant kingdoms.

The ferrous forms of the family of P-450 cytochrome can bind carbon monoxide, the resulting complex absorbing maximally at 446-454nm, hence the name cytochrome P-450 (Omura and Sato, 1964). These cytochromes can serve as terminal oxygenases in mixed-function oxidation reactions, where molecular oxygen is split into two atoms, one being incorporated into the substrate, and one reduced to water (Fig. 4.1). To facilitate the binding of substrate and oxygen, the iron haem unit of P-450 cycles between ferrous and ferric states. This is accomplished by electron transfer from an associated reductase entity (Lu et al., 1969; Prough and Burke, 1975).

In mammalian systems, cytochrome P-450 serves as the terminal oxidase for lipid-soluble, foreign organic compounds (xenobiotics), and endogenous fatty acids, lipids, steroids and prostaglandins (reviewed in Ortiz de Montellano, 1986). A variety of reactions are catalysed,

including N-,O-, and S- dealkylations, C- and N- oxidations and hydroxylations, oxidative deaminations, and sulph-oxidations. In the case of xenobiotics, the reaction products are almost invariably more polar than the parent compound, and are, thus, more readily excreted. However, there are cases when these products are more reactive than their parent compound, and bioactivation by cytochrome P-450 can lead to organ-specific toxicity. For example, hydroxylation of cyclophosphamide leads to mutagenicity and teratogenicity (Hales, 1981), whilst desaturation of valproic acid leads to hepatotoxicity (Rettie et al., 1987).

Originally the versatility of P-450 in metabolising such a wide range of substrates was attributed to a low substrate specificity for a small number of enzymes (Klinger et al., 1981). However, this versatility is now attributed to a multiplicity of structurally and functionally distinct cytochromes with restricted, but possibly overlapping, substrate specificity (Nebert and Gonzalez, 1985).

The ancestral P-450 gene is thought to have diverged over 200 million years ago, probably in response to changes in demand for different endogenous steroids, prostaglandins and biogenic amines, as membrane structure evolved. The production of a number of cytochromes with varying substrate specificity possibly conferred a selection advantage on some individuals now capable of metabolising environmental chemicals which the species encountered. Thus, a gene producing enzymes critical to endogenous function acquired a new role in the production of detoxification enzymes (Nebert

and Gonzalez, 1985; Wolf, 1986). Continued divergence of the P-450 gene superfamily has led to the existence of at least fifty mammalian P-450 genes at the present time.

The emergence of the concept of a P-450 gene superfamily is recent, and previous to this concept, the identification of many P-450 isoenzymes with apparent species and substrate specificity led to Levin, Guengerich and Waxman adopting different classification systems (Haniu et al., 1984; Waxman, 1986). The isoenzymes were often designated by their response to inducing agents. Many P-450 isoenzymes, especially those involved in bioinactivation of foreign compounds, exhibit an increased rate of reaction in conditions of exposure to a wide variety of chemicals (Conney, 1967). Such increases in rate appear to be attributable to enzyme induction, since they are often accompanied by an increase in de novo enzyme synthesis. However, mechanisms such as enzyme stabilisation or activation may also play a role in the induction phenomenon (Juchau, 1981). Although there are a wide variety of chemicals that act as P-450 inducers, they can all be broadly classified in four major categories: phenobarbitol-type, polycyclic hydrocarbon-type, steroid and peroxisome proliferator-type and ethanol-type (Conney, 1967; Juchau, 1981; Sharma et al., 1988).

In only one of the four major classes of inducing agent is the mechanism of induction partially understood. Inducers of the polycyclic hydrocarbon class, such as 3-methylcholanthrene, β-naphthoflavone, 2,3,7,8-dioxin and polychlorinated

biphenyls bind to a specific cytosolic receptor, and this receptor complex crosses into the nucleus and binds to a locus on the specific P-450 gene (Ah locus). This binding is correlated with rapid transcriptional activation of the gene (Lambert and Nebert, 1977; Nebert and Gonzalez, 1985; Wolf, 1986).

Simplification of the variety of different nomenclatures of P-450 isoenzymes has recently been achieved by introducing a nomenclature based on the gene superfamily theory (Nebert et al., 1987). The recommended nomenclature incorporates Roman numerals for distinct gene families, capital letters for subfamilies and Arabic numerals for individual genes. Thus, corresponding P-450 isoenzymes found in rat, rabbit or human, and previously referred to by different names, can now be categorised as products of a single gene. For example, rat P-450c, rabbit P-4506, mouse P-450P, and human $P\text{-}450P_4$ are now all products of P450IA1 (Appendix D).

4.1.2 Prenatal xenobiotic metabolism

Early studies in rabbits, mice and guinea-pigs appeared to indicate that drug metabolising P-450 enzymes were not present during embryonic and fetal stages of development. This was held to account for the lack of observable xenobiotic metabolism (Fouts and Adamson, 1959; Jondorf et al., 1959). Following this finding, a number of studies were carried out, in an attempt to induce P-450 levels in

fetal tissues by pre-treating the mothers with phenobarbitone or 3,4-benzpyrene (Feuer and Liscio, 1964; Basu et al., 1971; Macleod et al., 1972; Muller et al., 1973). The results of these studies indicated that P-450 enzymes were refractive to induction until at least two weeks post partum, and the expression of the enzymes was postulated to be under the control of female gonadal hormones.

Over the same time period, other studies revealed the presence of both steroid-metabolising and drug-metabolising P-450 enzymes in the placentae of rats and man (Chakraborty et al., 1971; Juchau et al., 1972), and this organ was presumed to be the sole organ responsible for protection of the fetus from maternal-borne toxicants.

However, as techniques of detection improved, low levels of P-450 activity have been reported at various stages of gestation. Wang et al. (1974) reported low levels of benzpyrene hydroxylation in hamster fetal liver and skin microsomes, whilst Martinez-Zedillo et al. (1979) showed parathion dearylation in late fetal tissues of the horse. At this stage, all reported non-primate mammalian P-450 activity in prenatal tissues occurred in late gestation, whereas low P-450 expression could be observed in humans and primates in early and midgestation (Neims et al., 1976). Further improvements in technique allowed Filler and Lew (1981) to detect benzpyrene metabolism in pre-implantation mouse blastocysts, whilst Faustman-Watts et al. (1983) showed rat embryos in whole-embryo culture could bioactivate acetylaminofluorine, in the presence of a hepatic enzyme

source. By the use of P-450 inducing agents, such as pregnenolone-16α-carbonitrile, Arochlor 1254 and 3-methylcholanthrene, it was shown that the embryo could produce its own bioactivation enzymes which were more potent in producing embryotoxicity than the maternal enzyme source (Juchau et al., 1985a; Juchau et al., 1985b; Faustman-Watts et al., 1985).

The teratogenicity of acetylaminofluorine can therefore be modulated by induction of embryonic P-450 isoenzymes. The teratogenicity of cyclophosphamide can also be modulated by induction of bioactivation via P-450. However, this bioactivation takes place exclusively in the maternal circulation (Fantel et al., 1979; Hales, 1981). This can be explained by the presence of polycyclic aromatic hydrocarbon-inducible forms of P-450 in the prenatal situations, these forms being responsible for hydroxylation of acetylaminofluorine. The corresponding absence of phenobarbitone-inducible forms, responsible for cyclophosphamide hydroxylation, until postnatal stages of development explains the absence of fetal-mediated cyclophosphamide teratogenicity (Lum et al., 1985; Henderson et al., 1986).

4.1.3 Xenobiotic metabolism potential of rat visceral yolk sac

The visceral yolk sac performs many fetal liver-like functions prior to the formation of the fully functional hepatic unit in the rat. These functions include haemo-

poiesis, carrier protein production, growth factor production and glycogen storage (Moore and Metcalf, 1970; Janzen et al., 1982; Beck et al., 1987; Padykula et al., 1966).

Taking into account these liver-like functions, and the visceral yolk sac-mediated uptake and transport of molecules from the maternal circulation throughout gestation, there is obvious potential for xenobiotic metabolism in the visceral yolk sac.

Sheth et al. (1982) reported production and metabolism of progesterone, pregnanediol and oestradiol in the rat visceral yolk sac. This metabolism was NADPH-dependent, indicating the role of a pyridine nucleotide/haemoprotein-dependent enzyme similar to cytochrome P-450. Danan et al. (1985b) also showed a steroid metabolism role of the visceral yolk sac in the catabolism of vitamin D. These findings indicate the presence of substrate-specific steroid metabolising forms of cytochrome P-450, but there is only one unpublished report of xenobiotic metabolic capacity of the rat visceral yolk sac. Holness (1984) reported biphenyl hydroxylase activity in visceral yolk sac, with peak activity at 14.5 days of gestation.

4.1.4 Methods of study of cytochrome P-450

The least sensitive measurement of the presence of cytochrome P-450 in a tissue homogenate, is the absorbance spectrum given by the carbon monoxide adduct (Estabrook

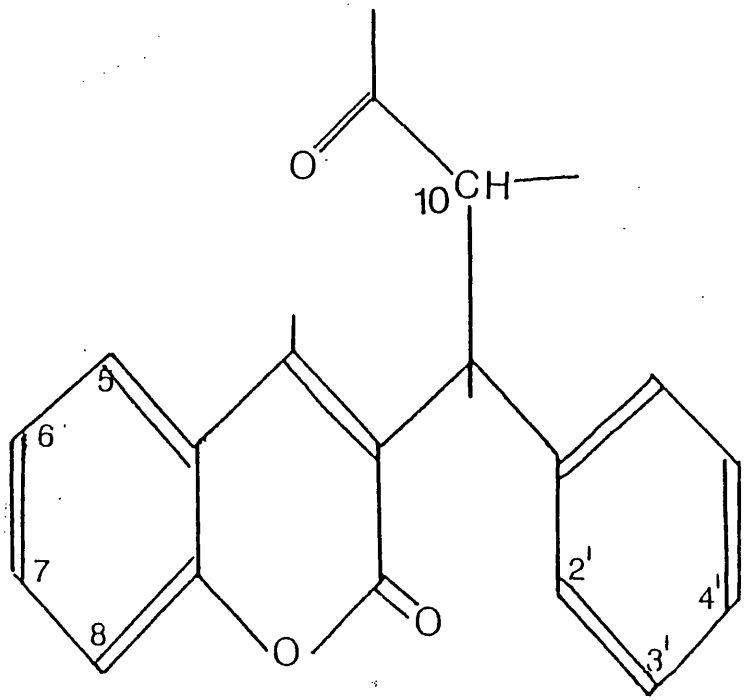
et al., 1972). This can be used to assess qualitatively the isoenzyme of P-450, since different isoenzymes exhibit different absorbance peak maxima. For example, in rat, the polycyclic aromatic hydrocarbon-inducible isoenzyme has a maxima at 448nm, the phenobarbitone-inducible isoenzyme has a maxima at 450nm, and the peroxisome proliferator-inducible form has a maxima at 452nm. The technique can also be used quantitatively by using dual-beam spectrophotometry to measure absorbance of the adduct in sample and reference cuvettes.

A more sensitive method of studying actual enzyme activity is to measure specific substrate turnover or co-factor turnover in microsomal preparations of a defined organ. By measuring NADPH turnover, a rough estimate of activity can be obtained, but pyridine nucleotide biochemistry is complicated by many competing reactions. NADPH is formed in mammalian systems by malate dehydrogenase and the pentose phosphate shunt. It is utilised in P-450-mediated reactions, cholesterol biosynthesis and production of deoxyribonucleotides (Stryer, 1981).

More accurate assessment of P-450 activity can be made by measuring specific substrate turnover using spectrophotometry (O'Brien and Rahimtula, 1978), fluorimetry (Burke and Prough, 1978), thin layer liquid chromatography (Burke and Bridges, 1975), gas-liquid chromatography (Wolff et al., 1979) or high performance liquid chromatography (Kaminsky et al., 1981).

Figure 4.2 P-450-mediated hydroxylation pattern of warfarin

Warfarin is regio- and stereo-selectively hydroxylated by different isoenzymes of cytochrome P-450. Hydroxylation can occur at ring positions 6, 7 and 8, ring position 4' or on carbon-10 (Kaminsky et al., 1983).



WARFARIN

Enzyme protein production can be assessed quantitatively and qualitatively by immunocytochemical techniques using polyclonal or monoclonal antibodies (Baron et al., 1986; Foster et al., 1986).

In this study, xenobiotic metabolising capacity of in vivo yolk sac microsomes was assessed using two highly specific fluorimetric substrate turnover assays. Ethoxycoumarin-O-deethylation is a reaction performed by the majority of drug-metabolising isoenzymes (Ullrich and Weber, 1972), whilst ethoxyresorufin-O-deethylation is a more specific reaction carried out exclusively by polycyclic aromatic hydrocarbon-inducible forms of P-450 (Burke and Mayer, 1974).

Because of the problem of the number of yolk sacs needed to produce a microsomal pellet from cultured yolk sacs, a different type of sensitive assay was used.

Visceral yolk sac is known to have a high affinity for the uptake of serum albumin (Agarval and Moore, 1979), and warfarin is known to be highly bound to serum albumin (Bachmann, 1974). Warfarin also has a well known pattern of P-450-mediated hydroxylation at 4^1,5,6,7,8 and C-10 ring positions (Fig. 4.2), for which different isoenzymes show regio- and stereo-selectivity (Fasco et al., 1978b). By using reverse-phase high performance liquid chromatography, it is possible to measure the hydroxylated metabolites of warfarin, qualitatively and quantitatively.

Because the assays for specific drug-metabolising isoenzymes differ between cultured and in vivo yolk sacs,

immunocytochemical techniques using specific polyclonal antibodies were used to support the biochemical studies, and to localise the enzymes histochemically.

4.2 METHODS

4.2.1 In vivo visceral yolk sac

a) Pretreatment

Timed-mated female Olac Wistar rats (200-350g) were maintained under conditions described in Chapter 2.2.1.

Six groups of rats, each consisting of three mothers, were injected intraperitoneally with sodium phenobarbitone (PB), β-naphthoflavone (BNF), pregnenolone-16α-carbonitrile (PCN), Arochlor 1254 (A-1254), corn oil or saline. Injections of PB (75mg/kg/day), BNF (100mg/kg/day), PCN (80mg/kg/day), or the appropriate saline or corn oil control, were administered once a day, for 3, 4 and 5 days respectively prior to the removal of the uteri. A-1254 (500mg/kg) was administered i.p. as a single dose, 5 days prior to removal of the uteri. These dose regimens were taken as accepted protocols for cytochrome P-450 induction (cf. Fantel et al., 1979; Juchau et al., 1985a).

b) Microsome preparation

Conceptuses were removed from the uterus and immediately placed in ice-cold SET buffer (0.25M sucrose, 5.4mM ethylenediamine tetraacetic acid, 0.1M Tris-HCl buffer, pH 7.4). The visceral yolk sacs were dissected from the fetuses and chorio-allantoic placentae, mixed and homogenised in three

equivalent volumes of SET buffer, using a Potter-Elvehjem homogeniser with Teflon pestle. The homogenate was spun at 10,500g (calculation from rpm using tables produced from equations of de Duve and Berthet, 1953) for 15 minutes at 4°C to remove nuclear and mitochondrial fractions, then respun at 100,000g for 60 minutes at 4°C (Du-Pont Sorvall OTD-65 ultracentrifuge) to obtain a microsomal pellet. This pellet was resuspended in half the original yolk sac displaced volume of SET buffer and stored at -40°C.

Microsomal preparations were produced from <u>in vivo</u> yolk sacs taken at 13.5 to 21.5 days of gestation, and also from 25% (w/v) homogenates of maternal liver and fetal liver from 16.5 to 21.5 days of gestation. These microsomal preparations were then assayed for P-450 activity using the fluorimetric substrate-turnover assays.

c) <u>Fluorimetric substrate-turnover assays for cytochrome P-450</u>

Ethoxycoumarin-O-deethylase (ECOD) activity was assessed by measuring the increase in fluorescence due to the product - 7-hydroxycoumarin (λex = 380nm, λem = 460nm) with time at 37°C, on a Perkin Elmar LS-5B spectrophotometer, according to the method of Ullrich and Weber (1972). Ethoxyresorufin-O-deethylase (EROD) activity was assessed by measuring the increase in fluorescence due to the product - resorufin (λex = 510nm, λem = 586nm) with time, under the same conditions, using the method of Burke and Mayer (1974).

Specific activities were evaluated in terms of mg of microsomal protein, estimated by the method of Lowry et al. (1959) as described in Chapter 2.2.7.

Using uninduced adult liver, both enzyme reactions were assessed for linearity with time over varying substrate and enzyme concentrations.

In vivo yolk sac activities were assessed for sensitivity to heat (65°C for 30 minutes) and carbon monoxide (bubbling for 3 minutes).

4.2.2 Cultured visceral yolk sac

a) Yolk sac culture

Yolk sacs were cultured by the method described in Chapter 2.5. The serum-based medium contained various concentrations of sodium warfarin ranging from 0-32µM. [Previous experimentation had revealed that warfarin had no deleterious effects on yolk sac growth below 64µM, and that this concentration range had no significant effect on pH or osmolality of the culture medium].

After 2, 5 and 8 days in culture, the yolk sacs were removed from the culture medium, pooled in groups of twelve and homogenised in 66mM Tris-HCl buffer (pH 7.4), using a hand held glass pestle. A 10.0µl aliquot was removed for protein assay by the method of Lowry et al. (1951). The remaining homogenate was spun at 3,000rpm for 2 minutes in a bench centrifuge, then the supernatant was filtered through

serial Millipore filters of 1.2, 0.66 and 0.22μm pore size. The filtrate was stored at -40°C, until being assayed for warfarin metabolites.

b) Warfarin metabolite assay by high performance liquid chromatography

Adult liver microsomes prepared from rats treated with PB or BNF were incubated with 2.5mM sodium warfarin (Aldrich, UK) for 5 minutes at 37°C before being placed on ice, in the warfarin hydroxylase assay described by Fasco et al. (1977). The reaction mixture was filtered through serial Millipore filters of 1.2, 0.66 and 0.22μm pore size, then 100μl of filtrate was concentrated on a Rainin Dynamax 4.6mm ID C-18 reverse phase HPLC column with pre-column. The filtrate was then eluted on a linear mobile phase gradient, run from 84% to 40% (w/v) acetic acid (pH 4.8) in acetonitrile, over 16 minutes at a flow rate of 1.0ml/min. The HPLC system was controlled by an LKB pump and controller, and hydroxylated metabolites of warfarin were detected in a flow cell, by absorbance at 313nm on an LKB 4050 Ultraspectrophotometer, and subsequent integration on a LKB integrator.

The adult liver microsomes were used to optimise the separation of 4-, 7- and 8-hydroxywarfarin on the column, using 0-2.5μg/ml standard solutions of the three hydroxylated metabolites (4-, 7- and 8-hydroxywarfarin were supplied by Central Toxicology Laboratory, ICI, Alderley Park), to

quantify the absorbance peaks, as described by Fasco et al. (1978b).

Cultured yolk sac filtrates were eluted through the column following the same protocol.

4.2.3 Immunocytochemical studies of P-450 isoenzymes

Cultured and in vivo yolk sacs, and sections of freshly dissected adult rat liver lobes, were embedded in OCT compound (Miles, UK) and frozen over solid CO_2 in hexane. 7µm sections were cut on a Leitz cryostat, picked up on 3-amino- propyltriethoxysilane-coated glass slides (Rentrop et al., 1986) and stored on dry ice for 15 minutes. The sections were then fixed in absolute acetone for 20 minutes at $0^{o}C$ and stored on dry ice.

The following method of immunocytochemistry is a modified version of that described by Boobis et al. (1985) and Foster et al. (1986).

Sections were treated with 5% (w/v) hydrogen peroxide in methanol for 30 minutes at $-20^{o}C$ to block endogenous peroxidases, thoroughly washed in 1% (w/v) bovine serum albumin in phosphate-buffered saline (pH 7.0), then treated overnight with rabbit anti-rat polyclonal antibodies to rat P-450c, P-450d and P-450PCN (IgG protein values of 25, 200 and 50ng/ml respectively). The polyclonal antibodies were a gift to Central Toxicology Laboratory, ICI, from Dr. J. Hardwick, Argonne National Laboratory, USA, and were assessed for specificity on rat liver by Western blotting

performed by Dr. R. Bars, Central Toxicology Laboratory, ICI, as described in Sesardic et al. (1986).

After thoroughly washing with albumin in saline, the sections were then incubated with goat anti-rabbit secondary antibody, and binding was visualised using the peroxidase anti-peroxidase/diaminobenzidine technique. The sections were then counterstained with Harris' haematoxylin (15 seconds), blued in tap water, then dehydrated in serial alcohols, cleared in xylene and mounted in DPX. Appropriate negative controls were incorporated using no primary antibody, or non-immune rabbit serum as primary antibody.

4.3 RESULTS

4.3.1 ECOD and EROD activities in in vivo visceral yolk sac microsomes

Using uninduced rat liver microsomes, specific activities of 97.2 ± 4.4 nmoles/min/mg protein for ECOD and 23.5 ± 2.1 nmoles/min/mg protein for EROD were observed (n = 8), when substrate concentrations were set at 500μM ethoxycoumarin and 1μM ethoxyresorufin. At these concentrations, rate of reaction was linear with respect to enzyme concentration.

In vivo yolk sac microsomes, prepared from control or PB-treated animals, did not exhibit any detectable ECOD or EROD activity (n = 12). Liver microsomes prepared from the PB-treated dams exhibited an increase in ECOD activity from 97.2 ± 4.4 to 6,710 nmoles/min/mg protein, and a small increase in EROD activity from 23.5 ± 2.1 to 40.1 ± 3.7 nmoles/min/mg protein.

Induction of both ECOD and EROD activities was seen in in vivo yolk sac microsomes prepared from BNF or A-1254-treated rats. A similar pattern of induction of both ECOD and EROD activity was observed with both inducers (n = 12). Fig. 4.3 shows specific activities of ECOD (a) and EROD (b) for yolk sac microsomes from BNF and A-1254-treated animals, and for fetal liver microsomes prepared from the same BNF-treated animals. Yolk sac activity appeared at 16.5 days, rose to a peak at 17.5 days, and then fell to below detectable levels by 19.5 days. Maximum values for the ECOD

assay were 39.8 ± 3.1 pmoles/min/mg protein with BNF as an inducer, and 56.4 ± 4.2 pmoles/min/mg protein with A-1254; for the EROD assay the maximum values were 29.4 ± 1.6 pmoles/min/mg protein with BNF, and 42.3 ± 2.9 pmoles/min/mg protein with A-1254.

At this stage of gestation (17.5 days), the ratios between the specific activities in yolk sac and fetal liver microsomes from the same litters treated with BNF, were 2.03 (ECOD) and 4.74 (EROD). These differences were statistically significant when Student's 't' test was applied ($p < 0.005$).

When PCN was used as an inducing agent, low levels of ECOD activity were measured at 15.5 and 16.5 days of gestation, a maximum value of 9.7 pmoles/min/mg protein being recorded. However, this activity was not consistently reproducible in every microsomal preparation, appearing three times in twelve microsome preparations.

ECOD activity in 17.5 day *in vivo* yolk sac was heat-labile and carbon monoxide-sensitive. Heating 100µl 17.5 day *in vivo* yolk sac microsomes at 65°C for 30 minutes caused a 82% drop in activity (n = 3), whilst bubbling CO through the same volume of microsomes suspended in 2ml of 66mM Tris-HCl buffer, pH 7.4, reduced activity by 93% (n = 3).

4.3.2 Warfarin hydroxylation in cultured visceral yolk sac

In the warfarin hydroxylation assay, microsomes from PB-induced maternal liver hydroxylated warfarin at the 4-, 7- and 8- ring positions, with preferential hydroxylation at

the 7- position. Microsomes from BNF-induced maternal liver microsomes metabolised warfarin almost exclusively at the 8-position (Fig. 4.4).

Filtrate from homogenised cultured yolk sac contained only one detectable warfarin metabolite - 8-hydroxywarfarin, which was detected at all three culture endpoints (2, 5 and 8 days of culture). Fig. 4.4 shows an increase in the concentration of this metabolite from days 2 to 5, but no change between days 5 and 8. There was a detectable amount of 8-hydroxywarfarin in the exocoelomic fluid at day 5 and 8 of culture (equivalent gestational ages 14.5 and 17.5 days) amounting to 12.4-16.6 pmoles/mg protein, but no detectable metabolites in medium incubated over the same culture period, in the absence of yolk sac material, or in yolk sac material cultured in the absence of warfarin.

Fig. 4.5 shows the increase in 8-hydroxywarfarin concentration with increasing warfarin concentration in the medium. The increase is linear over the concentration range 0.32μM sodium warfarin for 11.5 day and 14.5 day cultured yolk sacs, but reaches a maximum concentration of 148 pmoles/mg protein for 17.5 day cultured yolk sacs, then plateaus.

BNF-induced and uninduced *in vivo* yolk sac microsomes at 15.5 days exhibited no warfarin hydroxylase activity when assayed by the method of Fasco *et al*. (1977). However, BNF-induced *in vivo* yolk sac microsomes at 17.5 days produced a peak of 8-hydroxywarfarin equivalent to 2.1 pmoles/mg protein (n = 2).

4.3.3 Immunocytochemical localisation of P-450 isoenzymes

Frozen sections of uninduced or PB-treated *in vivo* yolk sac did not bind polyclonal antibodies to rat P-450s c, d and PCN.

BNF-induced *in vivo* yolk sac at 14.5 days did not bind anti-rat P-450 c, d or PCN (Fig. 4.6), but BNF-induced *in vivo* yolk sac at 17.5 days bound anti-rat P450c exclusively in the endodermal layer (Fig. 4.7).

Cultured yolk sac bound anti-rat P-450c in the endodermal cells, at equivalent gestational ages of 14.5 days (Fig. 4.8) and 17.5 days (Fig. 4.9), with no evidence of any antibody binding in the adjacent mesoderm.

No detectable binding of anti-rat P-450s d and PCN was observed in either *in vivo* or cultured yolk sac, although at the concentrations used, these antibodies strongly bound to BNF- or PCN-induced maternal liver (Fig. 4.10).

Figure 4.3 O-deethylation activities in *in vivo* visceral yolk sac microsomes

a) Ethoxycoumarin-O-deethylase activity was measured by the increase in fluorescence of the product - 7-hydroxycoumarin (Ullrich and Weber, 1972).

b) Ethoxyresorufin-O-deethylase activity was measured by the increase in fluorescence of the product - resorufin (Burke and Mayer, 1974).

- ▲ represents Arochlor-1254-induced yolk sac microsomes.
- ■ represents β-naphthoflavone-induced yolk sac microsomes.
- □ represents β-naphthoflavone-induced fetal liver microsomes.

ND = no product detectable.

Values are means ± s.e.m. of twelve determinations from different microsomal preparations.

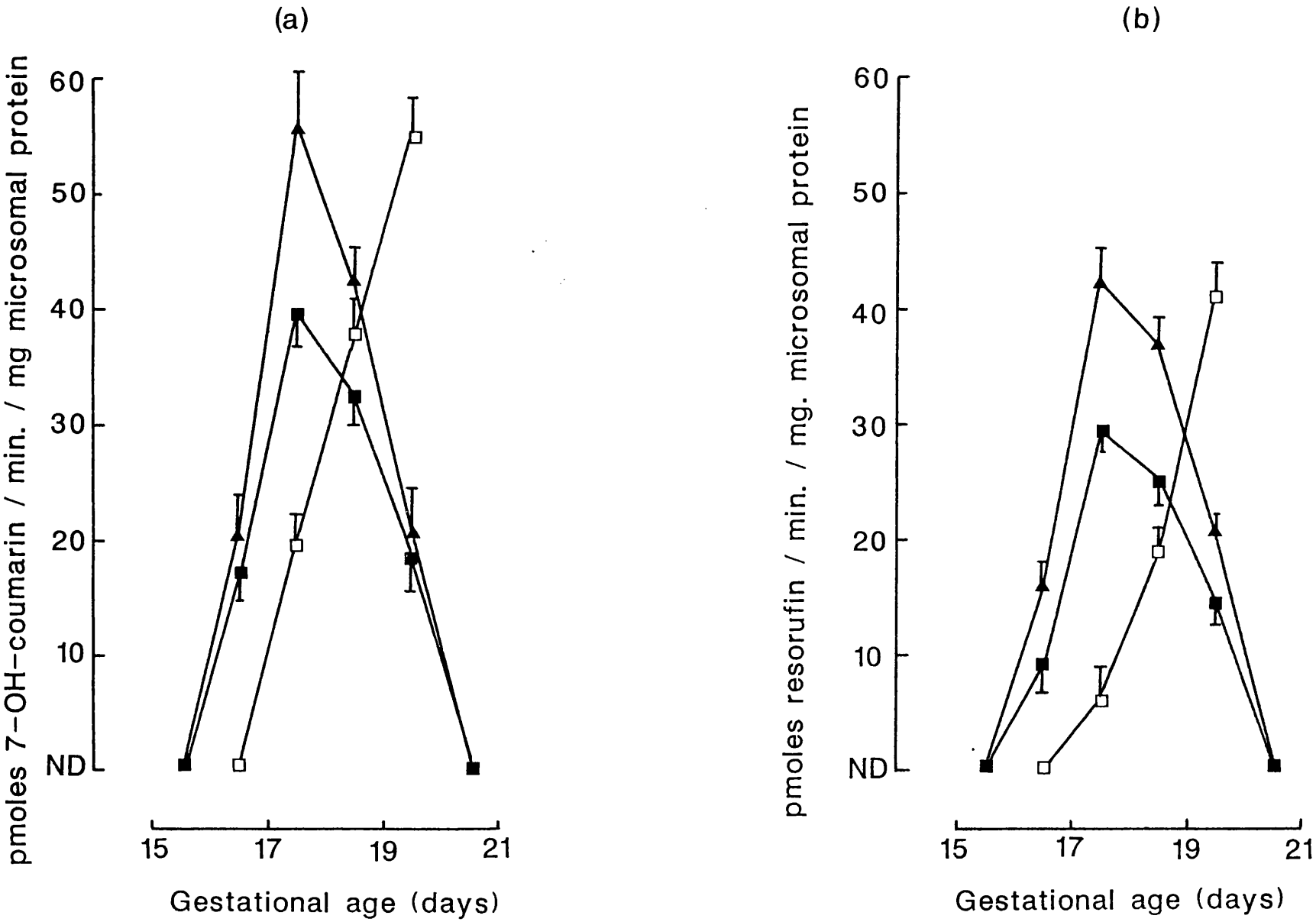

(a)
pmoles 7-OH-coumarin / min. / mg microsomal protein
60
50
40
30
20
10
ND
15
17
19
21
Gestational age (days)
(b)
pmoles resorufin / min. / mg. microsomal protein
60
50
40
30
20
10
ND
15
17
19
21
Gestational age (days)

Figure 4.4 Hydroxylation of warfarin in cultured yolk sacs and maternal liver

4-(), 7-() and 8-() hydroxywarfarin were detected by u-v absorbance at 313nm after HPLC separation by reverse-phase chromatography.

The values are means ± s.e.m. of thirteen determinations.

In culture, sodium warfarin (128 nmoles) was present in the culture medium, whilst, in maternal liver sodium warfarin (80 µmoles) was added for 5 minutes with a NADPH-generating system.

ND = no product detectable.

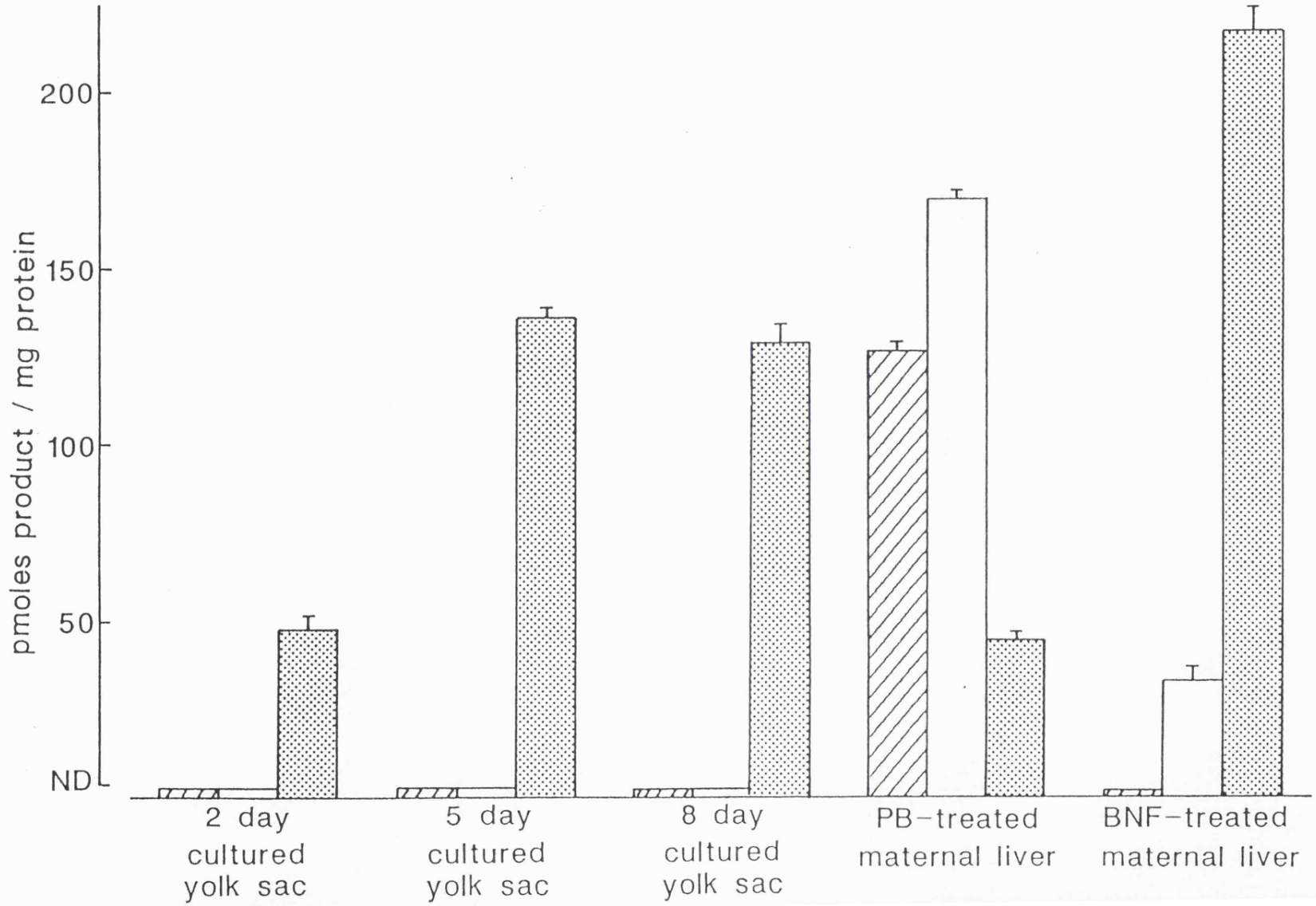

pmoles product / mg protein
200
150
100
50
ND
2 day cultured yolk sac
5 day cultured yolk sac
8 day cultured yolk sac
PB-treated maternal liver
BNF-treated maternal liver

Figure 4.5 Concentration of detected 8-hydroxywarfarin in cultured yolk sacs with increasing warfarin content of the medium

8-hydroxywarfarin was detected by u-v absorbance at 313nm, after HPLC separation by reverse-phase chromatography.

The values are means ± s.e.m. of six determinations.

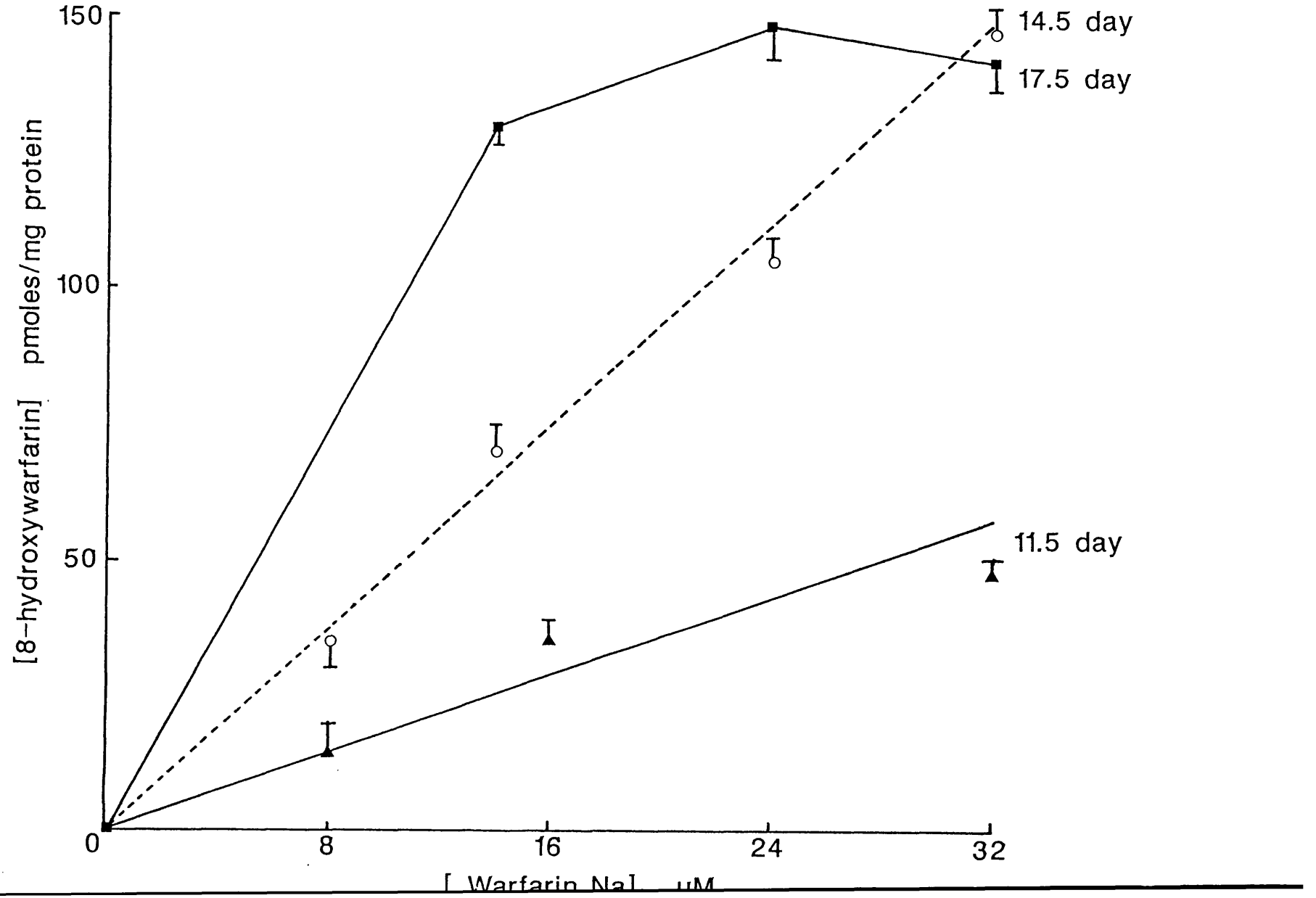

150
100
50
0
8
16
24
32
[8-hydroxywarfarin] pmoles/mg protein
[Warfarin Na] uM
14.5 day
17.5 day
11.5 day

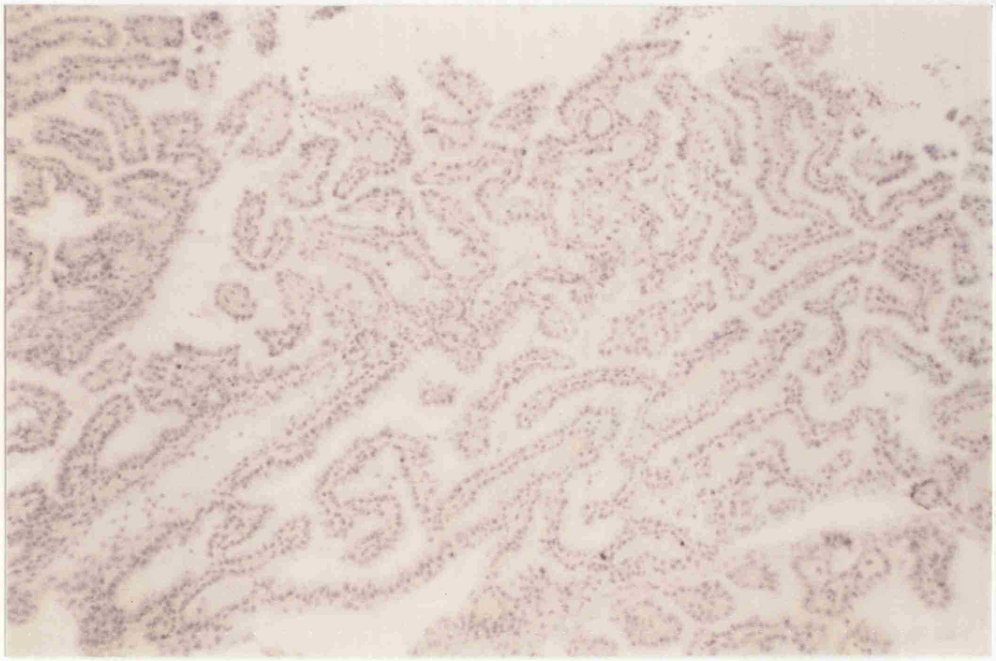
Figure 4.6 14.5 day *in vivo* yolk sac (BNF-treated) (x 95)

7μm frozen sections were fixed in absolute acetone for 20 minutes, then incubated overnight with rabbit anti-rat polyclonal antibodies to:

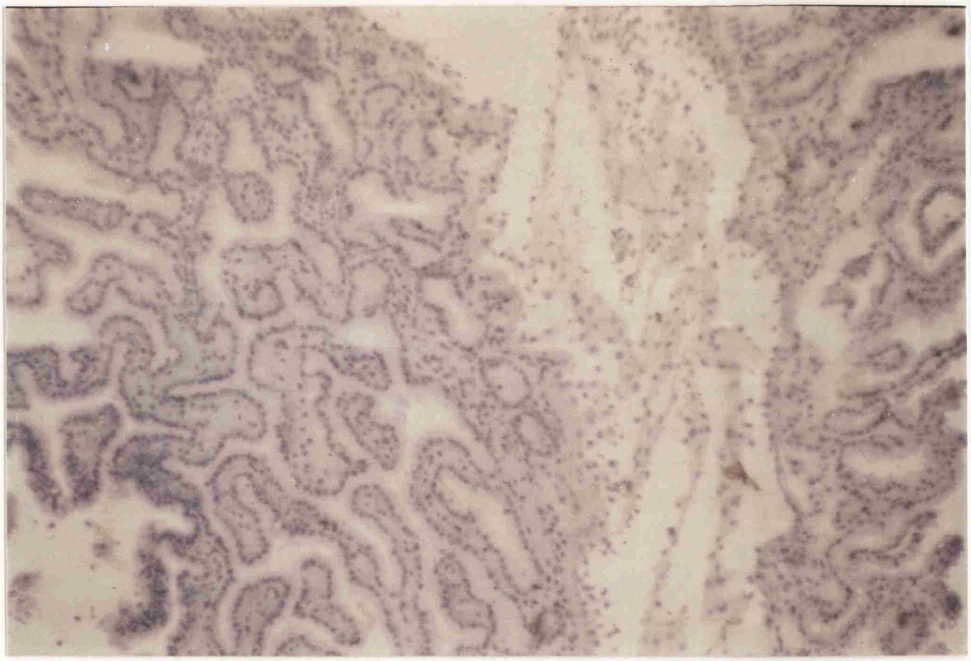
a) rat P-450c
b) rat P-450d
c) rat P-450 PCN

Binding was visualised by peroxidase-linked anti-rabbit antibody reaction with diaminobenzidine. This reaction leaves a brown precipitate over the site of binding, and can not be seen in these photomicrographs. The sections were then counterstained with Harris' haematoxylin.

a



b



c

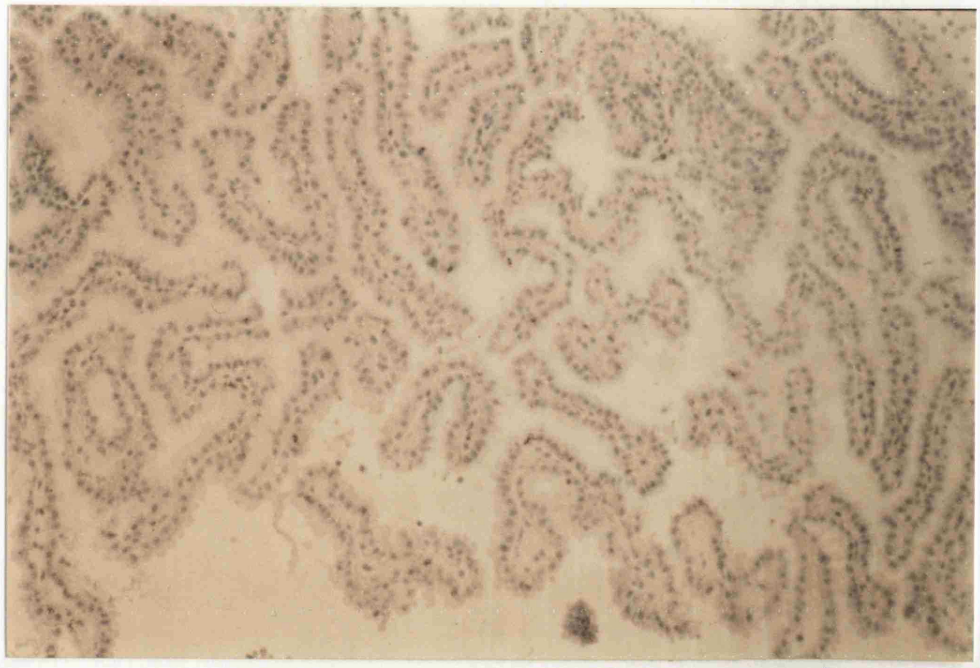


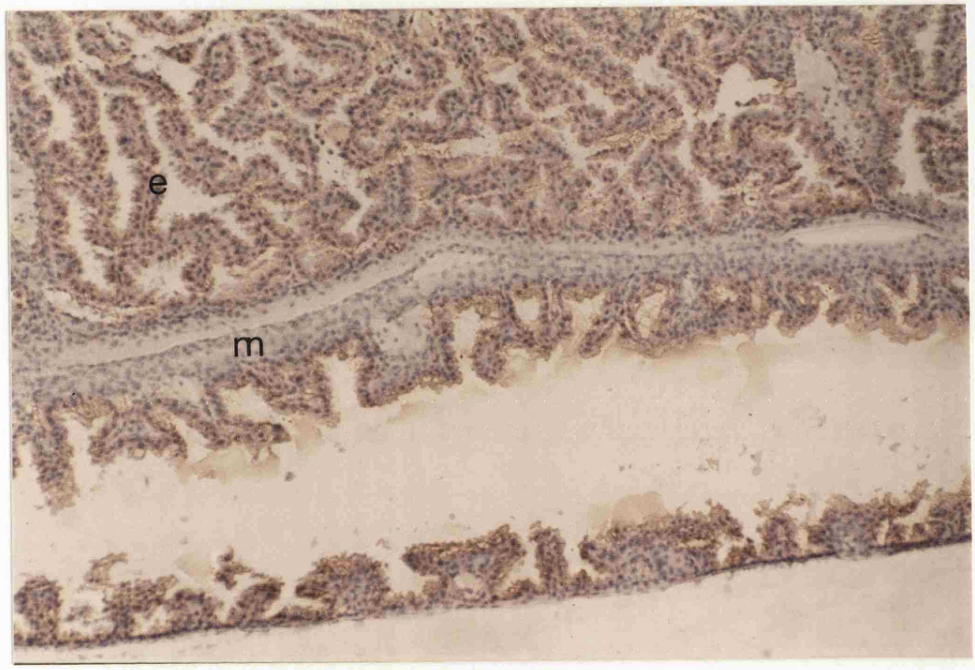
Figure 4.7 17.5 day *in vivo* yolk sac (BNF-treated) (x 95)

7μm frozen sections were fixed in absolute acetone for 20 minutes, then incubated overnight with rabbit anti-rat polyclonal antibodies to:

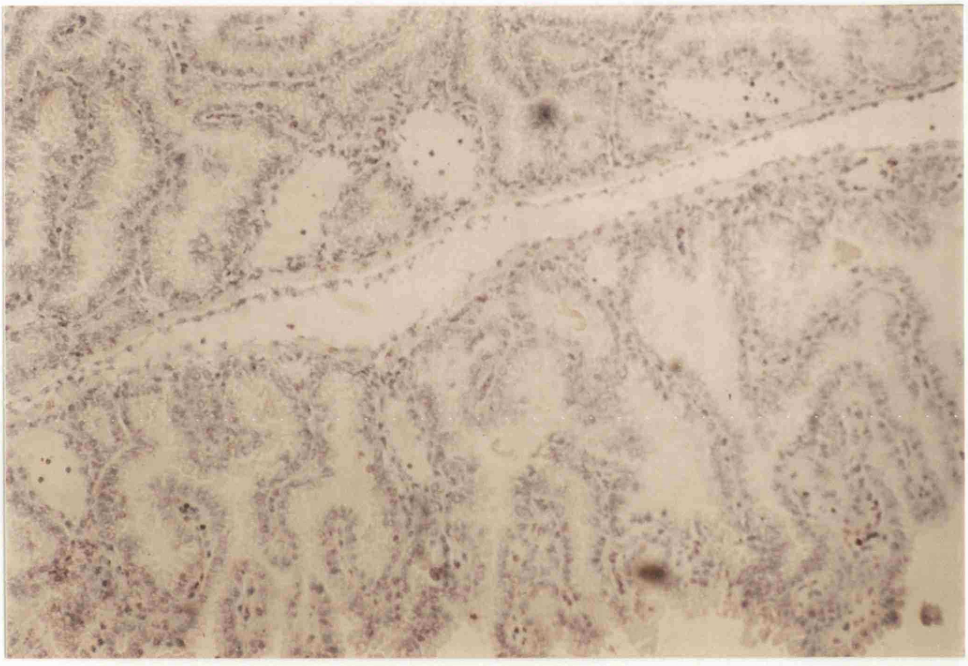
a) rat P-450c
b) rat P-450d

Binding was visualised by peroxidase-linked anti-rabbit antibody reaction with diaminobenzidine. This reaction leaves a brown precipitate over the site of binding, and can be observed in the endoderm (e) but not in the mesoderm (m). The sections were then counterstained with Harris' haematoxylin.

a



b



.1mm

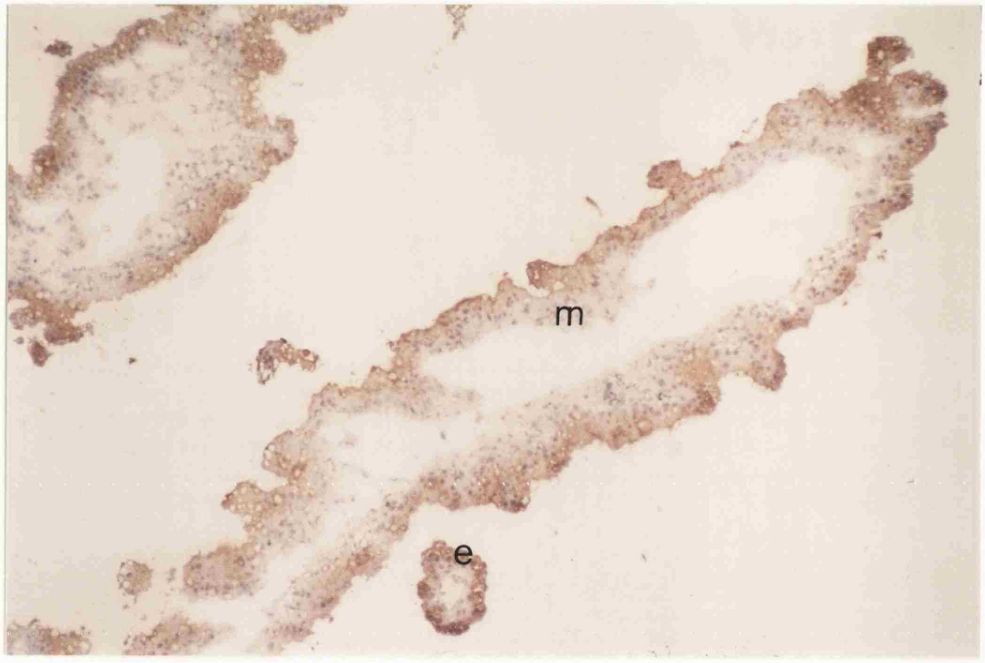
Figure 4.8 14.5 day cultured yolk sac x 95 (a and b) or x 380 (c)

7μm frozen sections were fixed in absolute acetone for 20 minutes, then incubated overnight with rabbit anti-rat polyclonal antibodies to:

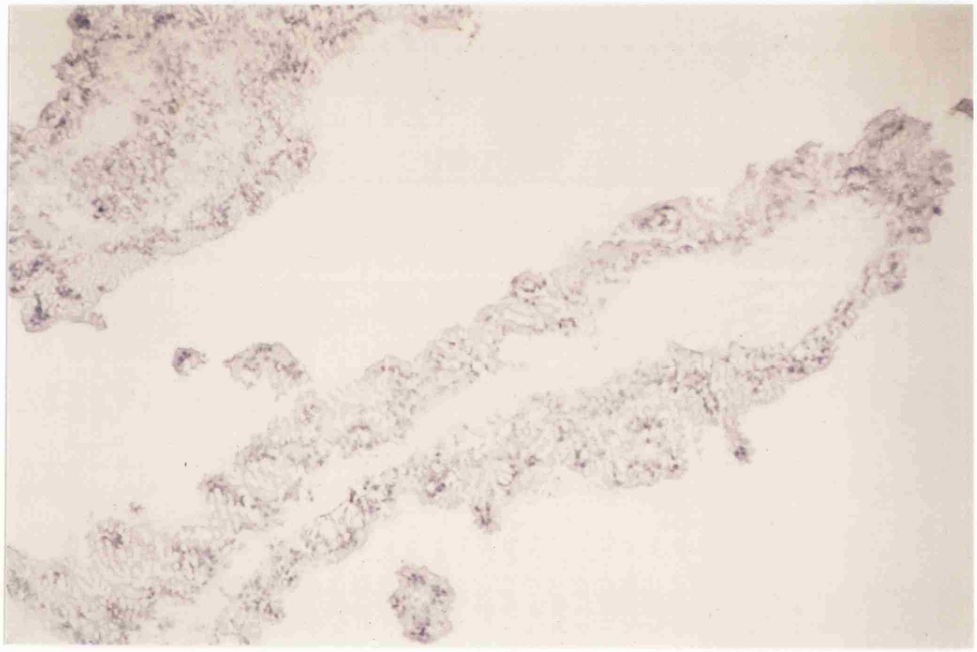
a) rat P-450c
b) no primary antibody
c) rat P-450c

Binding was visualised by peroxidase-linked anti-rabbit antibody reaction with diaminobenzidine. This reaction leaves a brown precipitation over the site of binding, and can be observed over the endoderm (e), but not the mesoderm (m). The sections were then counterstained with Harris' haematoxylin.

a



b



c

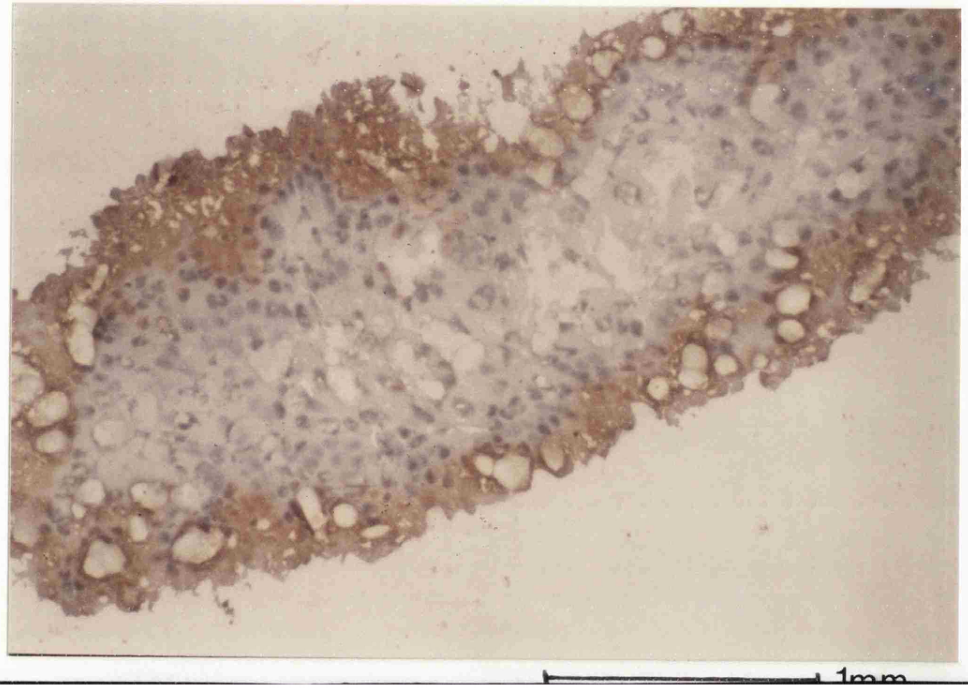


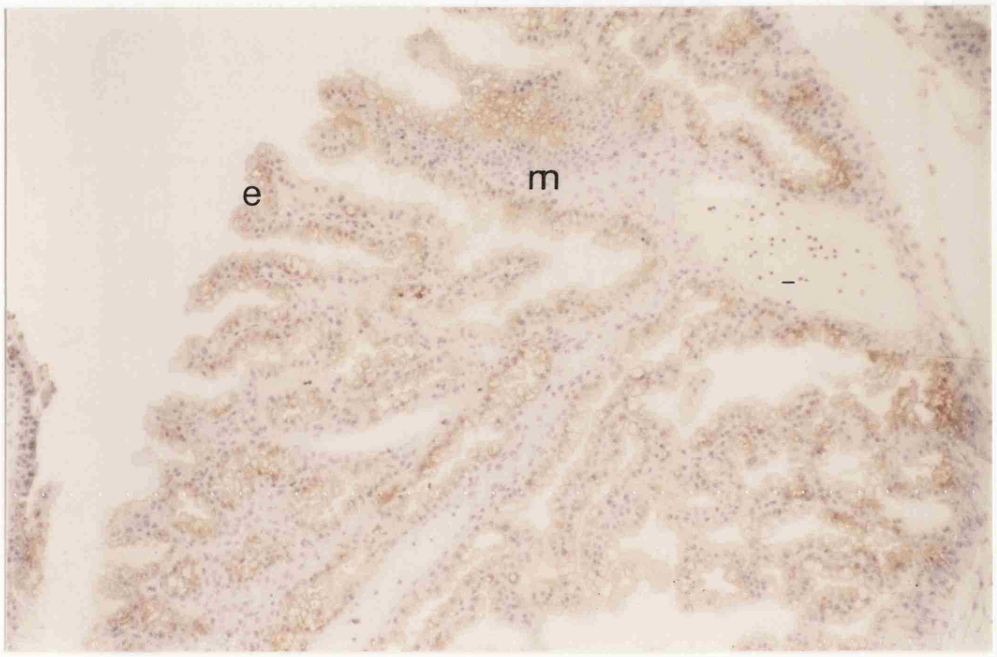
Figure 4.9 17.5 day cultured yolk sac x 152 (a and b) or x 380 (c)

7μm frozen sections were fixed in absolute acetone for 20 minutes, then incubated overnight with rabbit anti-rat polyclonal antibodies to:

a) rat P-450c
b) rat P-450d
c) rat P-450c

Binding was visualised by peroxidase-linked anti-rabbit antibody reaction with diaminobenzidine. This reaction leaves a brown precipitation over the site of binding, and can be seen over the endodermal cells (e), but not the mesoderm (m). The sections were then counterstained with Harris' haematoxylin.

a



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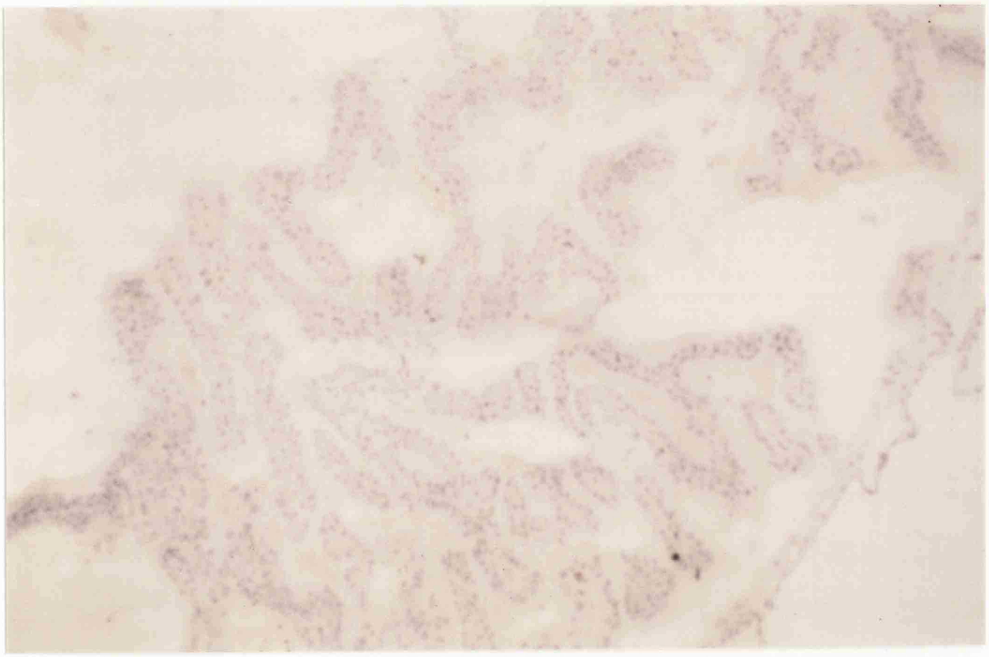
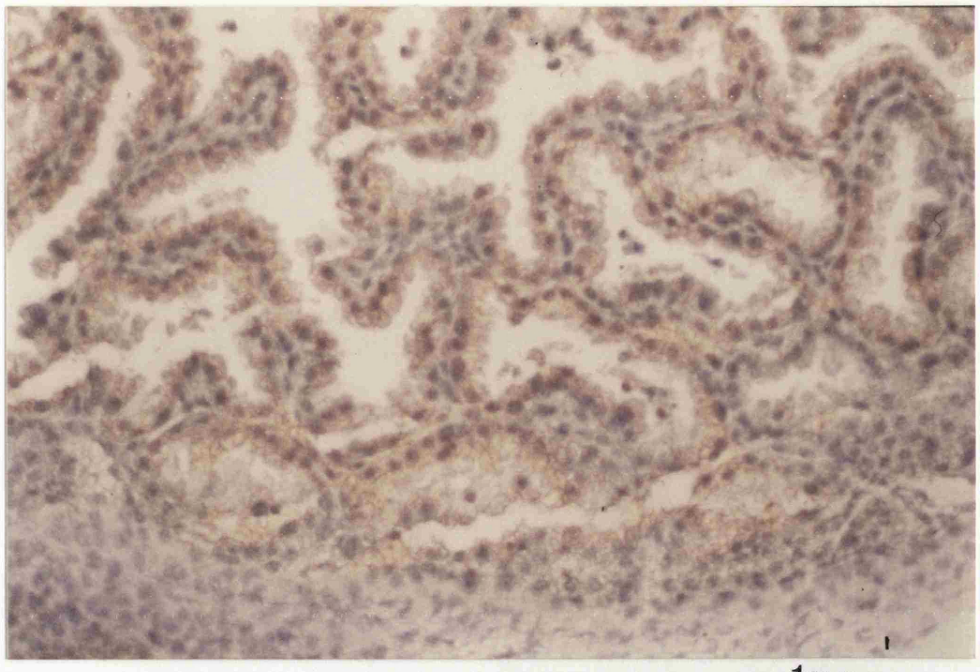
b



c



1mm

Figure 4.10 Maternal liver (x 152)

7μm frozen sections of BNF-induced (a and b) and PCN-induced (c and d) maternal liver were fixed in absolute acetone for 20 minutes, then incubated overnight with rabbit anti-rat polyclonal antibodies to:

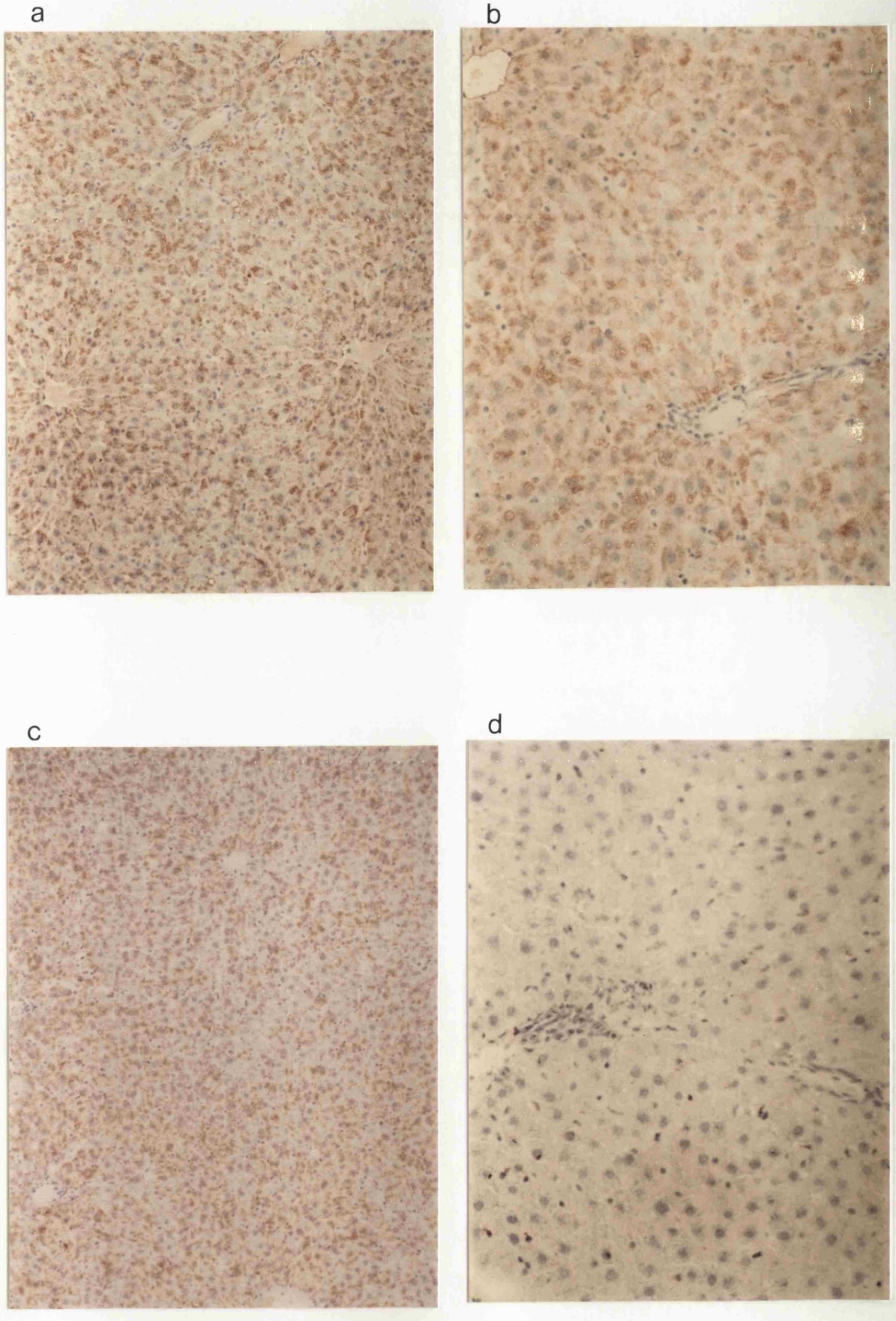
a) P-450c

b) P-450d

c) P-450 PCN

d) Non-immune rabbit serum as primary antibody

Binding was visualised by peroxidase-linked anti-rabbit antibody reaction, observed as a brown precipitate over the site of binding. The sections were then counterstained with Harris' haematoxylin.


a
b
c
d
.1mm

4.4 DISCUSSION

The O-dealkylation of ethoxycoumarin provides an assay for P-450 activity, with the potential for detecting a wide range of the xenobiotic metabolising isoenzymes (Matsubara et al., 1986; Pacifici et al., 1988). The uninduced ECOD activity of maternal rat liver microsomes obtained in this study compares well with the value of 115 ± 8 nmoles/min/mg protein reported by Matsubara et al. (1986).

Since yolk sacs removed from uninduced rats did not exhibit any detectable ECOD activity, it may be concluded that these structures have, at best, a very limited potential to metabolise ingested macromolecules. This contrasts with the detectable uninduced P-450 activities found in chorio-allantoic placentae of many mammals (Symms and Juchau, 1973; Gottlieb and Manchester, 1986).

However, when pregnant rats were pretreated with multiple injections of the polycyclic hydrocarbon, β-naphthoflavone, yolk sac microsomes were capable of dealkylating both ethoxycoumarin and ethoxyresorufin, between 15.5 and 18.5 days of gestation. The dealkylation of both substrates indicated the specific presence of P-450 enzyme produced by the IA gene subfamily (Nebert et al., 1987), since ethoxyresorufin-O-deethylation is a reaction specific to polycyclic hydrocarbon-inducible enzymes (Burke and Mayer, 1974; Gulyaeva et al., 1986).

The initial finding of specific P-450-mediated activity in the rat visceral yolk sac, indicated by the specificity

of the assay data and the carbon monoxide sensitivity of the activity, was the first published xenobiotic metabolising capability of this organ (Rowlands and Beck, 1987; Rowlands et al., 1988). The presence of the polycyclic-inducible activities in rat visceral yolk sac paralleled similar findings in mouse blastocysts, mouse embryos, mouse fetal liver and cultured rat embryos (Filler and Lew, 1981; Lambert and Nebert, 1977; Burki et al., 1973; Juchau et al., 1985; Neubert and Tapken, 1988). By contrast, other studies using 3-methylcholanthrene as a polycyclic hydrocarbon have failed to detect any induced P-450 activity in mammalian fetal forms (Lum et al., 1985; Giachelli and Omiecinski, 1987). The overwhelming weight of evidence indicates that genes of the subfamily P-450IA are expressed prenatally, and that evidence to the contrary is due to pharmacokinetic variability in the dose of inducing agent reaching the fetal target organ (Neubert, 1988). Indeed, in the case of 3-methylcholanthrene as an inducing agent, Marie et al. (1988) showed that transplacental induction of IA gene production was controlled by the concentration of cytosolic binding protein. Since cytosolic binding proteins for polycyclic hydrocarbons are induced by both phenobarbitone- and polycyclic hydrocarbon-type P-450 inducers (Okey and Vella, 1984), this may explain the improved efficiency of Arochlor 1254 (a mixed inducer) to induce P-450 isoenzymes in the yolk sac, compared to β-naphthoflavone.

At the stage of gestation when visceral yolk sac was exhibiting induced P-450 activity, fetal liver was only

slightly responsive to induction, and it was found that specific activities of EROD and ECOD in the yolk sac were much higher than corresponding activities in fetal liver. As the fetal liver became responsive to P-450 induction, the visceral yolk sac activities showed a concommitant decrease. The fetal liver pattern of response to BNF-induction closely paralleled the inductive response to Arochlor 1254 reported by Wong et al. (1987). The decrease in P-450 activity of the yolk sac from 18 days of gestation is not a novel phenomenon, in terms of rat visceral yolk sac enzyme functions. Morphological and biochemical changes are widespread in this organ as parturition approaches, and similar decreased activities have been reported for other enzymes with a wide range of functions (Padykula, 1958; Johnson and Spinuzzi, 1966). Examples include alkaline phosphatase, adenosine triphosphatase, succinate dehydrogenase and non-specific esterases, and the fall in these activities is often paralleled by a large rise in fetal liver activities.

The visceral layer of the rat yolk sac is open to exposure to maternal-borne xenobiotics, either directly, by uptake from the uterine fluid, or via the fetus, by passage through the chorio-allantoic placenta, and then through the vitelline vasculature. Therefore, it is apparent that, under certain conditions of chemical exposure, the biotransformation capacity of the rat visceral yolk sac may be raised to a level where significant activation or inactivation of a potential embryonic toxicant may occur.

Since this finding of yolk sac P-450 activity, Juchau et al. (1988) reported low levels of O-depentylation, O-debenzylation and O-deethylation reactions in rat visceral yolk sac, cultured as part of the whole embryo culture technique from 9.5 days to 11.5 days of gestation.

From the warfarin hydroxylation assay results, the visceral yolk sac in culture appears to be capable of hydroxylating warfarin exclusively at the 8-ring position, at a stage of culture equivalent to gestational ages between 9.5 days and 15.5 days, when no P-450 activity was apparent in vivo. To substantiate this finding, microsomes from uninduced and BNF-induced visceral yolk sac at 15.5 days and 17.5 days were assayed by the warfarin hydroxylase assay employed for maternal liver. No activity was observed at 15.5 days, whilst at 17.5 days, BNF-induced in vivo yolk sac microsomes exhibited a low hydroxylation of warfarin at the 8-position.

There is considerable evidence that this selective hydroxylation at the 8-ring position is an exclusive property of products of the IA gene subfamily (Kaminsky et al., 1983; Rettie et al., 1985). The loss of a proportional increase in 8-hydroxywarfarin production with increasing warfarin concentration, between 5 and 8 days of culture, may be partly explained by further metabolism of hydroxylated warfarin metabolites. Phase II conjugation reaction enzymes have been demonstrated to be present in rat visceral yolk sac (Harris et al., 1986; Harris et al., 1987), and reactions such as glutathione conjugation may

play an important role in the elimination of P-450-metabolised xenobiotics from the yolk sac.

The immunocytochemical findings provided evidence to back up the biochemical results, both in terms of the isoenzyme profile of the visceral yolk sac, and the ontogenic pattern of expression of P-450 *in vivo* and in culture.

The P-450IA gene subfamily is the simplest of the subfamilies in the P-450 gene superfamily, since it appears to contain only two genes - A1 and A2. In the rat, these two genes generate two protein types, previously termed P-450c and P-450d, with 70% amino acid homology and a major difference in ferric iron spin state (Nebert *et al.*, 1987). The immunocytochemical findings indicated that neither β-naphthoflavone nor Arochlor 1254 induced enzymes that bound a polyclonal antibody to P-450d in *in vivo* or cultured visceral yolk sac, although both compounds induced such enzymes in maternal liver. Therefore, our results indicate the expression of only one of the IA genes - A1, in the visceral yolk sac, the expression being polycyclic hydrocarbon-inducible *in vivo*, but apparently constitutive in culture, where no exogenous induction was required.

There are other cases of P-450 isoenzymes being expressed differently *in vivo* and in culture; for example, polycyclic hydrocarbon-induced fetal hepatocytes lose the ability to express the normal ontogenic pattern of isoenzymes *in vivo* (Bollinne *et al.*, 1987; Cresteil *et al.*, 1986). There are also cases of a P-450 isoenzyme being expressed constitut-

ively in one tissue and only when induced in another; for example, P-450IIB enzymes in lung and liver (Wolf, 1986).

Visceral yolk sac also shows differences in enzyme expression between *in vivo* and *in vitro* environments; ornithine decarboxylase activity in cultured yolk sac at 10.5 days is only a fraction of the corresponding *in vivo* activity (Huber and Brown, 1982), whilst cathepsin B expression in cultured yolk sac is also lower than the corresponding *in vivo* activity after 11.5 days (Decatris, 1988).

The exclusive expression of P-450IA gene in visceral yolk sac is matched by the results of Shiverick *et al*. (1986) in rat fetal liver. They reported that P-450c and not P-450d was inducible by β-naphthoflavone, and it is interesting to note that they found this to be true in haemopoietic cells, whose progenitor cells may originate in the rat visceral yolk sac (Moore and Metcalf, 1970).

Mathis *et al*. (1986) reported that the polycyclic hydrocarbon induction of P-450c in fetal liver hepatocytes could be potentiated by co-culture with dexamethasone, a synthetic glucocorticoid. Using Southern blotting and exonuclease footprinting methodology, Mathis *et al*. (1989) reported that a glucocorticoid responsive element, which binds the glucocorticoid-receptor complex, was present in the first intron of the P-450IA gene, and positively regulated the expression of this gene. By an extension of the hypothesis that fetal liver expression and visceral yolk sac expression are inversely linked, and using the indirect evidence of

maternal and fetal transcription factors affecting visceral yolk sac function, the cultured yolk sac may act as an important model in the study of the regulation of this P-450IA gene in prenatal xenobiotic metabolism. The particular advantages of this organ culture system include: the availability of the serum-based medium for analysis of putative maternal or fetal transcription factors, the differences in IA1 enzyme expression in vivo and in culture, and the presence of two types of cell, one type expressing the enzyme (endoderm) and one type being non-expressive (mesoderm). A single experimental attempt to identify the subcellular localisation of the P-450 isoenzymes in rat visceral yolk sac, using unfixed tissue in a cryo-immuno-histochemistry technique failed due to poor retention of yolk sac morphology (see Appendix E).

A further development in the prenatal expression of P-450 isoenzymes has been the identification of a human P-450 isoenzyme - P-450HFLa in human fetal liver, which catalyses testosterone-6β-hydroxylation, but also catalyses limited drug oxidations (Kitada et al., 1987; Wrighton et al., 1988). This isoenzyme type was found to be present in fetal liver of rats, rabbits, guinea-pigs, dogs and monkeys (Kitada et al., 1988), and may be related to the PCN-inducible isoenzymes produced by the P-450IIIA gene family. The findings of this study with PCN as an inducer were non-correlative. Immunocytochemistry using anti-rat P-450PCN antibody produced no positive binding in yolk sac material, but occasional low ethoxycoumarin-O-deethylase activity was

recorded with 15.5 and 16.5 day in vivo yolk sac microsomes. The lack of antibody binding may be explained by the polyclonal antibody not cross-reacting with the protein produced by the IIIA3 gene. The lack of reproducibility in the PCN-induction of O-deethylation activity may be due to the low activity generated, or less likely, to the expression of these isoenzymes being controlled by an autosomal recessive genetic polymorphism, as is seen in humans with the P-450 isoenzyme responsible for debrisoquin metabolism (Gonzalez et al., 1988).

Further study of yolk sac P-450 isoenzyme expression may therefore benefit from using molecular biological techniques to study gene transcription. Gene and nucleotide structures to rat P-450 isoenzymes responsible for xenobiotic metabolism are available for probe manufacture (Hines et al., 1985; Gonzalez et al., 1985; Black and Coon, 1986).

The assays and techniques employed in this study of xenobiotic metabolising capacity of the rat visceral yolk sac would only detect production of gene subfamilies IA, IIA, IIB and IIIA, and therefore exclude other P-450-mediated activities such as endogenous steroid production and cholesterol metabolism, which have been reported previously (Sheth et al., 1982).

CHAPTER FIVE

Use of the Cultured Visceral Yolk Sac in Studies of Prenatal Gene Expression

5.1 INTRODUCTION

5.1.1 Transcription and translation of genetic information

DNA, the molecule of heredity in all prokaryotic and eukaryotic organisms, expresses its genetic information by determining the protein synthesis of a cell (Stryer, 1981). The nuclear DNA is packaged in condensed chromosomes which unravel to reveal distinct genes. The DNA consists of large non-coding regions interspersed with introns and exons, which in varied combination code for specific messenger RNAs.

Jacob and Monod (1961) introduced the concept of mRNA as a short-lived polynucleotide intermediate, between the DNA template and the cytosolic protein synthesis units of the ribosomes. The intermediate completely conserved DNA base composition, and was quite large, since they correctly assumed that three nucleotides coded for one amino acid, in the eventual translation of genetic information into protein structure.

Single stranded mRNA is transcribed from either DNA strand, in a 5'-> 3' direction, to produce heterogenous nuclear mRNA.

The transcription of DNA to RNA in eukaryotes, is regulated by a complex series of promoter, enhancer and suppressor sites upstream of the transcription initiation site (Maniatis and Ptashne, 1976).

Eukaryotic primary gene transcripts are rarely used directly as mRNA, and are usually post-transcriptionally

modified by extensive splicing, methylation capping of the 5' guanine residues, and polyadenylation of the 3' tail (Shatkin, 1976).

A proportion of these secondary nuclear mRNA species are then transported into the cytoplasm, where they are linked to the endoplasmic reticulum to code for exported proteins, or to the free ribosomal compartment for cytosolic protein production.

Although post-transcriptional control of protein production can occur, the main regulation of protein synthesis occurs in the nuclear transcription phase, thus by studying messenger RNA production in a cell type or organ, some conclusions can be drawn about the synthetic functioning of the cells.

5.1.2 Techniques for measuring mRNA expression

A specific mRNA can be detected in the cytoplasm of cells, either in solution, on filters or in tissue sections, by hybridisation to a probe, containing the complementary nucleotide sequence to a portion of the mRNA. The probe can be labelled by radioactive or biotinylated markers (Maniatis et al., 1982; Pringle et al., 1987).

Three main types of probe are used in the detection of mRNA: double-stranded cDNA probes, single stranded RNA probes (riboprobes), or single stranded synthetic oligonucleotide probes (Hudson et al., 1981).

To prepare a cDNA probe specific to a mRNA, total mRNA must be extracted from a tissue known to synthesise the encoded protein. This may be done by differential precipitation of the RNA in lithium chloride and urea (Auffray and Rougeon, 1980) or by the guanethidium-isothiocyanate separation method (Maniatis et al., 1982).The small fraction of this sample which is mRNA is extracted as poly(A)+ RNA, by making use of the fact that the majority of eukaryotic mRNAs are 3'-polyadenylated, and can therefore be column separated by binding to polymers of thymidine (Maniatis et al., 1982). Using an oligo-dT primer, a cDNA strand can be synthesised by reverse transcriptase, and a complementary cDNA strand can then be synthesised by DNA polymerase I, using labelled or unlabelled nucleotides. The naturally formed hairpin loop of paired cDNA is then cleaved by S1 nuclease to form double stranded cDNA. The cDNA is then inserted into a plasmid vector and taken up in a suitable bacterial or bacteriophage host. Insert-containing colonies are selectively cultured (Watson et al., 1983), then the plasmid is cut using appropriate restriction enzymes, electrophorised on agarose gel, and the insert is extracted, diluted and labelled by either the random primer or nick-translation method (Feinberg and Vogelstein, 1983; Rigby et al., 1977).

The double stranded cDNA probe has several hundred residues, and may therefore be labelled to a high specific activity (Coghlan et al., 1987).

However, the double-stranded probes have to be denatured at high temperature leading to some uncontrolled degradation, and the possibility of re-annealing during hybridisation. The production of cDNA is a complicated, long procedure limited by the culture productivity, whilst impurities can be incorporated from the cloning vector (Coghlan et al., 1987).

Riboprobes are produced from cDNA by subcloning into a plasmid vector containing promoter sequences for specific bacteriophage RNA polymerases (Melton et al., 1984). By linearisation of the plasmid twice, using opposing promoters, two transcription templates may be produced, which, using the appropriate polymerases, will produce two RNA probes, one of which is complementary to the mRNA, and one which is not and can be used as the control (sense). Riboprobes are produced from cDNA and therefore suffer from the conferred disadvantages, but they are single stranded and therefore cannot re-anneal, and their size can be controlled by alkaline hydrolysis (Cox et al., 1984). Non-specific probe binding can also be removed by RNase A digestion, leaving the RNA/RNA hybrids intact, however digestion of the RNA probe by endogenous RNAse activity can be a problem during RNA preparation. Because of the number of enzymes and radioactive nucleotides required in the preparation of riboprobes, this technique is expensive, and is best used only when high sensitivity is required, in cases of mRNAs of low copy number.

Abundantly transcribed RNAs can be detected using synthetic oligonucleotide probes. These probes can be manufactured using the published nucleotide sequence of cDNA (Caruthers et al., 1982). The oligonucleotide probes are usually 30-70 bases long, and therefore penetrate more easily into cross-linked tissue sections than cDNA probes. The shortened length also confers an ability to selectively hybridise to variable regions on two or more related mRNAs (Coghlan et al., 1985), and synthesis of control complementary sequences (sense probes) is possible.

Oligo-nucleotides are manufactured by 3' binding the initial mononucleotide to a phosphoramidite column (Caruthers et al., 1982). Selected 3'-protected mononucleotides are then sequentially condensed onto the synthetic chain. The protective groups are removed with thiophenol, then the oligonucleotide is ammonia-cleaved from the column, purified by ion-exchange HPLC or acrylamide gel electrophoresis, then 5'-end labelled with the chosen label.

The disadvantages of oligonucleotide hybridisation are that the technique does not work for some mRNAs perhaps due to small fixative-associated changes in tertiary structure, whilst the single label on the 5' or 3' end of the probe produces lower specific activity compared to cDNA or riboprobes, therefore limiting sensitivity (Coghlan et al, 1987).

In situ hybridisation of fragments of DNA to complementary DNA sequences in chromosome spreads (Gall and Pardue,

1969) formed the basis for the development of a number of techniques for the detection and localisation of nucleic acid sequences. Southern (1975) developed a method of detecting specific sequences of DNA restriction fragments, which were complementary to DNA, whilst cDNA probes were hybridised to isolated cell types in solution to identify RNA species (Brahic and Haase, 1978). The techniques were further developed so that immobilised mRNA could be detected, either by Northern or dot blotting on nitro-cellulose filters (Kafatos et al., 1979; Auffray and Rougeon, 1980) or by fixing in tissue sections (Hudson et al., 1981). The latter technique of in situ hybridisation of DNA or RNA probes to fixed tissue sections was defined as hybridisation histochemistry (Coghlan et al., 1985), due to its similarity to the identification of proteins in tissue sections by immunohistochemistry (cf. Baron et al., 1986 for review). The histological identification of mRNA or protein in a tissue section by binding to a labelled probe or labelled antibody respectively, are analogous techniques, whilst characterisation of the probe or antibody is made by observing the specificity of its binding to RNA or protein species, electrophoretically separated by molecular weight (Northern or Western blotting). The combined use of these two techniques of hybridisation histochemistry and immunocytochemistry yields results which indicate the cellular expression of a gene, and the resulting level of transcriptional and translational activity producing the protein (Kashkin et al., 1987).

The first procedure to be carried out in *in situ* hybridisation to tissue sections, is the fixation of tissue RNA. Tissue sections may be prepared from frozen or paraffin-embedded blocks, depending on the requirements for retention of morphology. Frozen sections are easier to prepare, requiring no elaborate processing or pretreatment before hybridisation, however paraffin-embedded sections provide better morphology and can provide serial sectioned material over a longer time period, since they have unlimited storage potential. For frozen section studies, the essential requirement for *in situ* hybridisation is rapid freezing of fresh tissue to prevent RNA digestion, therefore tissues are frozen, then post-fixed in fixative at $4^{o}C$. The fixative of choice for *in situ* hybridisation is a cross-linking fixative such as glutaraldehyde (Sabatini *et al*., 1963), which maintains a higher level of nucleic acid retention by covalent binding, than precipitation fixatives, such as acetone (Cox *et al*., 1984). In contrast, immunocytochemistry results are often enhanced by the use of precipitating fixatives, which precipitate the protein without altering tertiary structure to an extent where antigenic sites are destroyed (Baron *et al*., 1986). With paraffin-embedded sections, adequate fixation of the tissue with a cross-linking fixative is required to prevent loss of nucleic acid by diffusion during processing.

The kinetics of hybridisation between labelled probe and specific mRNA vary with each individual study, therefore the

temperature of hybridisation, and the ionic strength of hybridisation buffer must be optimised for each study. The optimum temperature for efficient hybridisation is about 30°C below the melt temperature of the hybrid (Watson et al., 1983). The melt temperature of the hybrid can be reduced by adding formamide (a helix destabiliser) to the hybridisation buffer (Coghlan et al., 1985). High ionic strength buffer will allow hybrid formation, but by lowering the ionic strength, the specificity of hybrid formation can be increased (Meinkoth and Wahl, 1984).

Blocking of non-specific binding of the probe is a property of a number of the macromolecules incorporated in hybridisation buffer, especially dextran sulphate and Denhardt's solution (Hudson et al., 1981). However, because hybridisation may occur between the probe and non-specific binding sites, matched control sections must be prepared, and hybridised under the same experimental conditions, to another specific probe (positive control), or to a sense probe to the complementary RNA sequence used (negative control). This is especially important if the guanine and cytosine content of the probe is high, since the increased hydrogen binding strength of these bases increases the chances of non-specific binding (Coghlan et al., 1987).

Hybridisation of radioactively labelled probes to mRNA is usually visualised by autoradiographic exposure to X-ray film or photographic emulsion. The emulsion consists of a suspension of silver halide crystals in a gelatine matrix,

and emitted particles dislodging electrons from the crystal lattice produce a latent image which, if retained, can be developed in photographic developer (Bogoroch, 1972). Increasing the emulsion grain size increases the sensitivity, as does increasing the emulsion thickness. However, these changes will be accompanied by increased background signals, and decreased cellular resolution.

The choice of radioactive probe label depends on the cellular definition required in the end result; probes can be labelled with ^{3}H, ^{32}P or ^{35}S. Tritium was the original isotope used in <u>in situ</u> hybridisation techniques (Gall and Pardue, 1969); its low activity emission giving excellent cellular localisation, and its long half-life (12.5 years) enabling replicate results to be obtained from one labelled probe sample, provided auto-digestion of the probe did not occur. However, the low energy emissions require long exposure times under autoradiography to produce results, whilst slight variations in emulsion thickness can lead to variable silver grain distribution patterns (Florance, 1989). ^{32}P emits high energy β-emission and therefore has short exposure times, but its short half-life (14.3 days) means that probes must be labelled for each experiment, and labelling procedures have to be carried out with extreme care, because of radioactive hazard. ^{35}S, although having lower energy emission, appears to require similar exposure times to ^{32}P, probably due to lower energy emissions dissipating more energy in the X-ray or emulsion (Florance,

1989). One disadvantage of ^{35}S over ^{32}P, is the relative expense of ^{35}S.

5.1.3 Expression of insulin-like growth factor II gene in the rat

The insulin-like growth factors (IGF-I and IGF-II) are a pair of growth factors with structural and amino acid sequence similarity to proinsulin. The amino acid sequences of the A and B domains of both IGFs show 45% homology with insulin (Froesch and Zapf, 1985) and are similarly linked by three disulphide bridges. The C-chain which links the A and B chains in proinsulin, is present in IGF-I and IGF-II, whilst both IGFs have a short extension on the carboxy-terminus of the A chain, termed the D domain (Bell et al., 1984). Multiple glycosylations on the C and D regions of both IGFs confer some protection against antibodies to the ligand binding site of insulin (Blundell et al, 1978).

IGF-I (67 amino acids) and IGF-II (70 amino acids) have 45 common amino acids, mainly in the A and B domains, with the main structural changes being found in the C and D domains, which confer the ligand binding specificity of IGF-I and IGF-II (Perdue, 1984; Froesch and Zapf, 1985).

Insulin and IGF-II genes have been localised to the short arm of chromosome 11 in the human (Bell et al., 1985) and to chromosome 5 in the rat (Soares et al., 1986). IGF-I gene is found on chromosome 12 in the human, which is linked

theoretically to chromosome 11, through common ancestral evolution (Rosenfeld and Hoffman, 1987).

Insulin-like growth factors, originally named because of their insulin-like activity which was resistant to insulin antibody (Froesch et al., 1963), exhibit various functions in vivo and in vitro (Baxter, 1986). Both IGF-I and IGF-II stimulate sulphate incorporation into cartilage in vitro, the IGF-I-mediated incorporation being growth hormone dependent. This incorporation was split into stimulation of sulphate and leucine uptake into glucosaminoglycans, and proline conversion to collagen hydroxyproline. Insulin-like effects of IGF-I and IGF-II have mostly been studied in adipose tissue, where their range of activities include stimulation of aerobic glucose metabolism, glycogen breakdown and inhibition of adrenaline-stimulated lipolysis. However, the insulin-like growth factors do not appear to effect glucose transport in the same manner as growth hormone itself. IGF-I and IGF-II stimulate DNA synthesis and cell proliferation in many cell types, including fibroblasts, myoblasts, lens epithelium, lymphocytes, Sertoli cells and pancreatic β-cells. These mitogenic effects on cultured cells are not as pronounced using purified IGF-I and IGF-II, as when serum is used as culture medium. This observation is postulated to be due to synergistic effects of other growth factors such as epidermal growth factor, transforming growth factor-α, and platelet-derived growth factor (Mercola and Stiles, 1988). In vivo, the insulin-like growth factors appear to exert some growth promoting effects

in dwarf mice and hypophysectomised rats (Schoelne et al., 1982).

IGF-I and IGF-II are bound to specific carrier proteins in the serum, one high molecular weight protein (150,000 mw) which is stimulated by growth hormone, and other low molecular weight proteins (40,000 mw) which are growth hormone independent, and which bind about a third of the IGFs that the heavy protein binds (Ooi and Herington, 1988). The biological role of the carrier proteins appears to be in providing a mobile store of IGFs, and one of the smaller proteins appears to be predominant in the fetus, whilst the larger protein appears to play a more important role in the adult.

Although the two IGFs appear to have similar in vivo and in vitro potency in their varied functions, there appears to be a difference in the temporal control of the two factors.

In the adult, IGF-I is the major peptide product produced predominantly in the liver, and also in kidney, lung, heart and testes, where synthesis is closely regulated by growth hormone derived from the adenohypophesis (d'Ercole et al., 1984; Froesch et al., 1985). Where growth hormone is deficient in hyposomatotropinism, IGF-I levels are low, whereas in gigantism, IGF-I and growth hormone levels are correspondingly high (Baxter, 1986).

IGF-II levels are high in many organs in the embryo and fetus, from gastrulation stages in mouse, rat and human (Smith et al., 1987; Florance, 1989; Ohlsson et al., 1989), matched by high transcription of mRNA in the human and rat

(Han et al., 1987; Beck et al., 1987). In the rat, the levels of IGF-II mRNA decline in the neonatal period, except in the choroid plexus and leptomeninges (Beck et al., 1988), and this decline is paralleled by an increase in IGF-I mRNA expression. Although IGF-II appears to be the major insulin-like growth factor in the human fetus, there does not appear to be the same neonatal decrease in IGF-II levels (Brice et al., 1989).

Pituitary-derived growth hormone does not appear to be involved in prenatal insulin-like growth factor activity regulation or growth regulation, since anencephalic neonates with low growth hormone levels are normal in birth size, and have normal IGF-I and IGF-II levels (Hill et al., 1987).

Other postulated regulatory factors for IGF-II in the fetus have included placental lactogen, prolactin, insulin and several growth factors (Adams et al., 1983; Underwood and d'Ercole, 1984; Adamson, 1986; Hill et al., 1987). These hypothesised factors are based on synergistic effects with IGF-II in cultured fetal cell types.

IGF-II mRNA transcription is switched off at 20 days postnatally in neonatal rats (Beck et al., 1988), which coincides with a sudden peak of serum glucocorticoid activity associated with gut closure (Patt, 1977). This change in the ileal epithelium prevents the passage of macromolecules in maternal milk directly into fetal circulation (Cornell and Padykula, 1969; Carlile and Beck, 1983). By administration of glucocorticoid to early rat neonates, Beck et al. (1989) showed that IGF-II mRNA levels

could be prematurely extinguished in liver, but expression of IGF-II mRNA in skeletal muscle was affected to a lesser degree, and the single administration of glucocorticoid had no effect on expression in choroid plexus. This finding not only indicated the role of glucocorticoids in the regulation of IGF-II, but also backed up earlier suggestions that both IGF-I and IGF-II exert endocrine and paracrine effects, depending on the site of synthesis (d'Ercole et al., 1984; Underwood et al., 1986). This hypothesis has been further supported by Senior et al. (1989) who showed that in some organs IGF-II mRNA expression corresponds to IGF-II receptor mRNA expression, for example skeletal muscle and perichondrium, indicating a local paracrine effect. However, in other tissues like liver, the IGF-II mRNA expression is in excess to receptor mRNA expression, corresponding to the situation with most endocrine hormones. The IGF-II receptor is a single transmembrane polypeptide with no tyrosine kinase activity (Morgan et al., 1987; MacDonald et al., 1988) showing no homology with insulin or IGF-I receptors (Rechler and Nissley, 1985) which resemble other tyrosine kinase-linked growth factor receptors (Mendelson et al., 1986).

The aim of this study was to examine the visceral yolk sac in culture using in situ hybridisation techniques, to test the hypothesis of glucocorticoid-mediated regulation of IGF-II. The visceral yolk sac transcribes mRNA for insulin (Muglia and Locker, 1984a) and insulin-like growth factor II (Beck et al., 1987), therefore if the cultured yolk sac

mimics this transcriptional activity, regulation of IGF-II mRNA by glucocorticoids can be examined.

5.1.4 Expression of alphafetoprotein gene in the rat

Serum albumin and its fetal counterpart α-fetoprotein share similar structure and function. They contribute to blood osmotic pressure, and bind and transport fatty acids, tryptophan, metal ions such as copper, and other ligands such as estrogen (Peters, 1985; Baker, 1988). Neither protein appears to have a life-critical role, as can be seen in the non-critical disorder - analbuminaemia (Ohno, 1981). However, the gene structure appears to have been highly conserved, so that there is still 33% homology between amino acid structures of human albumin and murine alphafetoprotein (Baker, 1988).

Both proteins contain three homologous domains encoded by three subgenes that consist of four exons each, and evolved by intragenic duplication of a common ancestor (Sargent et al., 1981a; Selten et al., 1982).

Alphafetoprotein (AFP) is expressed in two fetal tissues as a major product - fetal liver and visceral yolk sac, and in five tissues as a minor product - intestine, heart, pancreas, lung and kidney (Jansen et al., 1982; Abelev, 1971; Nahon et al., 1988).

In mouse, AFP is initially synthesised in visceral endoderm at 7 days of gestation (Dziadek and Adamson, 1978), but is gradually restricted to the central band of embryonic

endoderm, where mesoderm conjoins to give rise to visceral yolk sac (Adamson, 1986). The mouse albumin and AFP genes are arranged in tandem on chromosome 5, and coordinate synthesis of AFP and albumin has been demonstrated during fetal liver and visceral yolk sac development (Tilghman and Belayew, 1982) and in mouse/rat hepatoma hybrid cells (Sellem et al., 1984). There appears, therefore, to be a cis-acting control mechanism at the 5' end of the tandem genes, that switches both genes on together. Albumin and AFP mRNAs are synthesised in fetal liver and yolk sac until birth, then the levels of AFP mRNA in neonatal liver decline whilst albumin mRNA levels remain constant (Tilghman and Belayew, 1982). The selective decline in postnatal AFP mRNA transcription is attributed to the interaction of a glucocorticoid-receptor complex with a 5' site adjacent to AFP gene transcription initiation site (Turcotte et al., 1986), although fetal liver expression is not glucocorticoid-repressed (Chou, 1988). Reactivation of AFP gene expression in some adult hepatoma cells may be correlated with hypomethylation of a 5' site, at least in the rat AFP gene (Schwarz et al., 1986; Schulz et al., 1988), although other hypomethylated sites appear to have no role in expression of the gene in other tissue cell lines (Nahon et al., 1987; Tratner et al., 1988).

In the rat, there is no detectable albumin gene expression in the visceral yolk sac, whereas the fetal liver synthesises both albumin and alphafetoprotein (Chiu et al., 1979; Liao et al., 1980). Muglia and Locker (1984) showed

that the yolk sac albumin gene was hypermethylated whilst the AFP gene was undermethylated. Muglia and Locker (1984b) and Sellem et al. (1984) both showed a decline in AFP mRNA transcription in rat visceral yolk sac from 16.5 days of gestation, as fetal liver transcription exhibited a parallel increase in transcription of both AFP and albumin mRNA. Visceral yolk sac, as the primary site of ceruloplasmin synthesis, exhibits a similar pattern of declining transcription rate at this stage of gestation, paralled by a rise in fetal liver synthesis (Baranov et al., 1986).

Using synthetic oligonucleotide probes selective to rat AFP and albumin mRNAs, manufactured from published nucleotide sequences (Sargent et al., 1981b; Liao et al., 1982), the aim of this study was to observe the switching on and off of AFP mRNA synthesis in in vivo yolk sac, and compare to transcription patterns in the cultured yolk sac. Because AFP mRNA in the yolk sac has a long half-life (2.2 days) (Andrews et al., 1982), the culture period must be extended in order to allow degradation of mRNA to occur.

5.2 METHODS

5.2.1 Tissue collection

Yolk sac tissue was collected at the appropriate age of gestation (Chapter 2.2.8), or harvested from culture at the appropriate equivalent gestational age (Chapter 2.5).

For the experiments studying AFP mRNA expression, the normal culture end-point was extended to 22.5 days (13 days of culture). To facilitate this extension, further medium changes were made at 17.5 days and 20.5 days. These serum changes were carried out using a sterile syringe and bent needle, taking care not to disturb the yolk sacs when introducing or withdrawing the fluid.

When studying the effects on yolk sac IGF-II mRNA expression, of culturing in concentrations of hydrocortisone, the culture medium contained 0-1.3mM hydrocortisone phosphate. Parallel studies on the *in vivo* effect of water-soluble glucocorticoids on neonatal IGF-II mRNA expression were carried out, using sterile preparations of hydrocortisone and dexamethasone. Either 0.3mg/g hydrocortisone phosphate, 0.5mg/g dexamethasone or an appropriate volume of saline control was injected (i.p.) into a 9 day neonatal Wistar rat. 9 day pre-injected and 11, 13, and 15 day postinjected neonates were killed by i.p. overdose of sodium pentobarbitone, and abdominal segments were dissected.

All tissues were washed twice in fresh Medium 199 at $0^{o}C$, and rapidly frozen in OCT compound at $-70^{o}C$ over solid CO_2

in hexane. The blocks were then stored at -40°C until sectioned.

7µm sections were cut on a Leitz cryostat, picked up on 3-aminopropyltriethoxysilane-coated glass slides (Rentrop et al., 1986), and placed on dry ice for 15 minutes to facilitate adhesion and minimise ribonuclease activity.

5.2.2 Fixation and prehybridisation

Tissue sections were fixed at 4°C in 4% glutaraldehyde/ 20% ethylene glycol/0.1M phosphate buffer, pH 7.3, for 5 minutes. The sections were then prehybridised in Hybridisation buffer (Appendix F) at 42°C for 20 minutes, washed briefly in 70% methanol followed by absolute ethanol, air-dried, then stored in ethanol vapour at 4°C until hybridised.

5.2.3 Synthesis of DNA oligonucleotide probes

Six synthetic 30-mer probes were manufactured by the solid-phase phosphoramidite process (Caruthers et al., 1982), at the Howard Florey Institute of Experimental Physiology and Medicine, Melbourne, Australia.

The sequences of the 30-mer probe used was as follows:

a) IGF-II (antisense)

5'-CTG ATG GTT GCT GGA CAT CTC CGA AGA GGC-3'

(complementary to the mRNA for the amino acid sequence 147 to 156 of the rat IGF-II precursor protein).

b) IGF-II (sense)

5'-GCC TCT TCG GAG ATG TCC AGC AAC CAT CAG-3' (opposing sequence to the antisense probe).

c) AFP (AFP1)

5'-CTT CCT TCT CTT AAT TCC TTT GCC ATT GAT-3'

(complementary to region 650-679 of rat AFP mRNA).

d) AFP (AFP2)

5'-ACC AGT TAC CAT CAA ACC GAA AAG CTC ACA-3'

(complementary to region 1907-1936 of rat AFP mRNA).

e) AFP (AFP3)

5'-TCA TAC TGA GCG GCT AAG TAA AGA ATG GTG-3'

(complementary to region 557-586 of rat AFP mRNA).

f) Serum Albumin

5'-TTG TTT TGC ACA GCA GTC AGC CAG TTC ACC-3'

(complementary to region 325-354 of rat serum albumin mRNA).

5.2.4 5'-end labelling of oligonucleotide probes

The oligonucleotide probes were end-labelled with ^{32}P by incubating 50ng of heat denatured probe with 10 pmoles of γ-^{32}P-ATP at pH 9.5, in the presence of 10 units of T_4 polynucleotide kinase and 100 nmoles of dithiothreitol for 90 minutes at 37^{o}C. After terminating the reaction with 2µmoles of ethylenediamine tetraacetic acid (EDTA), pH 8.0, the reaction volume of 29.0µl was diluted to 125.0µl with 1mM Tris-buffered EDTA, pH 8.0.

A 2µl pre-precipitation sample of the ^{32}P-labelled probe was taken for electrophoresis, then 277µl sterile 'Q' water, 500µg glycogen, 120 µmoles NaAc and 1ml of absolute ethanol were added to the probe, and frozen on dry ice for 60 minutes. The precipitated probe was then centrifuged at 14,000rpm for 30 minutes and the supernatant was decanted and discarded as radioactive waste. 1ml of 70% ethanol (made with absolute ethanol and sterile Q) was added, vortexed, then centrifuged at 14,000rpm for 6 minutes. This washing procedure with 70% ethanol was repeated six times to remove all unincorporated ^{32}P, then the remaining pellet was vacuum desiccated, and resuspended in 100µl of hybridisation buffer. 2µl of postprecipitated probe solution was taken, and in conjunction with the pre-precipitation sample, mixed with an equal volume of formamide dye, and run on a 12% acrylamide gel equilibrated with 1x Tris-buffered boric acid/EDTA, at 1500V/<20mA.

After allowing the bromophenol blue marker to run two thirds of the way down the gel, the gel plates were separated, and the gel was autoradiographed using Kodak X-Omat AR film for 30 minutes, at room temperature (Fig. 5.1).

If the postprecipitation probe sample had no appreciable levels of unincorporated nucleotides, and labelled as a single band, a 1µl sample was blotted on a Whatman glass fibre filter paper, dried for 15 minutes at 70°C, and immersed in 5ml Fisofluor 1 scintillation fluid. Average counts per minute of the probe were measured on a 3 minute cycle using an automated Minaxi scintillation counter. From these results, the probe was further diluted with hybridisation buffer to give a specific activity of 5-20 µCi/µl.

5.2.5 Hybridisation histochemistry

The probe was heat-denatured at 80°C for 2 minutes, then 20µl labelled probe were pipetted onto repelcoted 22x22mm coverslips, and each coverslip picked up onto a section by inverting the slide. Hybridisation was performed at 42°C, in a humidified chamber for 24 hours.

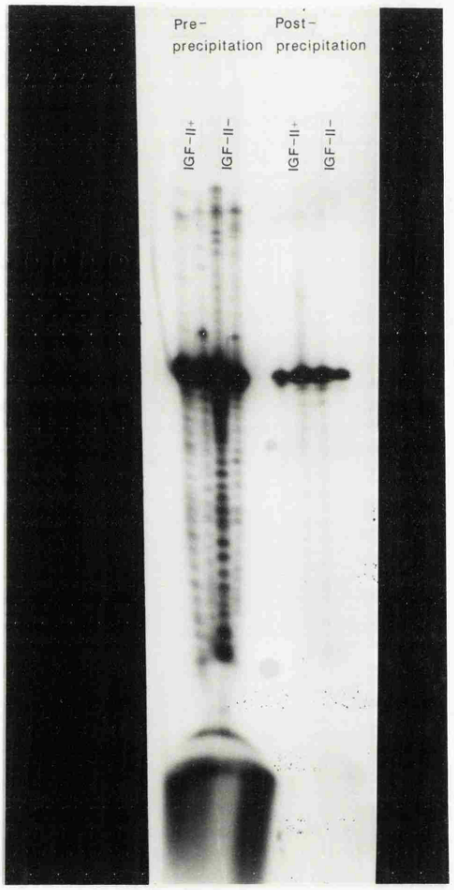
Slides removed from the chamber were immersed and agitated in 4x Standard Saline Citrate (0.6M NaCl/0.6M Na citrate) to dislodge the coverslips. The slides were placed in slide racks immersed in 2x SSC, then washed in 1x SSC for 2 hours in a shaking water bath at 37°C. After a final 30 minute wash in 0.5x SSC at 37°C, the slides were rinsed in

Figure 5.1 5'-end labelling and purification of oligonucleotide probes

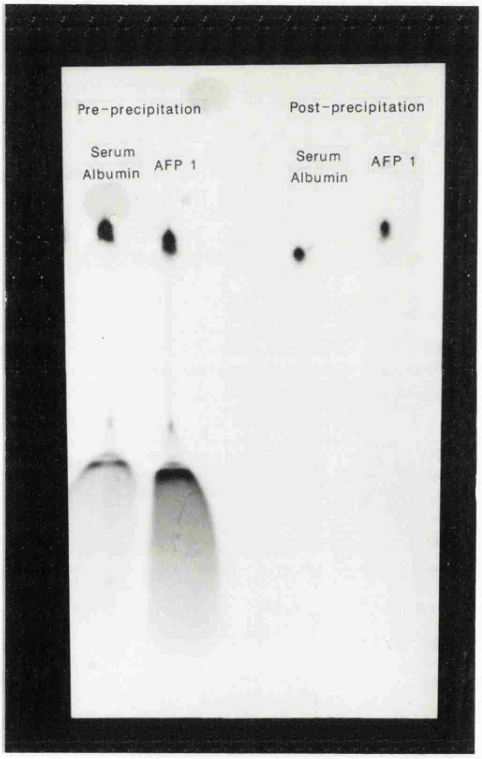
Synthetic oligonucleotide DNA probes to rat insulin-like growth factor II, serum albumin and alphafetoprotein were 5'-end labelled by incubating heat-denatured probe with γ-^{32}P-ATP, in the presence of T_4 polynucleotide kinase. The labelled probes were then purified by absolute ethanol precipitation, and repeated removal of unincorporated nucleotides by reprecipitation in 70% ethanol.

Preprecipitation and postprecipitation fractions of probes to IGF-II (antisense + sense -) (a) and serum albumin and alphafetoprotein (b) were then electrophorised on acrylamide gel, and visualised on Kodak AR X-Omat film, exposed for 30 minutes.

a


Pre-
precipitation
Post-
precipitation
IGF-II+
IGF-II−
IGF-II+
IGF-II−

b


Pre-precipitation
Post-precipitation
Serum
Albumin
AFP 1
Serum
Albumin
AFP 1

two washes of absolute ethanol and vacuum dried. The sections were then exposed to Kodak X-Omat AR film for 18-20 hours. The developed X-ray was used to estimate the exposure time for thin layer emulsion autoradiography.

Ilford K5 emulsion was dissolved in an equal volume of 1% glycerol in Q water at 40°C for 2 hours. While the emulsion was dissolving, the slides were pre-warmed to 40°C on a hot plate, then dipped for 5 seconds in emulsion, and air-dried horizontally for 20 minutes. The slides were then racked up and placed over desiccant in a sealed, dark chamber. Autoradiography was performed over 3-7 days at 4°C, then the emulsion was developed at 15°C for 3 minutes in 14% (w/v) Kodak D19 developer, stopped in 1% (v/v) acetic acid in distilled water, and fixed for 2 minutes in 20% (v/v) Ilford Hypam fixer. The sections were then rinsed in distilled water, and refixed in 0.5x formyl saline for 30 minutes.

The sections were stained with Harris's haematoxylin for 1 minute, rinsed in acid alcohol and blued in tap water for 2 minutes. A brief exposure to eosin (2-3 seconds) was then followed by sequential dehydration in graded alcohols, clearing in two washes of xylene, and coverslipping with DPX.

A photographic record of the silver grain development was made using a Leitz Diaplan photomicroscope with fitted dark field condenser. Bright field photomicrographs were taken on Kodak Ektachrome 50 colour film, while dark field photomicrographs were taken on Kodak Panatomic-X black and white film.

5.2.6 Semi-quantitative estimation of mRNA expression using Northern blotting

Tissue samples were collected as described in Chapter 5.2.1, but instead of freezing down in OCT compound, the samples for Northern blotting were immediately immersed in liquid nitrogen, then stored at -40^{o}C until homogenised.

Total tissue RNA was prepared using a modification of the method of Auffray and Rougeon (1980). All apparatus and non-Tris-containing solutions used, were washed or prepared using sterile Q water autoclaved three times in the presence of 1ml/litre diethyl pyrocarbonate (DEP/Q).

Tissue samples were homogenised on ice, in 10ml/g of lysis buffer (6M Urea/4M LiCl/3M NaAc, pH 5.2/0.1% sodium dodecyl sulphate), using a polytron, and the homogenate was transferred to sealed Corex tubes and stored at 4^{o}C overnight. The tubes were then spun at 10,000rpm for 30 minutes at 4^{o}C in a Sorvall RC-5B ultracentrifuge, the supernatant carefully discarded, and the pellet resuspended in 5ml of 6M Urea/4M LiCl by vortexing. The centrifugation step was repeated, and the resulting pellet resuspended in 10mM Tris-HCl, pH 7.5/0.5% SDS by vortexing. The RNA was then extracted twice in equal volumes of phenol-chloroform-isoamyl alcohol by shaking in sterile glass universals on an orbital shaker for 15 minutes, spinning in a bench centrifuge at 3,000rpm for 5 minutes and decanting off the aqueous layer. The extraction procedure was then repeated using chloroform-isoamyl alcohol and shaking for 5 minutes.

The RNA in the resulting supernatant was ethanol-precipitated overnight at -40°C in Corex tubes with 0.1x volume of 3M NaAc and 2x volume of ethanol. The tubes were centrifuged at 4°C in a Sorvall RC-5B ultracentrifuge at 10,000rpm for 45 minutes. The supernatant was decanted, replaced with an equal volume of 70% ethanol, and respun at 10,000rpm for 45 minutes. The ethanol was decanted, the pellet vacuum desiccated, and resuspended in sterile DEP/Q.

RNA yield was estimated by absorbance of 1µl solution in 999µl Q, in quartz cells at 260nm (40µg/ml RNA gave an absorbance of 1.0 unit).

100µg of total RNA from each sample were electrophoresed on a 1.2% agarose gel containing formaldehyde at 150V/<90mA along with 5µl of an RNA molecular weight ladder (BRL). The RNA was transferred to pre-soaked Hybond, N (Amersham) from the gel by drawing 20x SSC through the gel by capillary action under light pressure for 18 hours. After blotting, the Hybond was dried thoroughly at 60°C, wrapped in Saran wrap and exposed to UV light for 2 minutes on a trans-illuminator, to cross-link the RNA to the membrane. The marker track was detached from the rest of the Hybond membrane and stained for 10 minutes in 0.5M NaAc, pH 5.2, containing 0.04% methylene blue. The track was then rinsed in strongly running tap water and dried.

The Hybond membrane was prehybridised for 45 minutes at 42°C in a shaking water bath. The prehybridisation and hybridisation solutions contained: 5x Standard Sodium Phosphate EDTA pH 7.7 (0.9M NaCl/0.05M Na_2PO_4/0.5mM Na_2

EDTA); 50% (v/v) deionised formamide; 5x Denhardt's solution; 0.5% SDS; and 0.2mg/ml heat-denatured salmon sperm DNA.

Oligonucleotide probes were 5'-end labelled with ^{32}P as described in Chapter 5.2.4, but were not precipitation-purified. Before hybridisation, the probe was heat-denatured at 80°C for 2 minutes, then added to the hybridisation chamber in a second volume of hybridisation solution, containing 6% (w/v) polyethylene glycol 6000. Hybridisation was performed overnight at 42°C in a shaking water bath.

Post-hybridisation washes of the hybridised membrane were carried out at 42°C in a shaking water bath, with three 20 minute washes in 2x SSPE and two 20 minute washes in 0.5x SSPE. The filter was blotted semi-dry on Whatman 3MM paper, then wrapped in Saran wrap, and exposed to Fuji RX X-ray film at room temperature for 3-5 days.

Molecular weight of exposed bands of RNA were estimated using the corresponding BRL molecular weight ladder.

The filter was then incubated in two 45 minute washes of stripping solution containing 5mM Tris-HCl, pH 7.5/1mM EDTA/ 0.1% Denhardt's solution, at 65°C. The filters were then blotted semi-dry on Whatman 3MM paper and rehybridised with a ^{32}P-labelled cDNA probe to human mRNA for hypoxanthine phosphoguanosyl transferrase (HPGT), to check that this ubiquitously expressed enzyme appeared in equal amounts in all lanes, indicating equal loading of the gel and transfer of the messenger RNAs.

process of ethanol-precipitation was repeated using a microultracentrifuge.

The final concentration of poly(A)+ RNA was checked by absorbance of a 2µl sample in 1ml of sterile Q, in a quartz cuvette at 260nm as described in Chapter 5.2.6. The concentration was adjusted to 2µg/ml with sterile Q.

The poly(A)+ RNA samples were then blotted onto a piece of Hybond membrane, presoaked in 3x SSC, using a specialised dot blot hybridisation kit, where the RNA is drawn onto the membrane by vacuum, forming dots of uniform diameter. This method is a modification of the original dot blot hybridisation procedure of Kafatos et al. (1979).

The procedure for hybridisation and autoradiography of the membrane was as described for Northern blotting in Chapter 5.2.6.

5.3 RESULTS

5.3.1 IGF-II mRNA expression in visceral yolk sac

Fig. 5.2 shows intense expression of IGF-II mRNA in 16.5 day *in vivo* yolk sac. The distribution of silver grains indicates the transcription of this gene in endodermal and mesodermal cells. This pattern of transcription is also seen in 11.5, 13.5, 15.5 and 17.5 day cultured yolk sac (Figs. 5.3-5.6). There appears to be no significant difference in intensity of expression over the age range studied.

5.3.2 Effect of glucocorticoids on IGF-II mRNA expression

Fig. 5.7 shows the X-ray autoradiograph of whole abdominal sections of neonatal rats, exposed to a single i.p. injection of hydrocortisone (2.5mg/g) or dexamethasone (0.1, 0.05 or 0.025mg/g). In Fig. 5.7a, hydrocortisone injection at 9.5 days causes a depletion of IGF-II mRNA in liver, so that after four days (13.5 days) levels are almost undetectable in hepatic tissue, whilst a signal persists in the surrounding skeletal muscle. Injection of half a litter with 0.1mg/g dexamethasone caused complete eradication of hepatic IGF-II mRNA expression in both control and treated pups. Repeating the experiment with lower levels of dexamethasone, and cross-fostering the treated and control litter mates to different weaning mothers, caused a pattern of IGF-II expression similar to that seen with hydro-

cortisone (Fig. 5.7b). Hepatic IGF-II mRNA was completely undetectable, 5 and 7 days after injection, whilst signal persisted in skeletal muscle.

Culturing yolk sacs from 9.5 days in serum-based medium containing various concentrations of hydrocortisone caused a severe decrease in the final yolk sac diameter attained. This decrease in growth was dependent on hydrocortisone dose.

Age (days)	Control	Hydrocortisone [0.33mM]	Hydrocortisone [0.65mM]	Hydrocortisone [1.30mM]
11.5	11.0mm	10.8mm	9.7mm	8.3mm
14.5	52.7mm	50.3mm	42.3mm	31.2mm
17.5	118.4mm	115.2mm	56.4mm	39.8mm

Each mean diameter is the mean of twelve yolk sacs cultured in six different experiments. Yolk sacs cultured in medium containing 2.6mM hydrocortisone phosphate died between 0 and 4 days of culture.

Culturing yolk sacs in hydrocortisone-containing medium did not appear to have any significant effect on IGF-II mRNA expression. Figs. 5.8, 5.10 and 5.12 represent cultured yolk sacs of 11.5, 14.5 and 17.5 days respectively, all cultured in the absence of hydrocortisone. These control yolk sacs express IGF-II mRNA in endodermal and mesodermal cells. Figs. 5.9, 5.11 and 5.13 represent yolk sacs of corresponding ages, treated with 0.65mM hydrocortisone (a and b) or 1.3mM hydrocortisone (c and d). Yolk sac tissue still

expresses IGF-II mRNA, although 11.5 day 1.3mM-treated yolk sac (Fig. 5.9d) appears to show an overall depletion of signal in the mesodermal layer.

Fig. 5.14a shows the Northern blot of 17.5 day control and 1.3mM hydrocortisone-treated yolk sac tissue hybridised with an oligonucleotide probe to IGF-II mRNA. Equivalent expression of transcripts of sizes 4.0, 2.4 and 1.75 kb were detected in both tissue samples, whilst the expression of HPGT mRNA was also equivalent (Fig. 5.14b).

5.3.3 Expression of AFP mRNA in visceral yolk sac

Fig. 5.15a shows the X-ray autoradiograph of sections of 13.5 and 20.5 day *in vivo* yolk sac, adult liver and 15.5 day embryo head and yolk sac, hybridised to all three AFP mRNA probes and the serum albumin mRNA probe. The serum albumin probe hybridised specifically to adult liver in a concentration-dependent manner, without any detectable hybridisation to prenatal tissues. All three AFP probes hybridised in a concentration-dependent manner to yolk sac tissue, and did not bind to embryonic head containing mesoderm and ectoderm-derived tissues. AFP3 did show low levels of hybridisation to adult liver.

Fig. 5.15b shows the X-ray autoradiograph of sections of adult liver, early and late fetal liver, and *in vivo* and cultured yolk sacs from 11.5 days of gestation to term, hybridised to the probe for serum albumin mRNA and AFP2 probe to AFP mRNA. At 15nCi/µl, the serum albumin probe

hybridises to adult and fetal liver, but not to yolk sac tissue, whilst AFP2 hybridises to fetal liver and not adult liver. AFP2 hybridised to both in vivo and cultured yolk sac, showing an apparent increase in hybridisation between 11.5 days and 13.5 or 14.5 days. There was an apparent decrease in hybridisation between 17.5 and 21.5 days in vivo, which was not paralleled in culture.

The apparent increase in hybridisation between 11.5 and 13.5 days in vivo and 11.5 and 14.5 days in culture, on the X-ray autoradiography was also seen in Figs. 5.16-5.19, which represent emulsion autoradiographs of yolk sacs of these ages.

The cellular distribution of silver grains in the emulsion autoradiographs indicated that AFP mRNA was transcribed in endodermal cells, but not in mesodermal cells. This pattern of expression was observed in in vivo and cultured yolk sacs, and is especially clear in Figs. 5.18, 5.19 and 5.21.

The apparent decrease in hybridisation between 17.5 days (Fig. 5.20) and 21.5 days (Fig. 5.22) in vivo, observed on the X-ray autoradiograph was not as clear on emulsion autoradiograph. Areas of Fig. 5.22b appear to be only slightly hybridised, whilst other areas appear to be strongly hybridised. There appeared to be no significant decrease in hybridisation between cultured yolk sacs of 17.5 days (Fig. 5.21) and 22.5 days (Fig. 5.23).

Fig. 5.24 shows X-ray autoradiographs of a Northern blot (a) and dot blot (c) of RNA prepared from 16.5 and 21.5 day *in vivo* and cultured yolk sacs, hybridised to AFP2.

The Northern blot shows the probe hybridised to a transcript of size 2.2kb in all four tissue mRNAs, but a slightly decreased hybridisation was seen with 21.5 day *in vivo* yolk sac.

These results were backed up by the results of the dot hybridisation (Fig. 5.24c) where a decrease in AFP mRNA production of between 1×10^{-3} and 2.5×10^{-3}g was observed between 16.5 day and 21.5 day *in vivo* yolk sac. No apparent change was observed between cultured yolk sacs at these ages. The loading of the dot hybridisation apparatus was shown to be equal for all tissue RNAs, by the equivalent hybridisation of the HPGT probe to the 2µg and 0.04µg dots of all tissues.

Figure 5.2 16.5 day *in vivo* yolk sac (x 76)

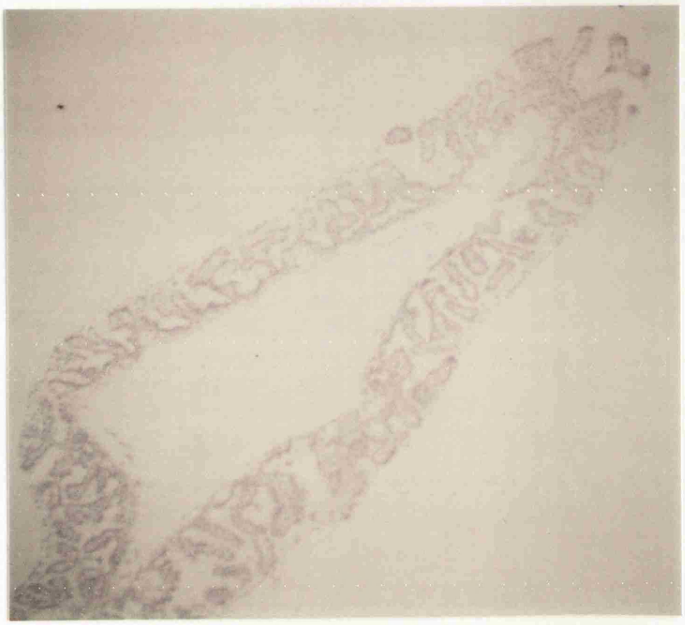
a) Bright field photomicrograph (H and E)

b) Dark field photomicrograph of hybridisation to IGF-II (antisense)

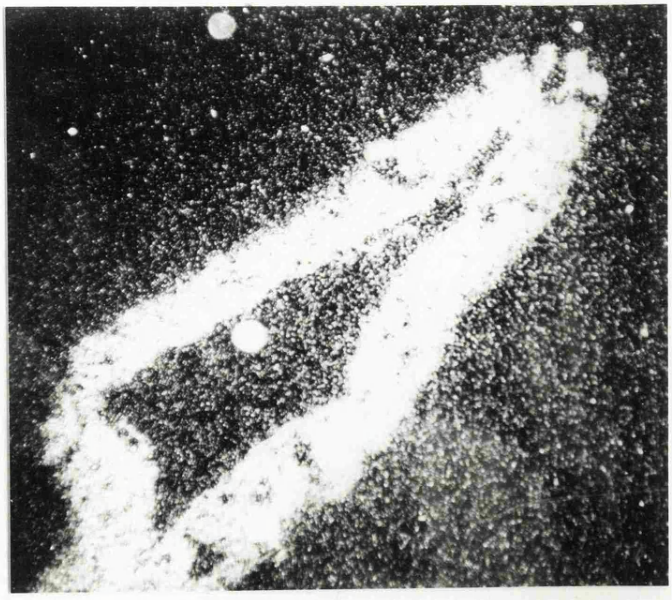
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours at 42^{o}C.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

a



b



.1mm

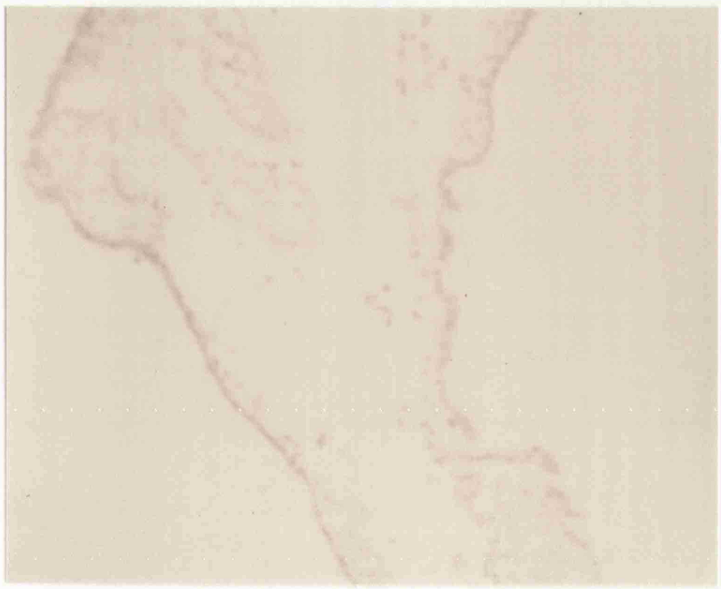
Figure 5.3 11.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph (H and E)
b) Dark field photomicrograph of hybridisation to IGF-II (antisense)
c) Dark field photomicrograph of hybridisation to IGF-II (sense)

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours at 42^{o}C.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

a



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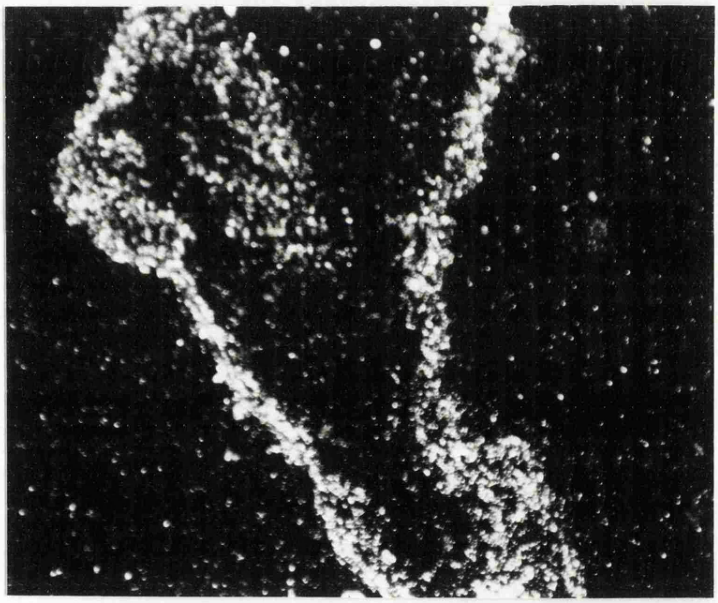
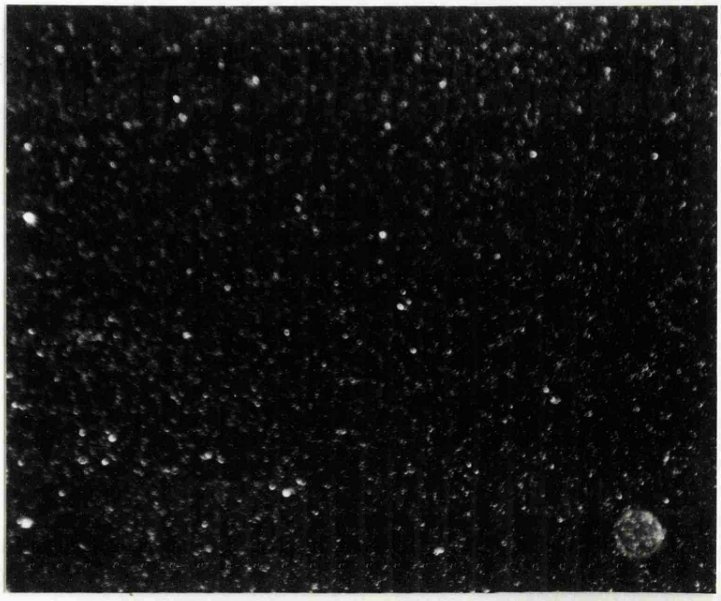


c



.1mm

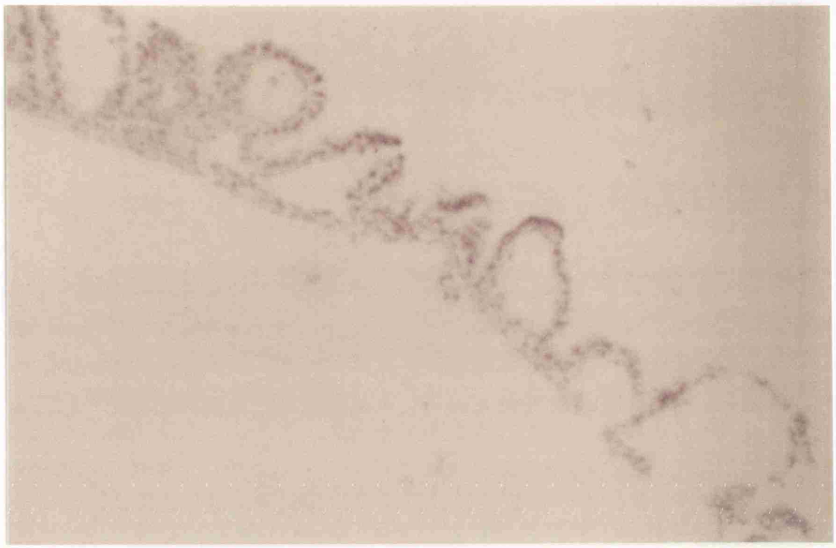
Figure 5.4 13.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph (H and E)
b) Dark field photomicrograph of hybridisation to IGF-II (antisense)
c) Dark field photomicrograph of hybridisation to IGF-II (sense)

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours at 42^{o}C.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

a



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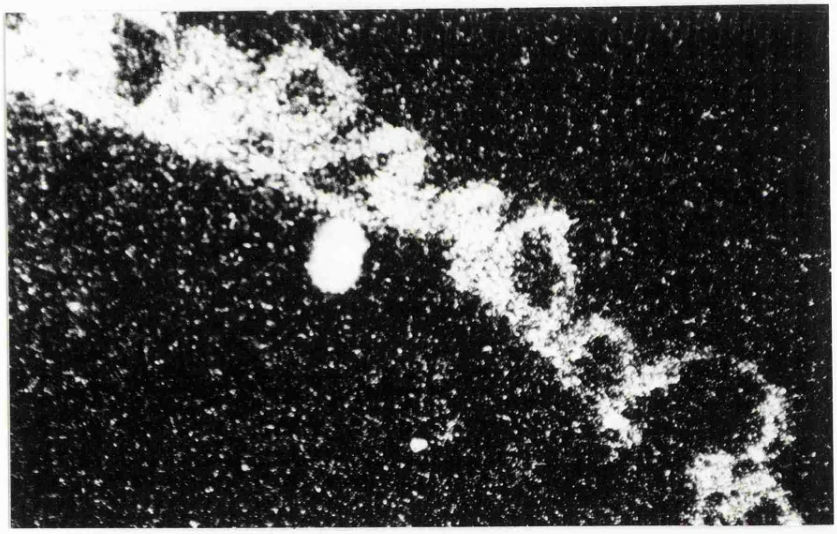
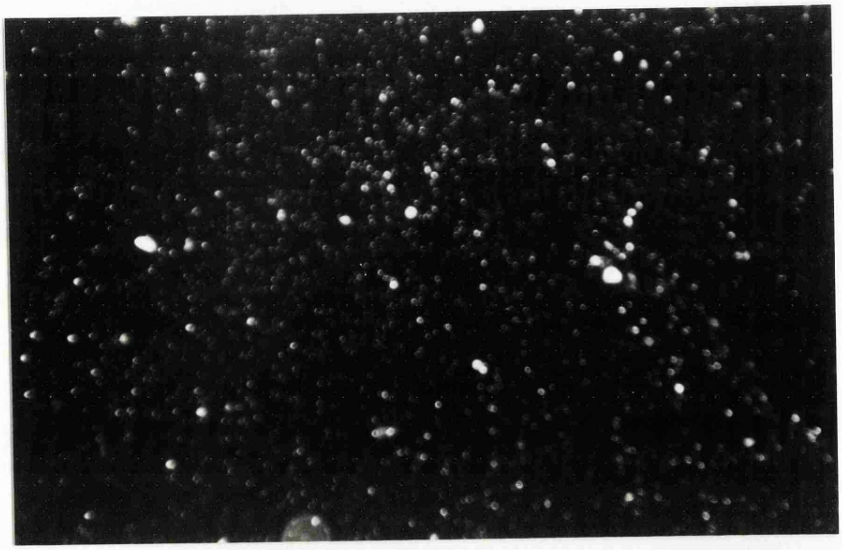


c



.1mm

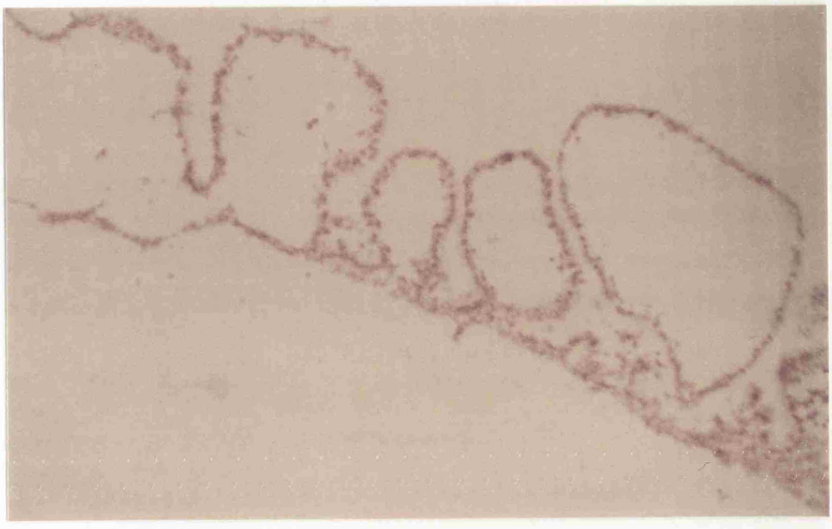
Figure 5.5 15.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph (H and E)
b) Dark field photomicrograph of hybridisation to IGF-II (antisense)
c) Dark field photomicrograph of hybridisation to IGF-II (sense)

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours at 42^{o}C.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

a



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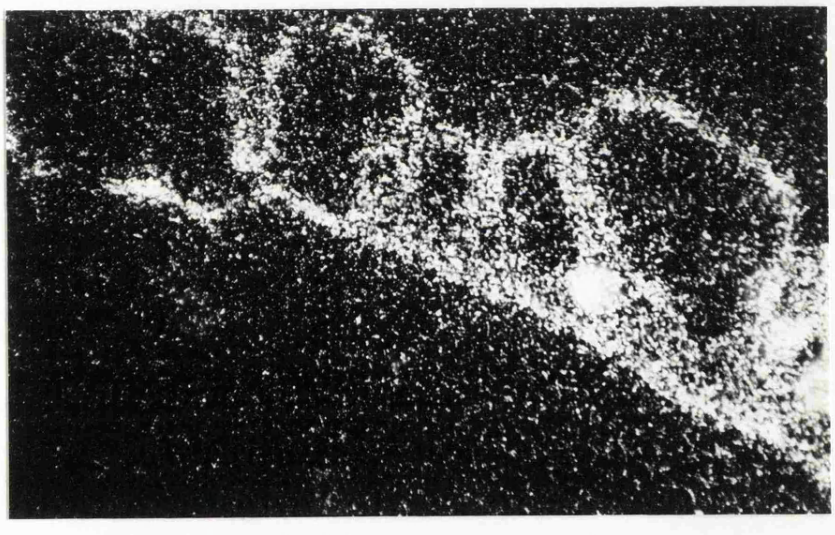


c

.1mm

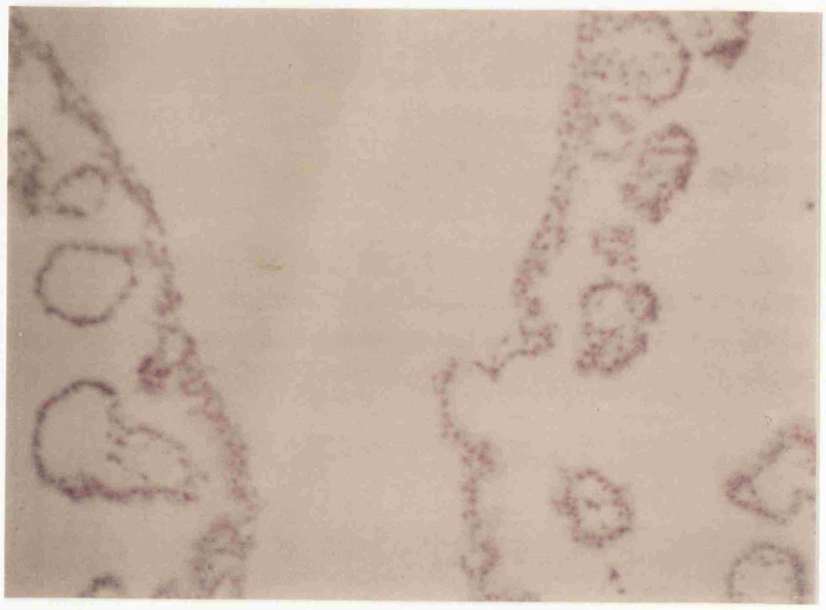
Figure 5.6 17.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph (H and E)
b) Dark field photomicrograph of hybridisation to IGF-II (antisense)
c) Dark field photomicrograph of hybridisation to IGF-II (sense)

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours at 42^{o}C.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

a



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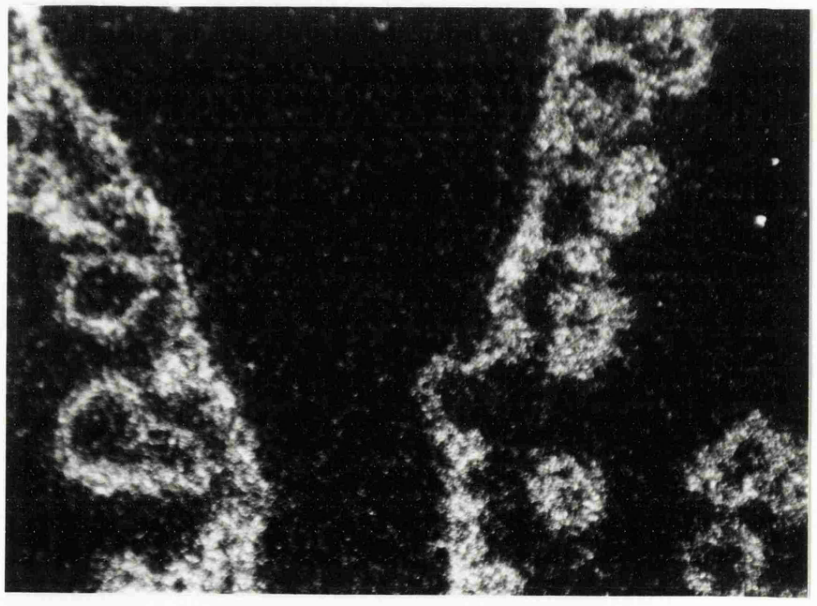

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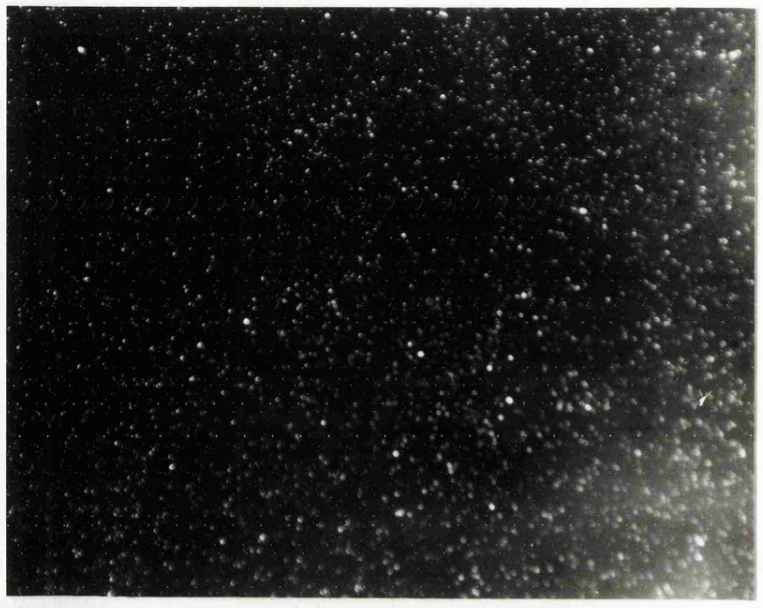
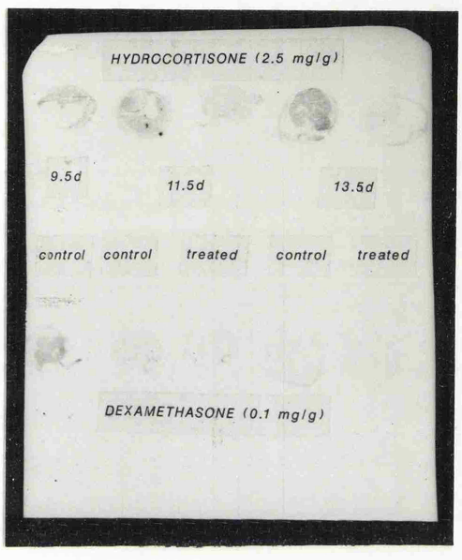


Figure 5.7 Neonatal expression of IGF-II mRNA after injection with hydrocortisone or dexamethasone

a) Dexamethasone (0.1mg/g) or Hydrocortisone (2.5mg/g) were administered i.p. to 9.5 day neonatal rats, which were then killed two or four days later. 7μm frozen abdominal sections of control (saline-treated) or treated animals were fixed in 4% glutaraldehyde, prehybridised for 20 minutes, then hybridised to IGF-II (antisense) with specific radioactivity (8nCi/μl). Hybridisation was visualised by X-ray autoradiography on Kodak AR X-Omat film overnight.

b) The experiment was repeated with dexamethasone (0.025mg/g and 0.05mg/g), cross-fostering control and treated neonates to separate weaning mothers. Neonates were killed 2, 5 and 7 days after injection.

a


HYDROCORTISONE (2.5 mg/g)
9.5d
11.5d
13.5d
control
control
treated
control
treated
DEXAMETHASONE (0.1 mg/g)

b

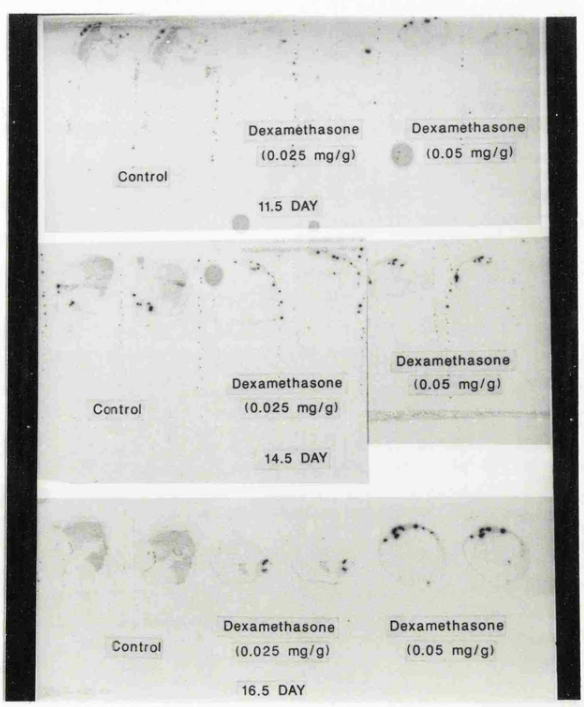

Control
Dexamethasone (0.025 mg/g)
Dexamethasone (0.05 mg/g)
11.5 DAY
Control
Dexamethasone (0.025 mg/g)
Dexamethasone (0.05 mg/g)
14.5 DAY
Control
Dexamethasone (0.025 mg/g)
Dexamethasone (0.05 mg/g)
16.5 DAY

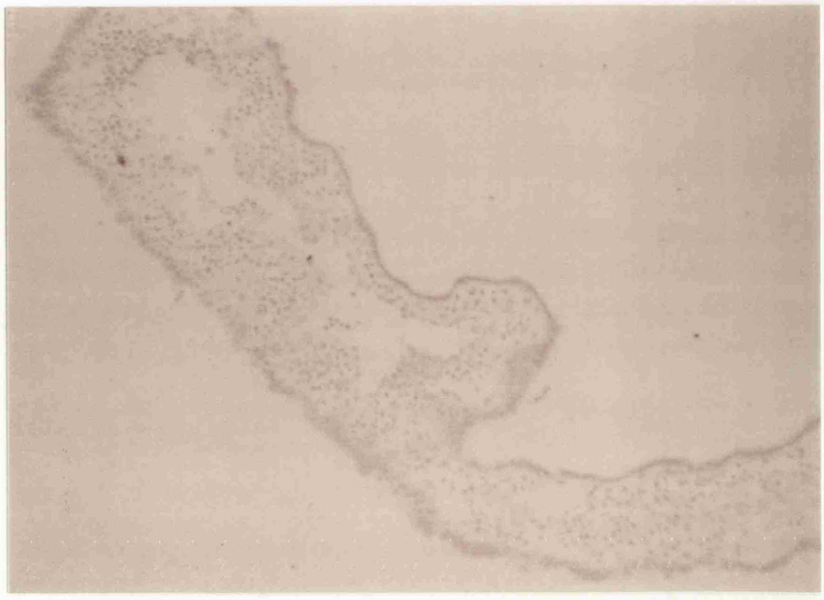
Figure 5.8 11.5 day cultured yolk sac (control) (x 95)

a) Bright field photomicrograph (H and E)
b) Dark field photomicrograph of hybridisation to IGF-II (antisense)
c) Dark field photomicrograph of hybridisation to IGF-II (sense)

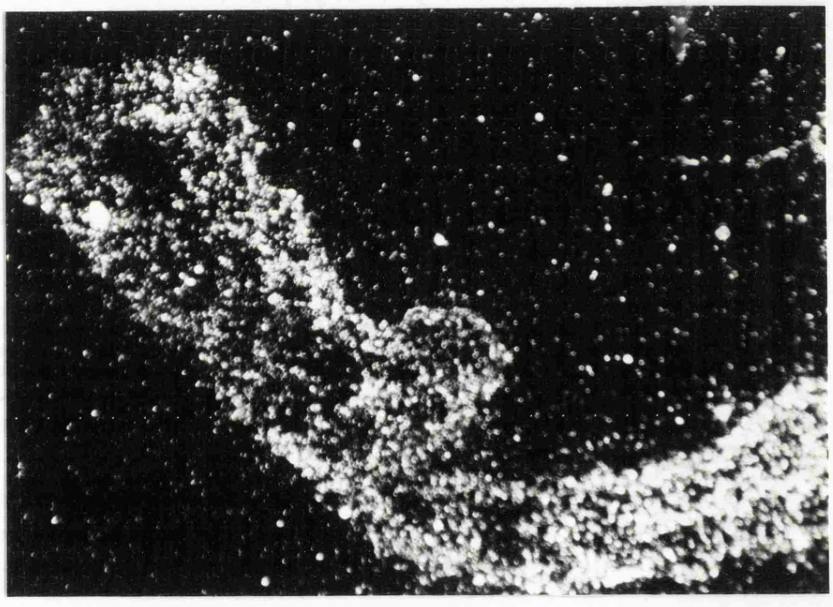
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

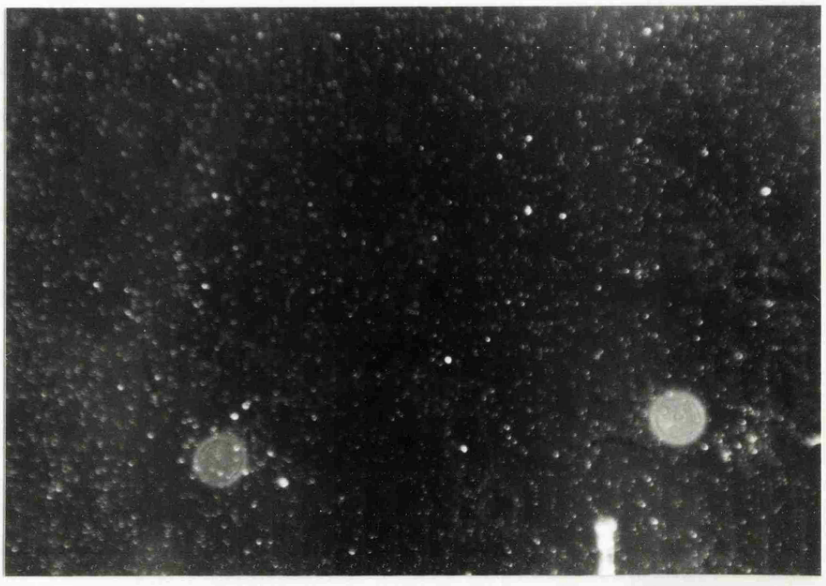
a



b



c



·1mm

Figure 5.9 11.5 day cultured yolk sac (hydrocortisone-treated) (x 95)

0.65mM hydrocortisone a) Bright field photomicrograph (H and E)

b) Dark field photomicrograph of hybridisation to IGF-II (antisense)

1.3mM hydrocortisone c) Bright field photomicrograph (H and E)

d) Dark field photomicrograph of hybridisation to IGF-II (antisense)

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

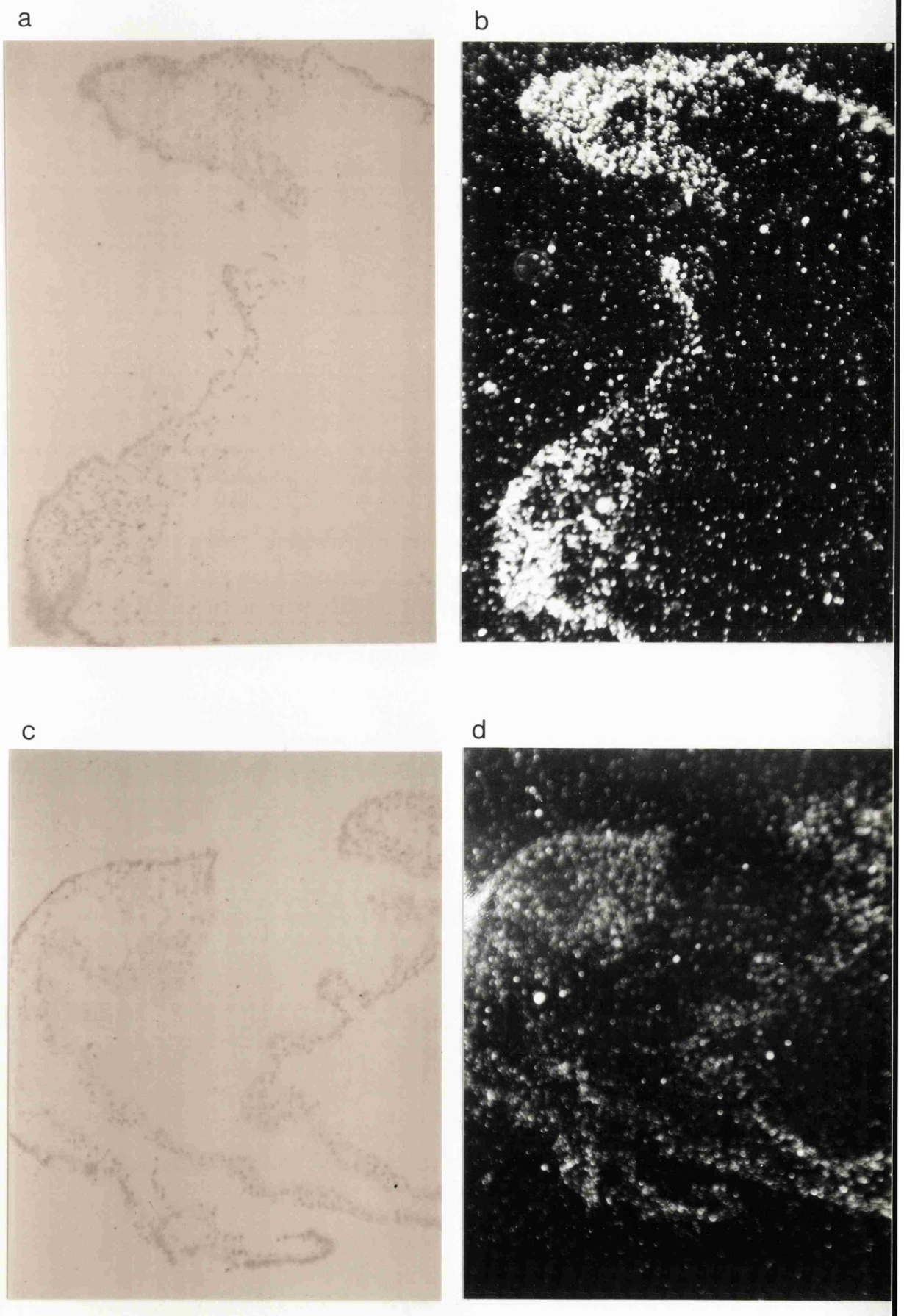

a
b
c
d
.1mm

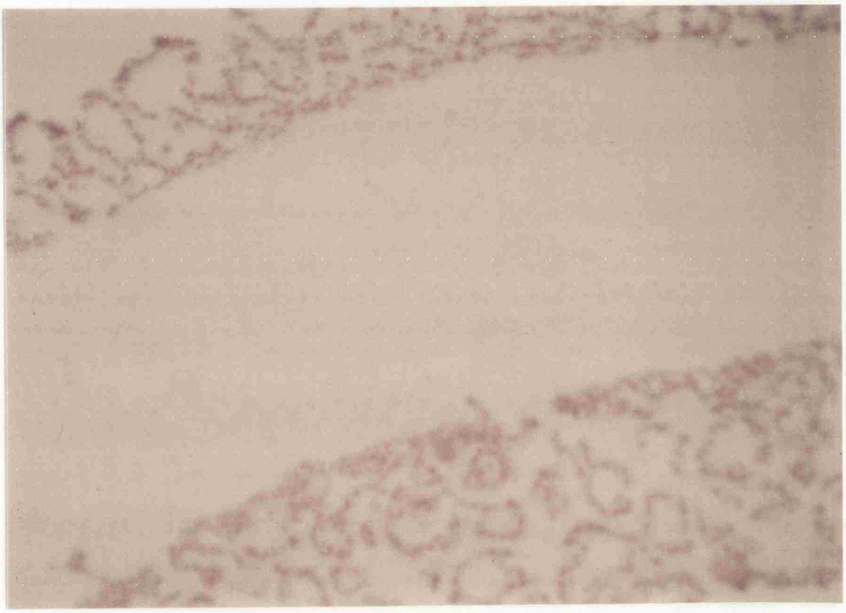
Figure 5.10 14.5 day cultured yolk sac (control) (x 95)

a) Bright field photomicrograph (H and E)
b) Dark field photomicrograph of hybridisation to IGF-II (antisense)
c) Dark field photomicrograph of hybridisation to IGF-II (sense)

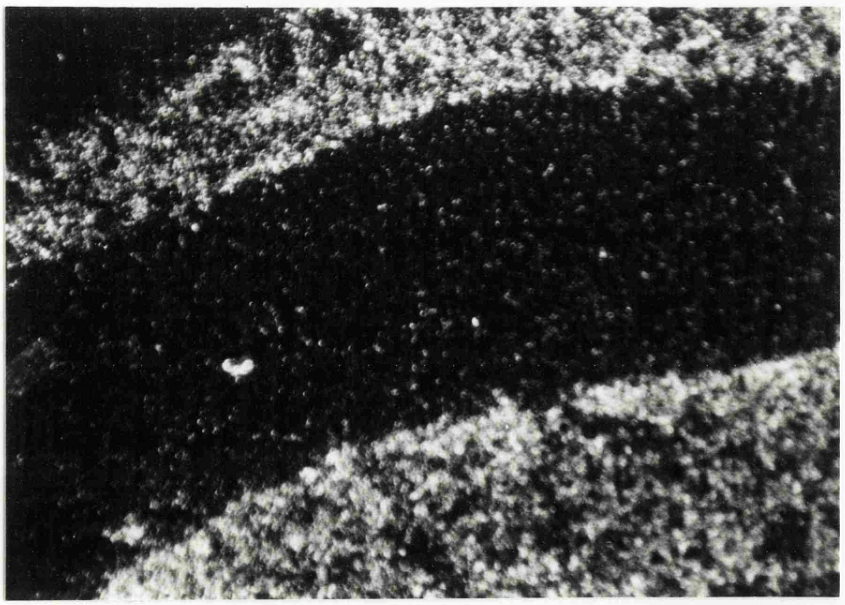
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

a



b



.1mm

Figure 5.11 14.5 day cultured yolk sac (hydrocortisone-treated) (x 95)

0.65mM hydrocortisone a) Bright field photomicrograph (H and E)

b) Dark field photomicrograph of hybridisation to IGF-II (antisense)

1.3mM hydrocortisone c) Bright field photomicrograph (H and E)

d) Dark field photomicrograph of hybridisation to IGF-II (antisense)

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

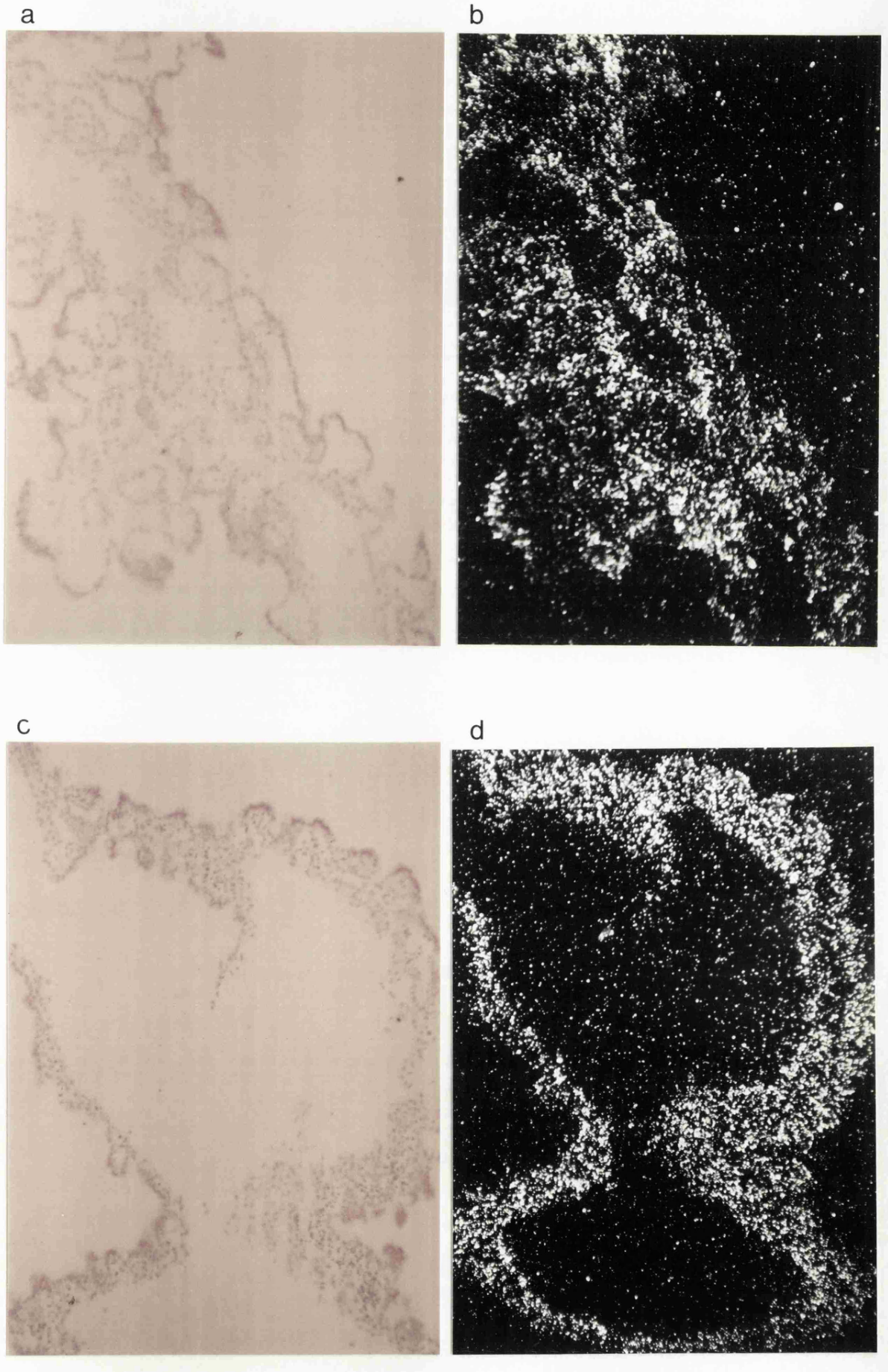

a
b
c
d
.1 mm

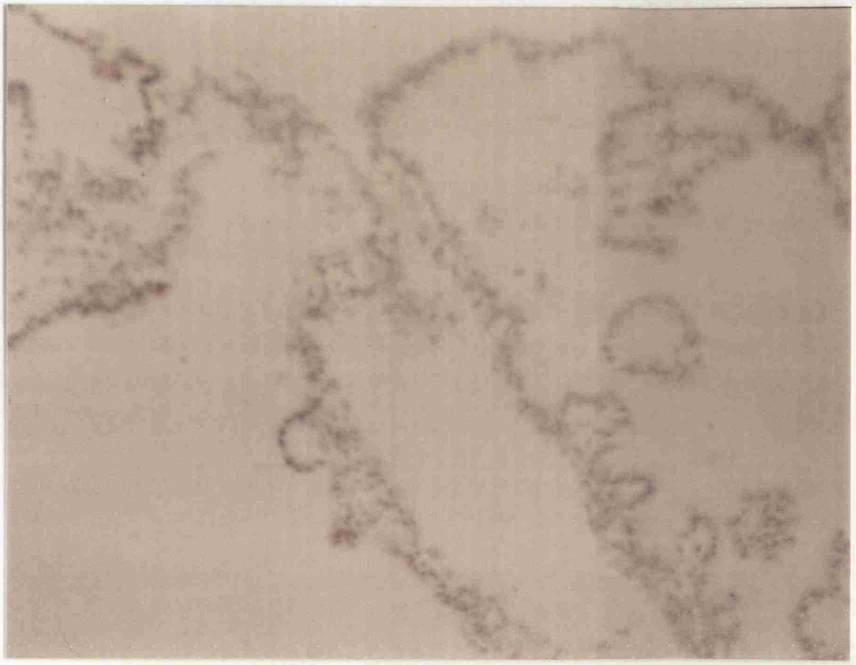
Figure 5.12 17.5 day cultured yolk sac (control) (x 95)

a) Bright field photomicrograph (H and E)
b) Dark field photomicrograph of hybridisation to IGF-II (antisense)
c) Dark field photomicrograph of hybridisation to IGF-II (sense)

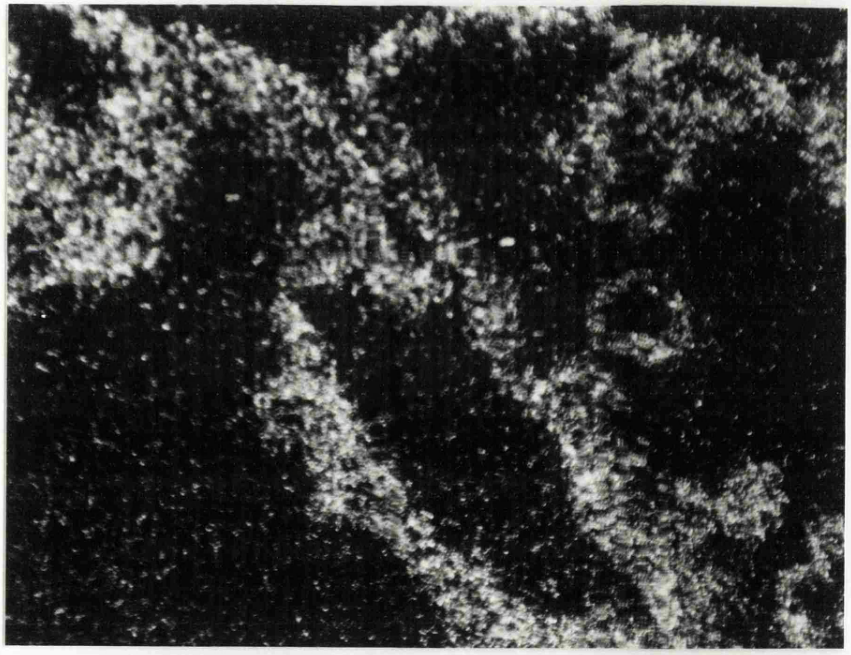
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

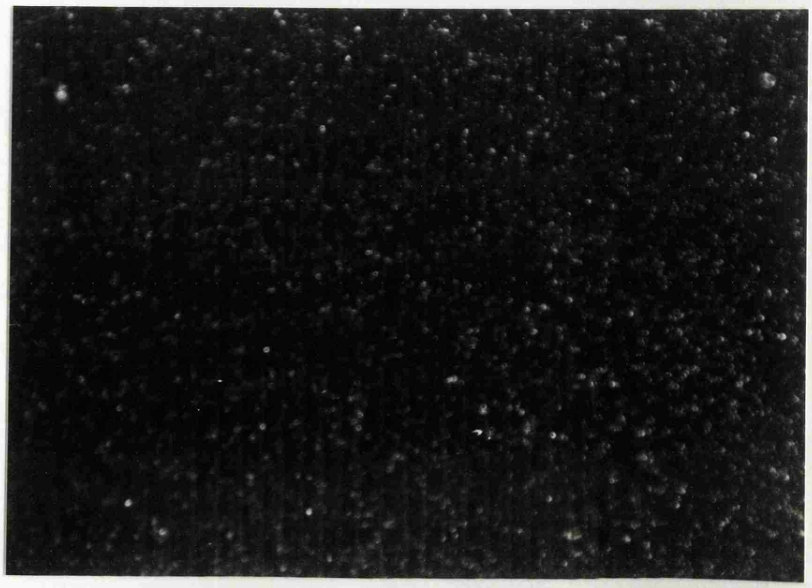
a



b



c



.1mm

Figure 5.13 17.5 day cultured yolk sac (hydrocortisone-treated) (x 95)

0.65mM hydrocortisone a) Bright field photomicrograph (H and E)

b) Dark field photomicrograph of hybridisation to IGF-II (antisense)

1.3mM hydrocortisone c) Bright field photomicrograph (H and E)

d) Dark field photomicrograph of hybridisation to IGF-II (antisense)

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (10nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

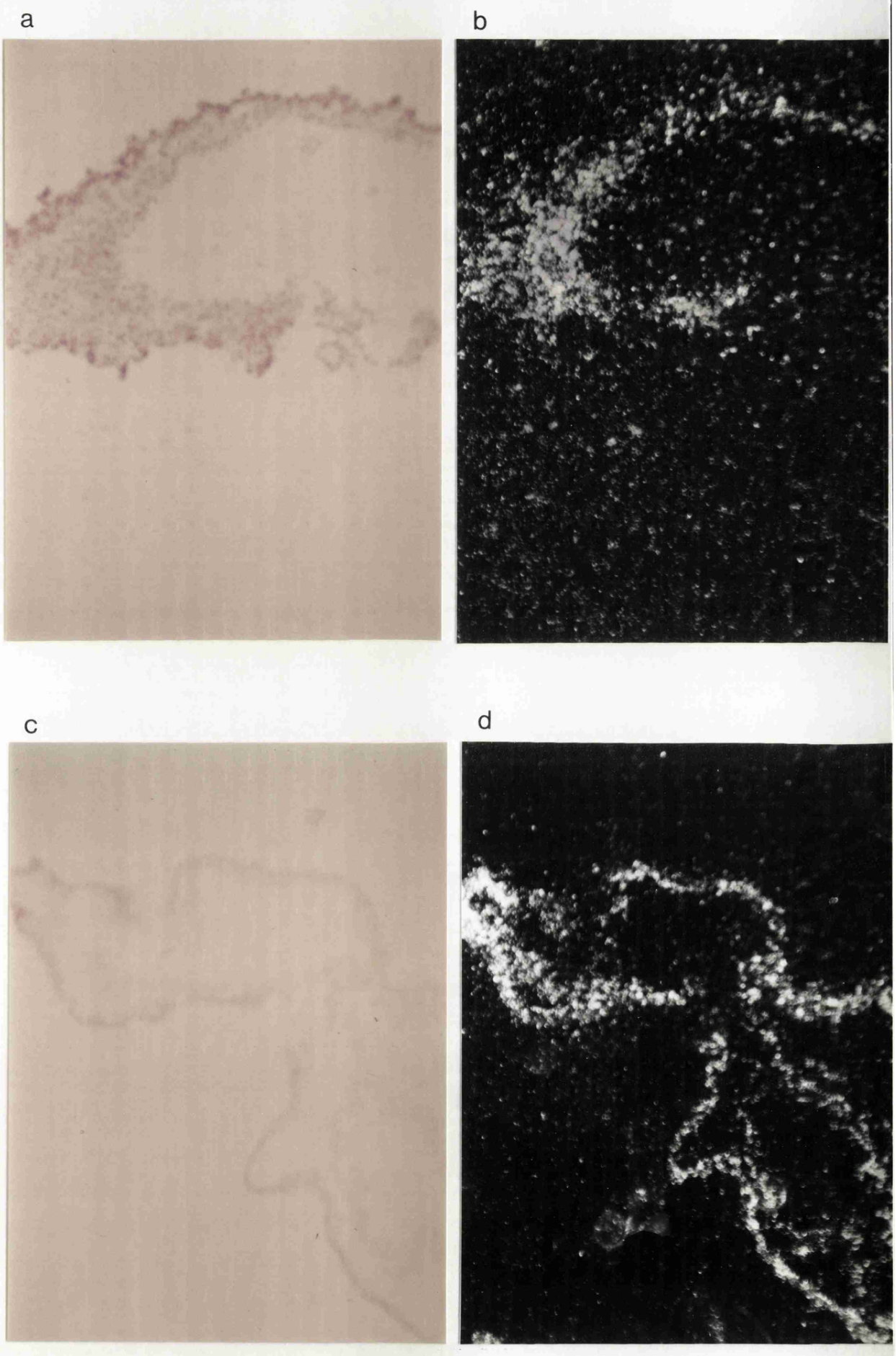

a
b
c
d
.1mm

Figure 5.14 Northern blot analysis of cultured yolk sac hybridisation to IGF-II mRNA probe

Total RNA was prepared from 9.5 days to 17.5 days, in either control medium, or in medium containing 1.3mM hydrocortisone phosphate, by polytron homogenisation and differential precipitation in LiCl/Urea.

100µg of total RNA was electrophorised on agarose gel, blotted onto Hybond and hybridised to either:

a) ^{32}P-labelled probe to IGF-II mRNA
b) ^{32}P-labelled probe to hypoxanthine phosphoguanosyl transferase (HPGT)

Hybridisation was visualised by exposing the hybridised membrane to Fuji RX film for 4 days (a) and 6 days (b).

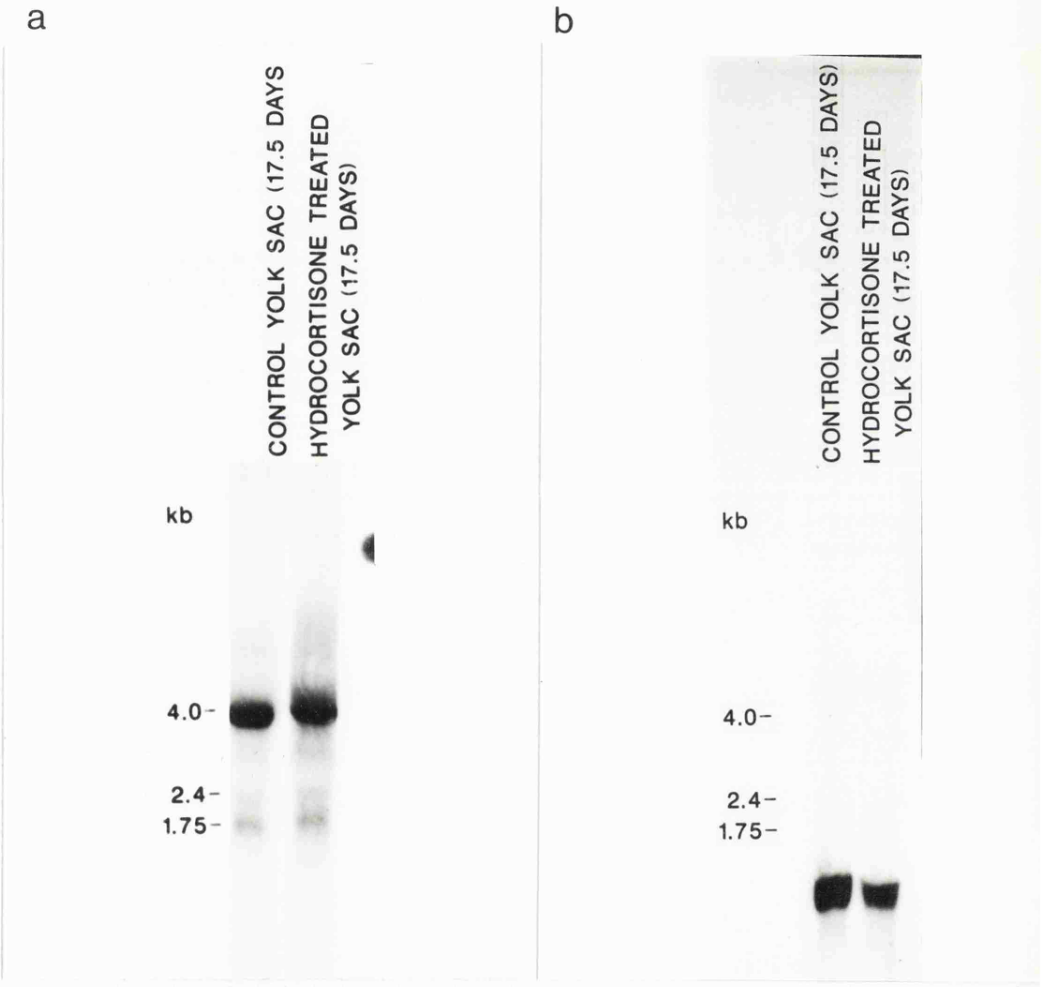
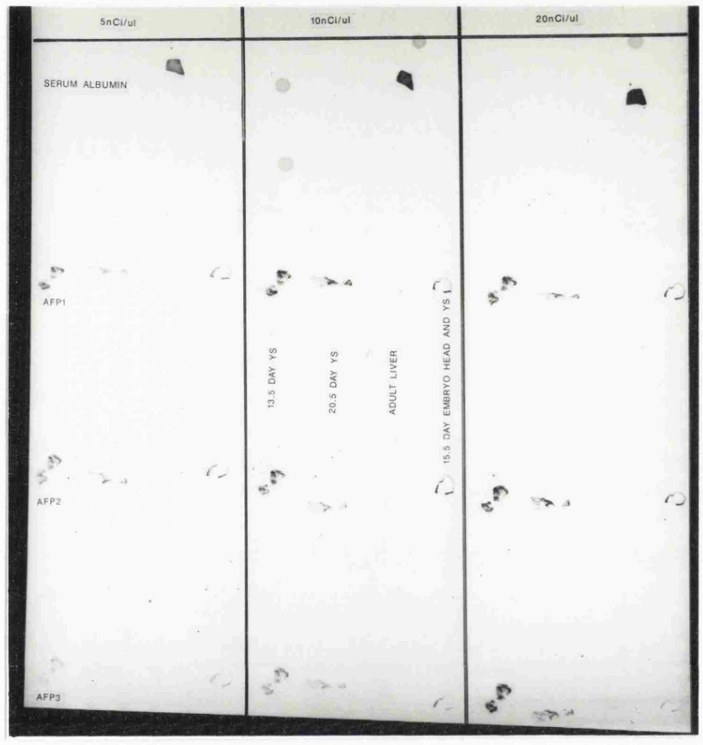

a
CONTROL YOLK SAC (17.5 DAYS)
HYDROCORTISONE TREATED YOLK SAC (17.5 DAYS)
kb
4.0
2.4
1.75
b
CONTROL YOLK SAC (17.5 DAYS)
HYDROCORTISONE TREATED YOLK SAC (17.5 DAYS)
kb
4.0
2.4
1.75

Figure 5.15 Characterisation of synthetic 30-mer oligonucleotide probes to rat alphafetoprotein mRNA and serum albumin mRNA

a) 7μm sections of 13.5 and 20.5 day *in vivo* yolk sac, adult liver and 15.5 day *in vivo* embryo head and yolk sac, were hybridised to three ^{32}P-labelled probes to AFP mRNA (AFP1, AFP2 and AFP3) and one ^{32}P-labelled probe to serum albumin mRNA, at three different specific radioactivities (5, 10 and 20 nCi/μl). Binding was visualised by X-ray autoradiography on Kodak AR X-Omat film overnight.

b) 7μm frozen sections of adult liver, fetal liver and 11.5 day to 22.5 day *in vivo* and cultured yolk sacs were hybridised to serum albumin probe and AFP2 (15nCi/μl), and binding was visualised by X-ray autoradiography on Kodak AR X-Omat film overnight.

a


5nCi/ul
10nCi/ul
20nCi/ul
SERUM ALBUMIN
AFP1
AFP2
AFP3
13.5 DAY YS
20.5 DAY YS
ADULT LIVER
15.5 DAY EMBRYO HEAD AND YS

b

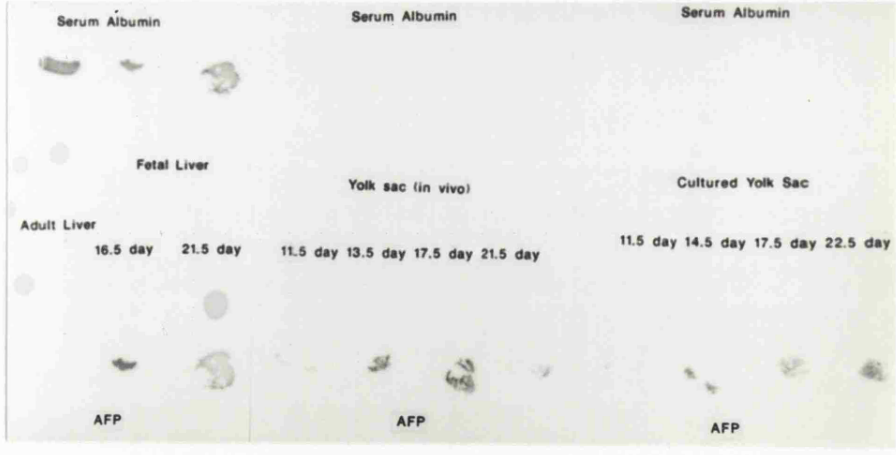

Serum Albumin
Fetal Liver
Adult Liver
16.5 day
21.5 day
AFP
Serum Albumin
Yolk sac (in vivo)
11.5 day 13.5 day 17.5 day 21.5 day
AFP
Serum Albumin
Cultured Yolk Sac
11.5 day 14.5 day 17.5 day 22.5 day
AFP

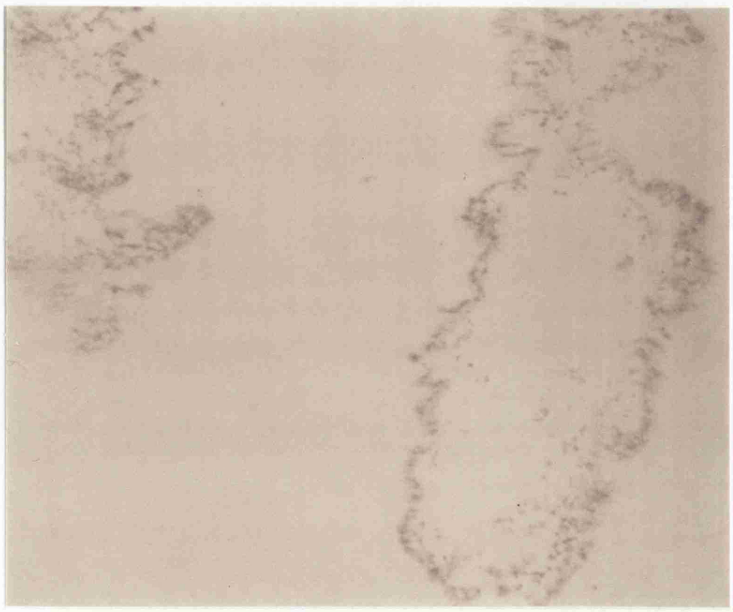
Figure 5.16 11.5 day *in vivo* yolk sac (x 95)

a) Bright field photomicrograph
b) Dark field photomicrograph of hybridisation to AFP2
c) Dark field photomicrograph of hybridisation to serum albumin probe

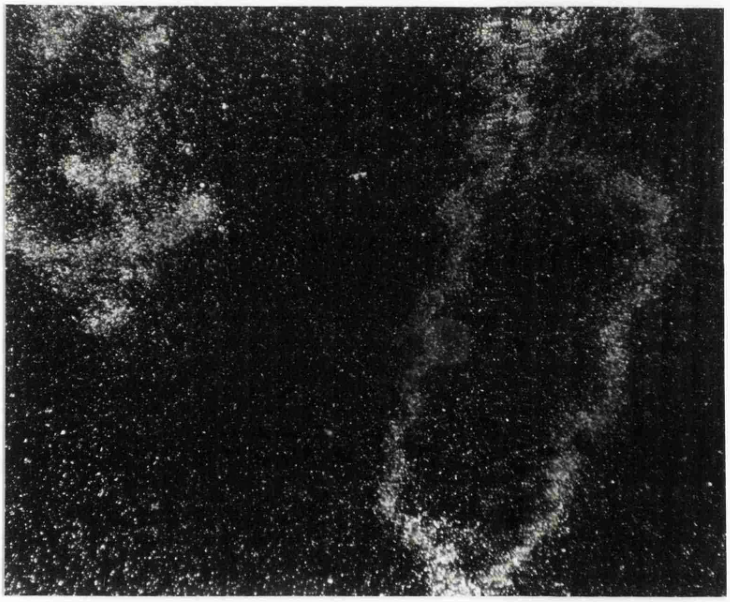
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

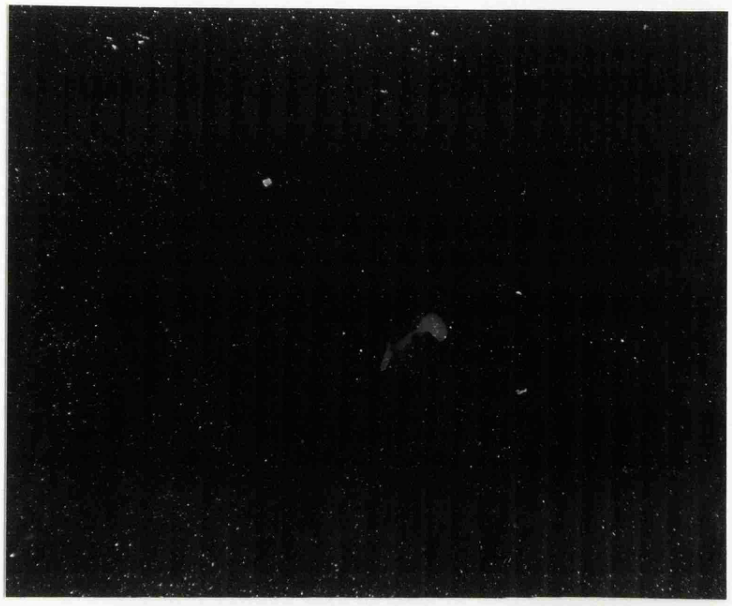
a



b



c



.1 mm

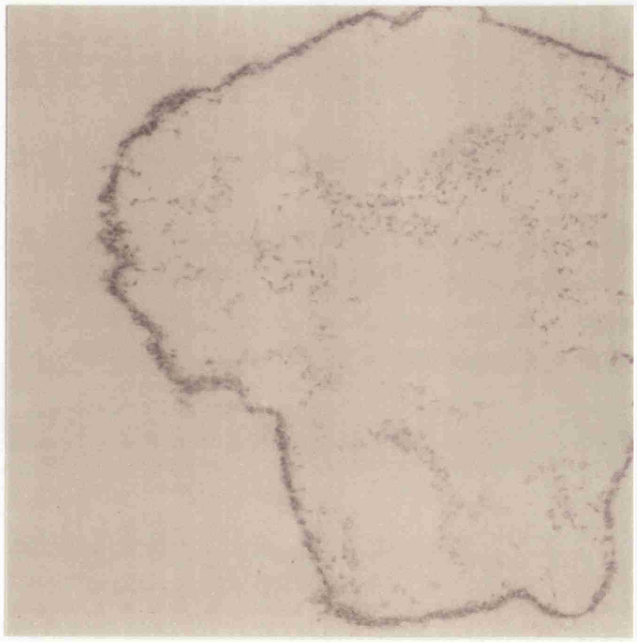
Figure 5.17 11.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph
b) Dark field photomicrograph of hybridisation to AFP2
c) Dark field photomicrograph of hybridisation to serum albumin probe

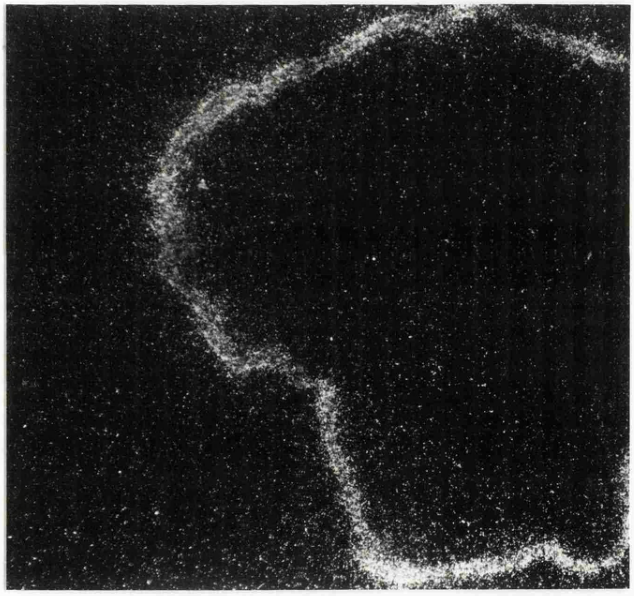
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

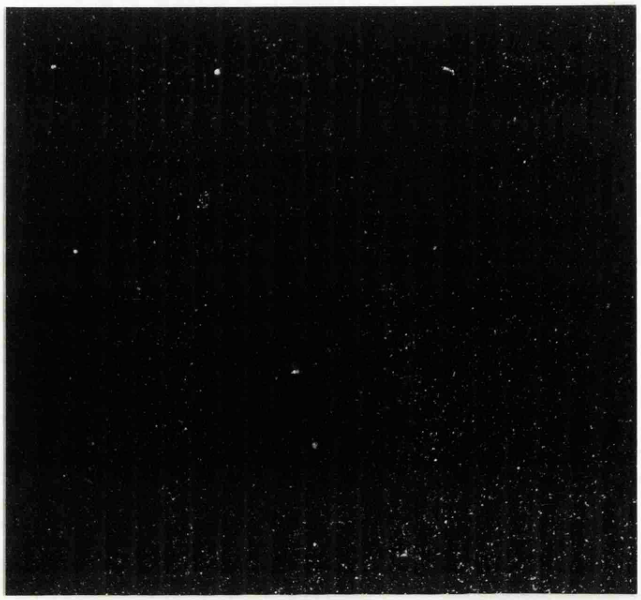
a



b



c



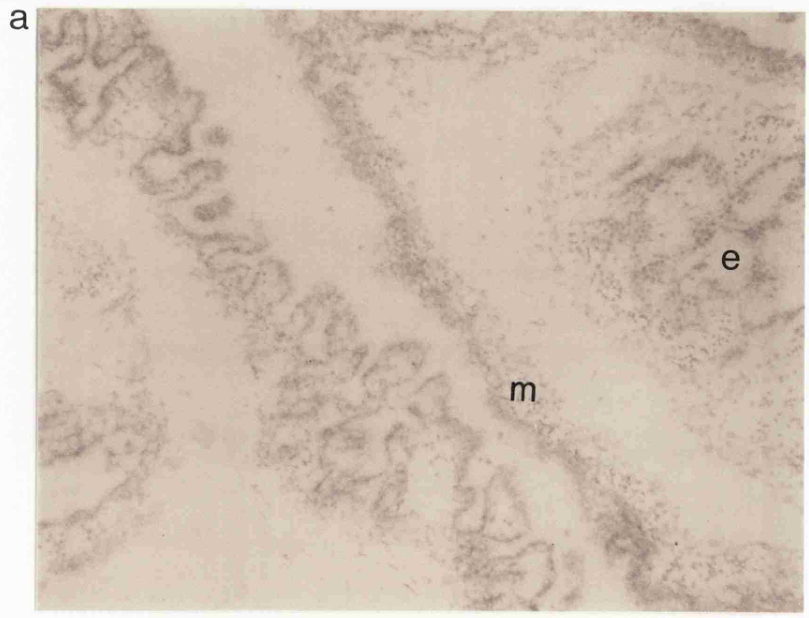
.1 mm

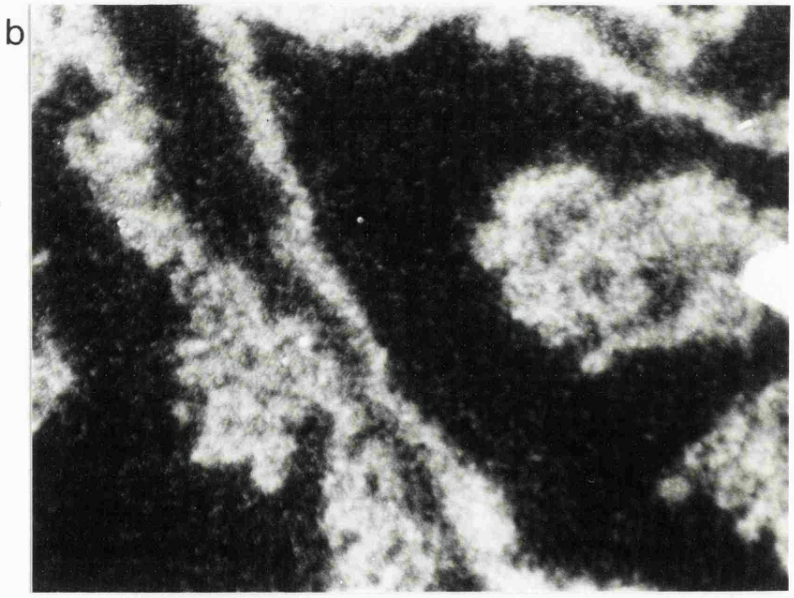
Figure 5.18 13.5 day *in vivo* yolk sac (x 95)

a) Bright field photomicrograph showing endoderm (e) and mesoderm (m)
b) Dark field photomicrograph of hybridisation to AFP2
c) Dark field photomicrograph of hybridisation to serum albumin probe

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.


a
e
m


b

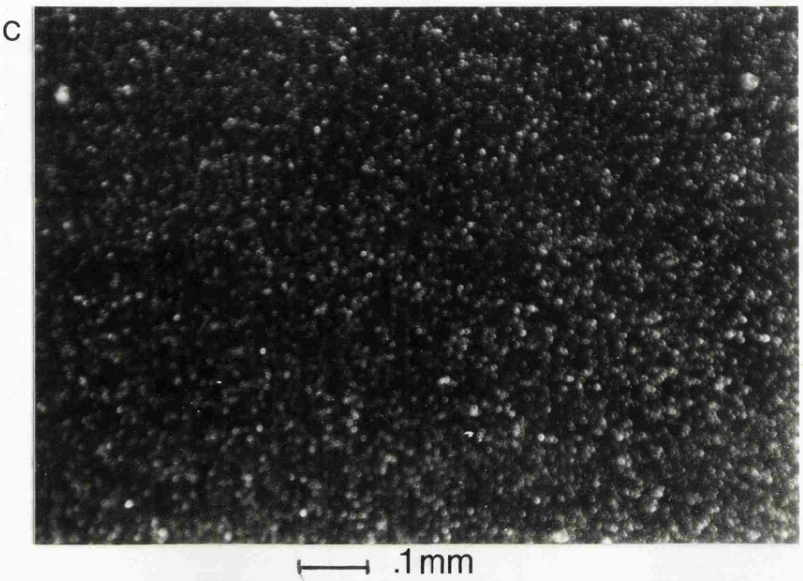

c
.1mm

Figure 5.19 14.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph showing endoderm (e) and mesoderm (m)
b) Dark field photomicrograph of hybridisation to AFP2
c) Dark field photomicrograph of hybridisation to serum albumin probe

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

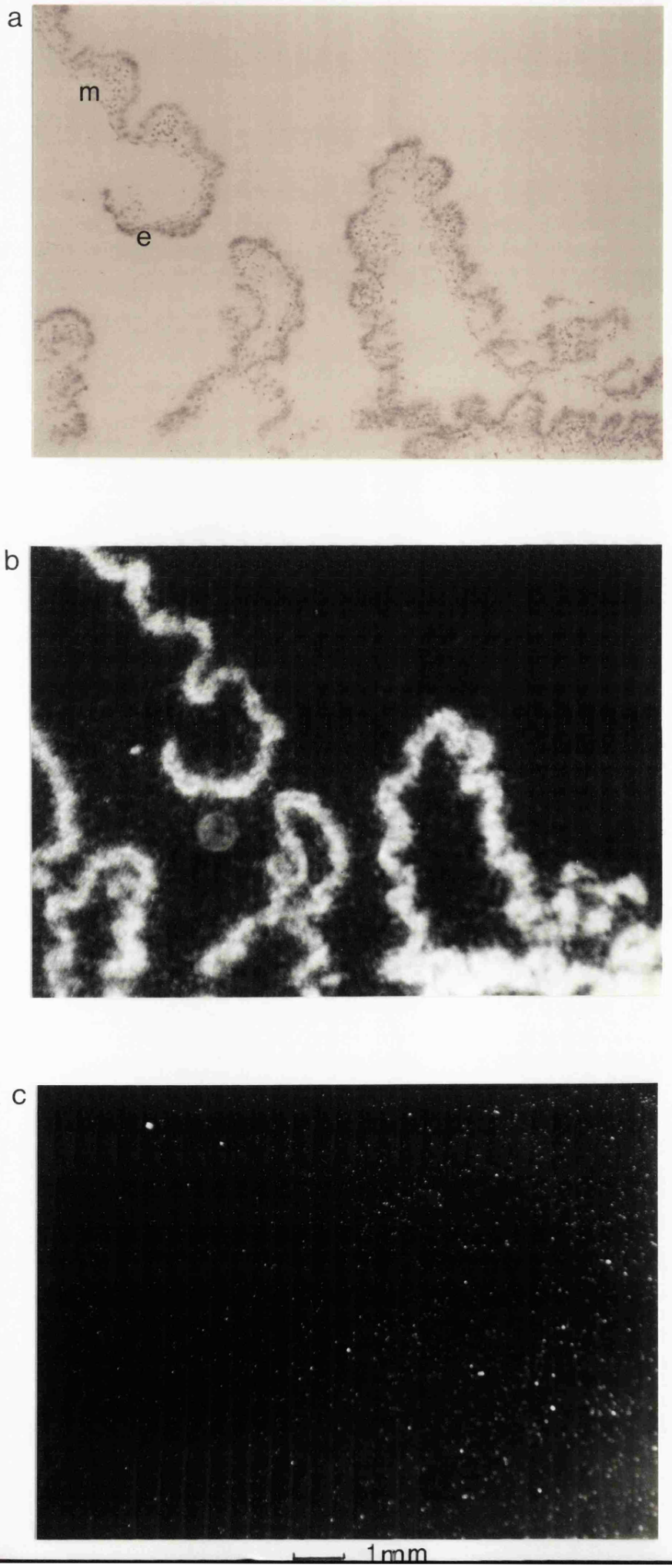

a
m
e
b
c
1mm

Figure 5.20 17.5 day *in vivo* yolk sac (x 95)

a) Bright field photomicrograph

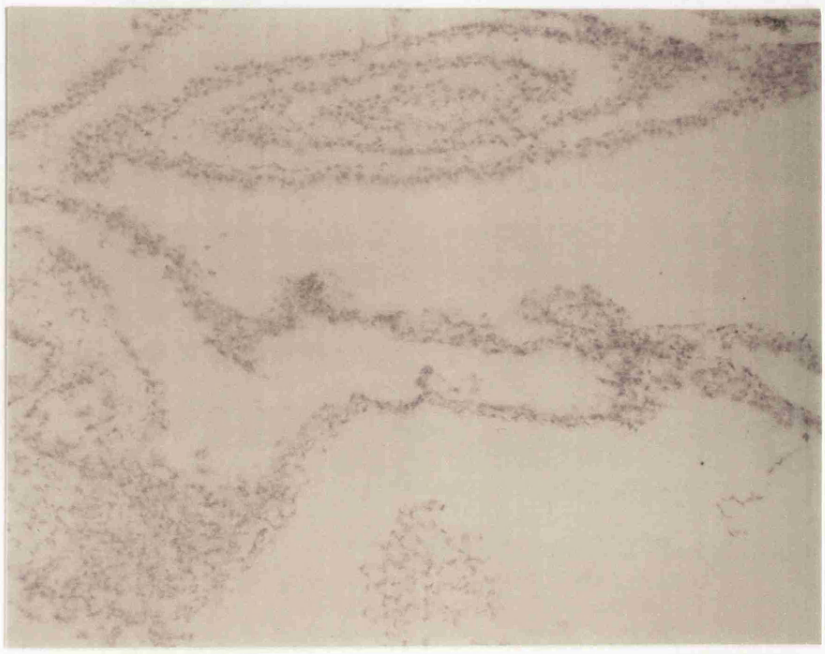
b) Dark field photomicrograph of hybridisation to AFP2

c) Dark field photomicrograph of hybridisation to serum albumin probe

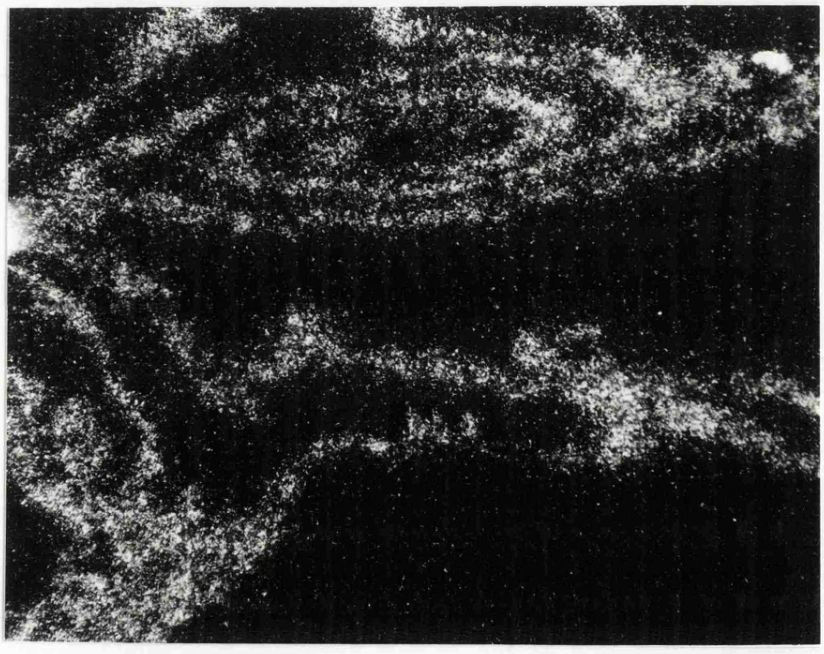
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

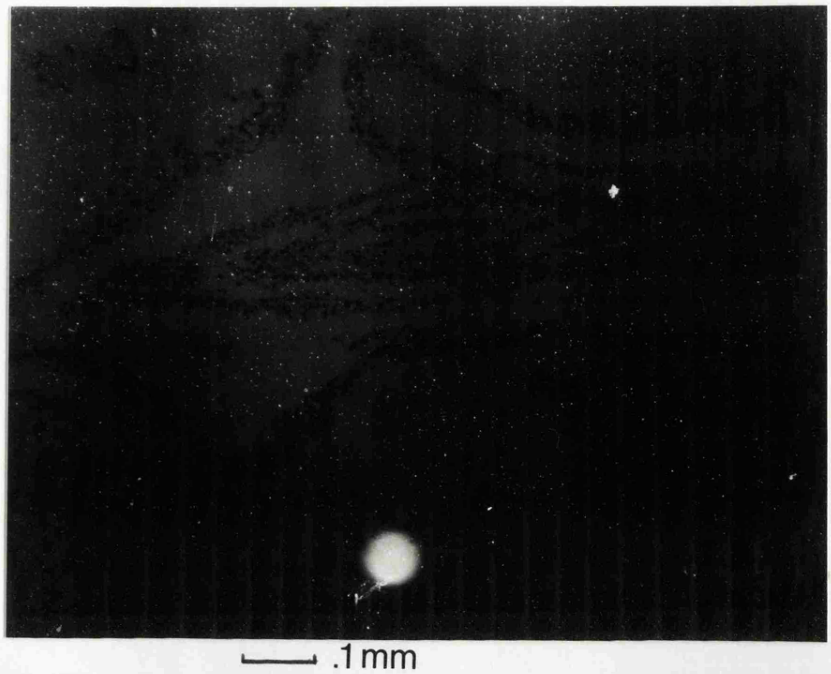
a



b



c



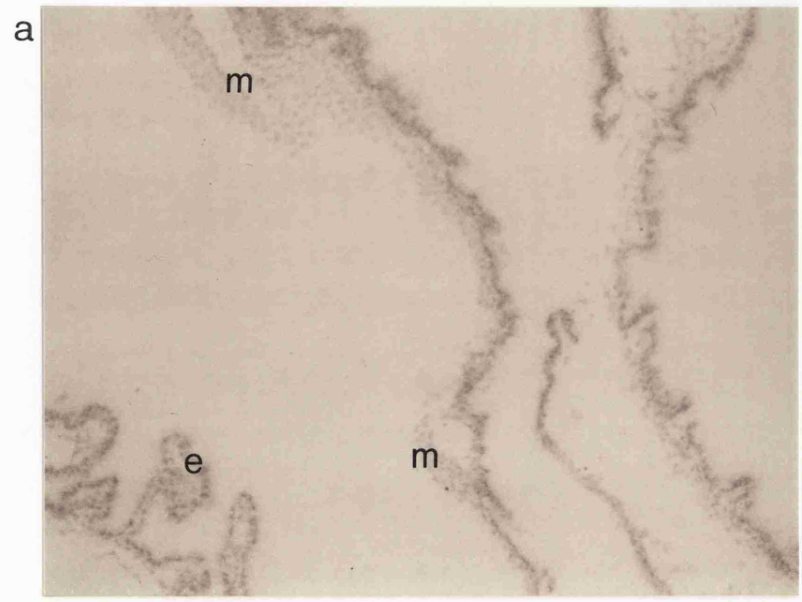
.1 mm

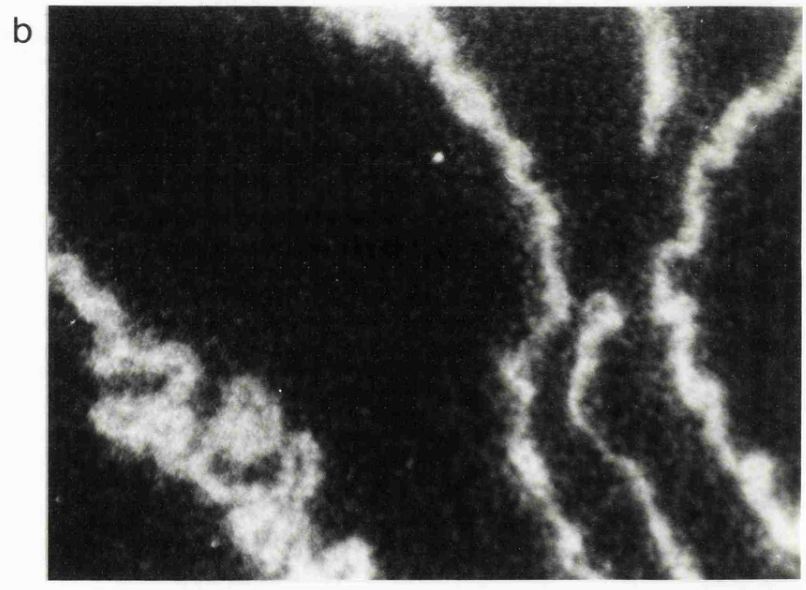
Figure 5.21 17.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph showing endoderm (e) and mesoderm (m)
b) Dark field micrograph of hybridisation to AFP2
c) Dark field photomicrograph of hybridisation to serum albumin probe

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.


a
m
e
m


b

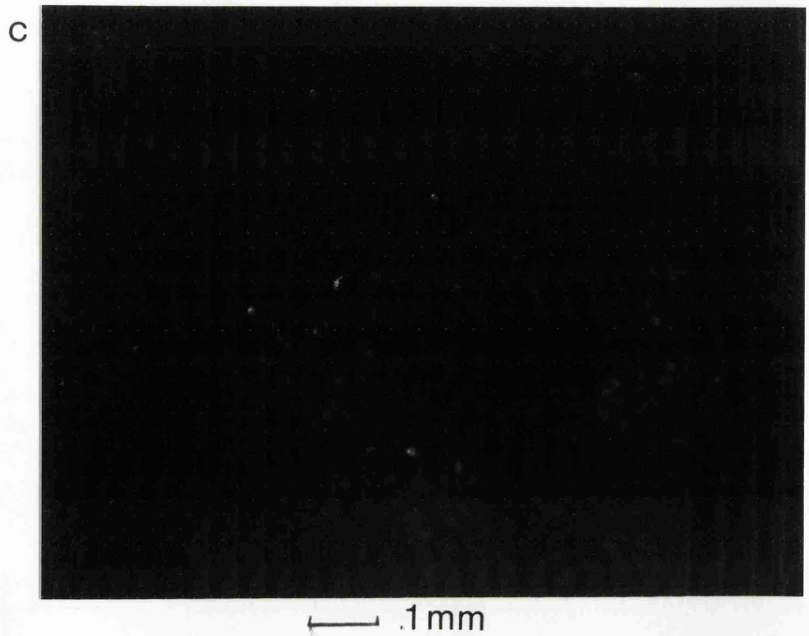

c
.1 mm

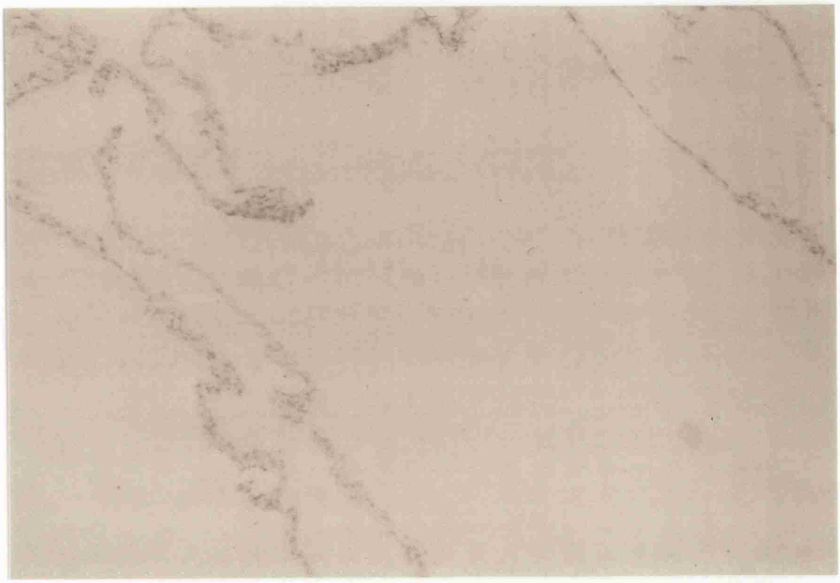
Figure 5.22 21.5 day *in vivo* yolk sac (x 95)

a) Bright field photomicrograph
b) Dark field photomicrograph of hybridisation to AFP2
c) Dark field photomicrograph of hybridisation to serum albumin probe

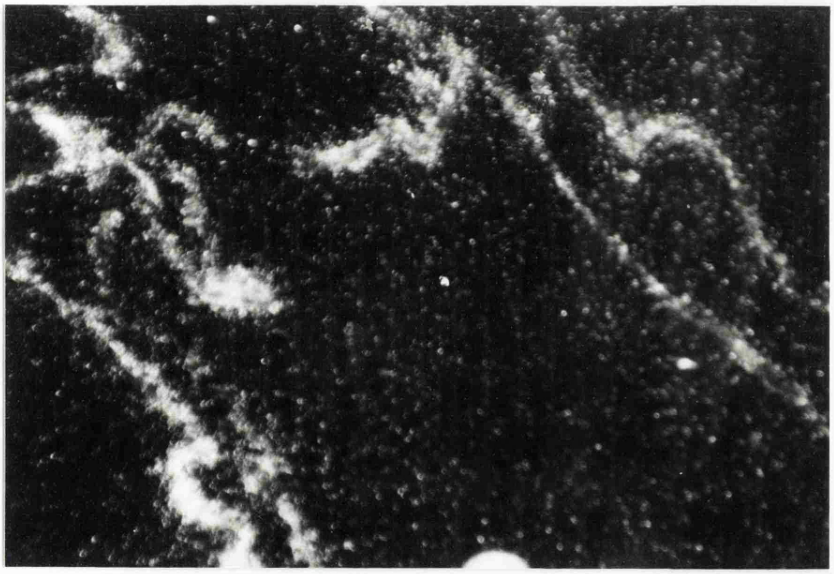
7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

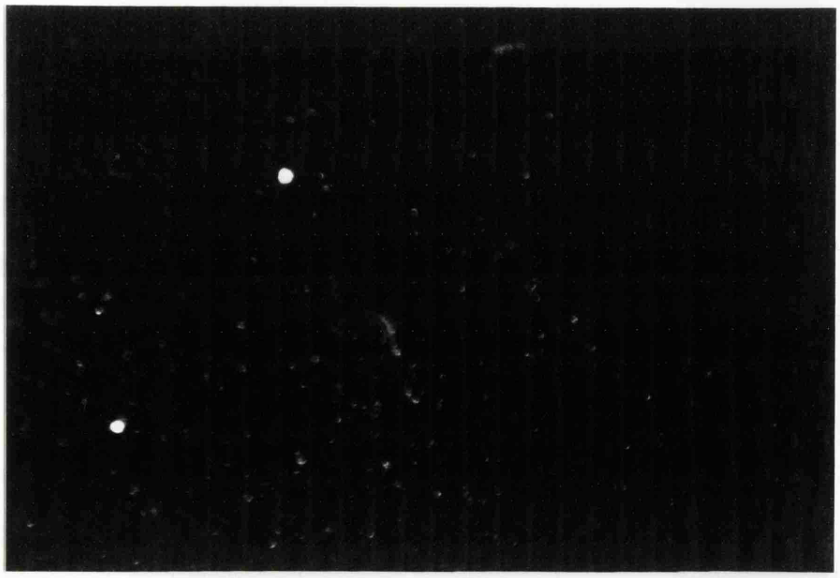
a



b



c



.1mm

Figure 5.23 22.5 day cultured yolk sac (x 95)

a) Bright field photomicrograph
b) Dark field photomicrograph of hybridisation to AFP2
c) Dark field photomicrograph of hybridisation to serum albumin probe

7μm frozen sections were fixed in 4% glutaraldehyde for 5 minutes, prehybridised for 20 minutes, then hybridised to ^{32}P-labelled oligonucleotide probes (15nCi/μl) for 18 hours.

Specific binding was visualised by thin layer K5 emulsion autoradiography.

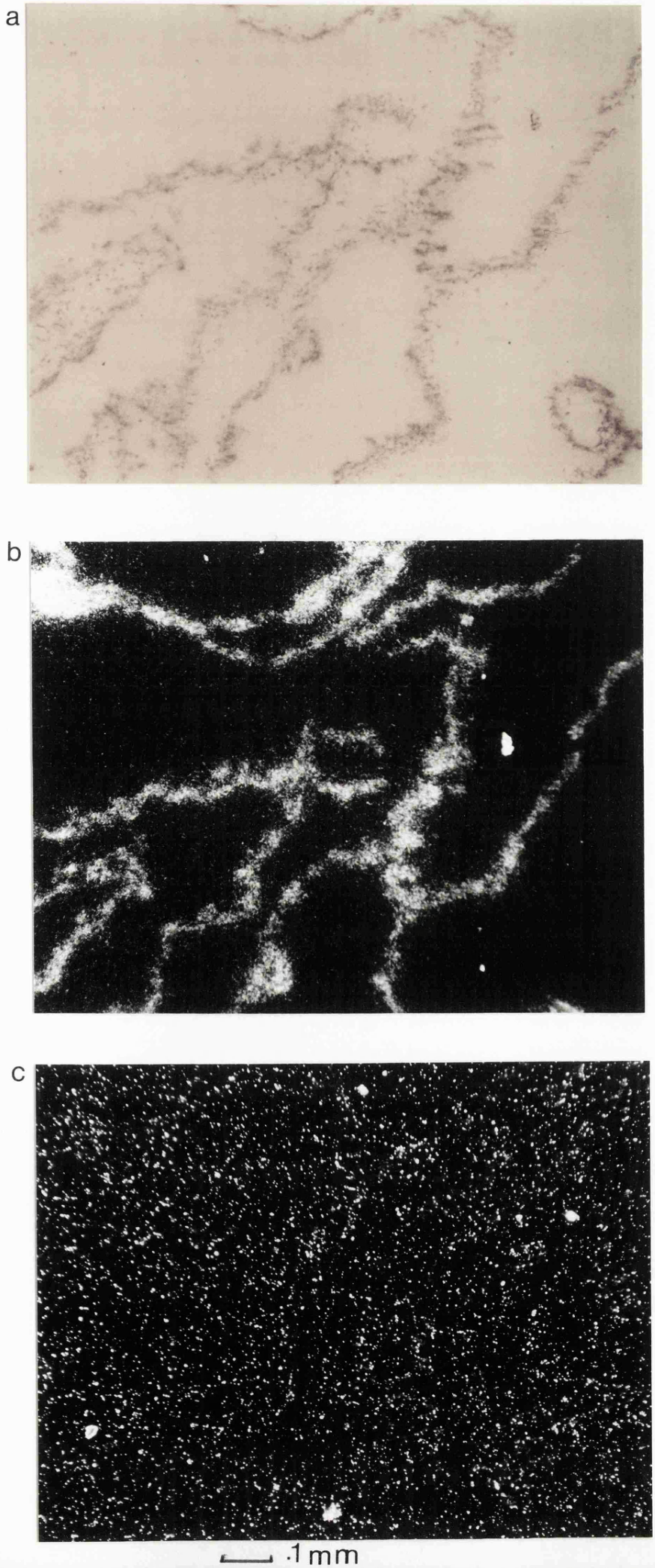

a
b
c
.1 mm

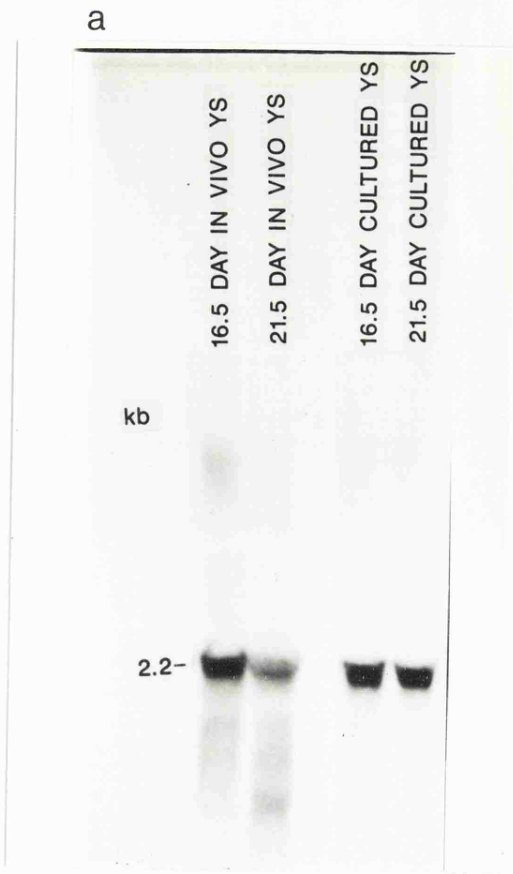
Figure 5.24 Northern and dot blot quantitative hybridisation of *in vivo* and cultured yolk sac RNA to a ^{32}P-labelled oligonucleotide to AFP mRNA

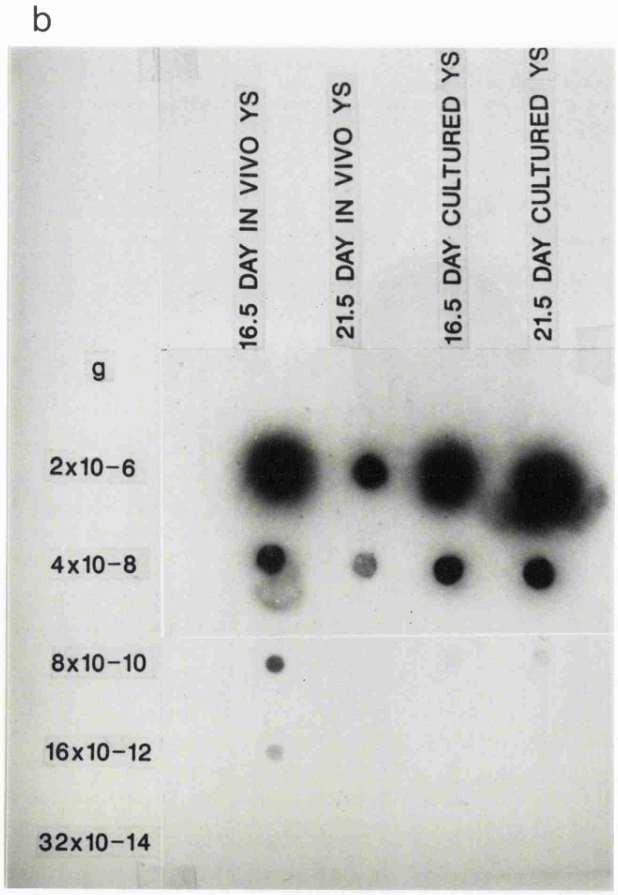
Total RNA from 16.5 day and 21.5 day cultured and *in vivo* yolk sac tissue, was prepared by polytron homogenisation and differential precipitation in LiCl/Urea.

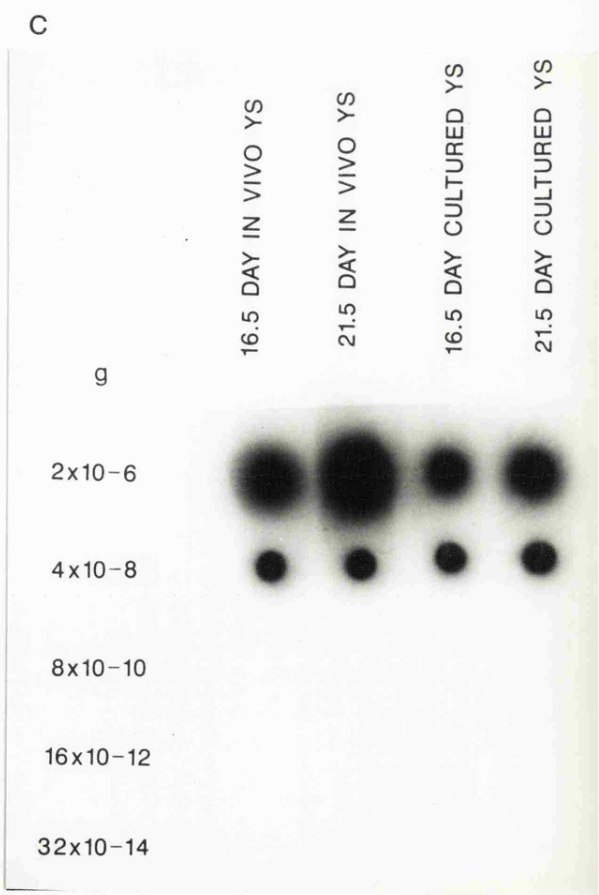
a) Northern blot v. AFP2
100µg total RNA was electrophorised on an agarous gel, blotted onto Hybond, and hybridised to AFP2 probe.

b) Dot blot v. AFP2
Serial dilutions of poly(A)+ RNA 2µg to 0.32pg were prepared from the total RNA samples by column separation. The dilutions were dot blotted on Hybond and hybridised to AFP2 probe. The hybridised membrane was exposed to Fuji RX X-ray film for 4 days.

c) Dot blot v. HPGT probe
The same membrane was stripped and reprobed with hypoxanthine phosphoguanasyl transferase mRNA probe. The hybridised membrane was exposed to Fuji RX X-ray film for 7 days.


a
16.5 DAY IN VIVO YS
21.5 DAY IN VIVO YS
16.5 DAY CULTURED YS
21.5 DAY CULTURED YS
kb
2.2-


b
16.5 DAY IN VIVO YS
21.5 DAY IN VIVO YS
16.5 DAY CULTURED YS
21.5 DAY CULTURED YS
g
2x10-6
4x10-8
8x10-10
16x10-12
32x10-14


c
16.5 DAY IN VIVO YS
21.5 DAY IN VIVO YS
16.5 DAY CULTURED YS
21.5 DAY CULTURED YS
g
2x10-6
4x10-8
8x10-10
16x10-12
32x10-14

5.4 DISCUSSION

The methodology of *in situ* hybridisation was introduced to the laboratory, shortly before this work was commenced. The protocol used was based on the published method of Coghlan *et al*. (1985). Over the time period covering this study, empirical observations have led to a number of small modifications to the protocol. The findings of co-workers in the laboratory have contributed to the subsequent development of the methodology. The first major change was a refinement of the prehybridisation step, whereby frozen sections were found to have substantially lower background levels of hybridisation, if a shorter time period was spent in prehybridisation buffer. Therefore this step was cut from one hour to 20 minutes. The second major change in protocol was changing from column purification of 5'-end labelled probe to ethanol-precipitation purification. This change had the dual advantage of increasing the probe yield and reducing the handling time of radioactive probe.

The *in situ* hybridisation technique has proved successful in both studies undertaken, but certain limitations have become apparent. The technique enables the location of gene expression at the cell layer level to be elucidated using ^{32}P-labelled oligonucleotide probes. This was conclusively shown in the two studies, as α-fetoprotein mRNA expression was located to endodermal cells of the yolk sac, whilst IGF-II mRNA expression was observed in both endodermal and mesodermal layers. However, this information is purely

qualitative, and quantitative assessment of mRNA expression cannot easily be made on frozen sections prepared for *in situ* hybridisation. This was made apparent in the study of AFP expression in late gestation *in vivo* yolk sacs, where a 10^3-fold decrease in poly(A)+ RNA between 16.5 day and 21.5 day yolk sacs was not shown conclusively on *in situ* sections.

Another problem revealed in some studies was the stability of the silver grains in commercially available emulsions. In some cases, apparently strong signals revealed on X-ray autoradiography were not translated into silver grain intensity after lengthy periods of incubation. In other cases, silver grain intensity appeared to diminish with time, despite double fixation in Hypam fixer and formyl saline.

However, in the two studies performed, looking at two high turnover signals, the technique enabled significant research progress to be made.

Insulin-like growth factor II (IGF-II) mRNA was shown to be expressed in cultured yolk sac by both endodermal and mesodermal cells, corresponding to results of *in vivo* studies showing the same expression of IGF-II mRNA in visceral yolk sac (Beck *et al.*, 1987). Northern blot analysis of total RNA from cultured yolk sac revealed three transcripts of 4.0, 2.4 and 1.75 kb size. These transcript sizes correlate with previous findings in fetal and neonatal

tissues. Beck et al. (1989) reported four transcripts, the additional transcript being a 1.25 kb size, whilst Soares et al. (1985) also reported four mRNAs with differing actual kb size, but the same relation to each other. Brown et al. (1986) reported six mRNA species in fetal and neonatal tissues, hybridising to a cDNA probe. All workers conclude that the different species reflect either alternate splicing of a single gene, or different promoter or polyadenylation sites, rather than expression of multiple genes sharing exons (Soares et al., 1986; Graham et al., 1986).

To study the potential of glucocorticoids to control mRNA expression in the cultured visceral yolk sac, a water soluble glucocorticoid was required, since the glucocorticoid used in the neonatal study (Beck et al., 1988b) - cortisone acetate - is lipid soluble, and therefore difficult to add to the culture medium. The effectiveness of hydrocortisone phosphate in down-regulating hepatic IGF-II mRNA expression in 11.5 day and 13.5 day neonates was clearly demonstrated using in situ techniques. The dose used (0.25mg/g) was the same dose of cortisone acetate used in the original paper. The initial finding using 0.1mg/g dexamethasone was that this dose was sufficiently high to completely switch off hepatic IGF-II mRNA expression in treated neonates, and their control litter mates. By cross-fostering the treated and control litter mates to separate mothers, whose litters had been removed at the same litter age, it was possible to show that dexamethasone effectively suppressed IGF-II mRNA expression at doses one hundred times

lower than observed with cortisone acetate or hydrocortisone. Thus, the initial finding with dexamethasone was explained by the phenomenon of coprophagy between the litter mates, where a high enough dose of dexamethasone was available to the control animals, through urinary excretion of the unchanged drug from the treated animals, to down-regulate their IGF-II mRNA expression.

Since the results from the neonatal dexamethasone study were initially inconclusive, hydrocortisone phosphate was chosen as the water soluble glucocorticoid to use in the study of potential prenatal regulation of IGF-II mRNA expression.

The culture of visceral yolk sacs in hydrocortisone-containing culture medium resulted in a dose-dependent suppression of growth, as measured by yolk sac diameter. This finding mimics the *in vivo* effects of repeatedly injecting pregnant rats i.p. with glucocorticoids, where a dose-dependent decrease in average litter weight is observed (Davis *et al.*, 1954; Parvez *et al.*, 1976).

The doses of glucocorticoid used in the studies on pregnant rats, both in this study and in previous publications (Parvez *et al.*, 1976; Beck *et al.*, 1989) are supraphysiological, but reflect the ability of the maternal and fetal liver to inactivate cortisol (Coufalik and Monder, 1974). In rats, the predominant glucocorticoid is corticosterone (Dupouy *et al.*, 1975), but as yet no evidence is available on the neonatal IGF-II expression after an induced surge in corticosterone levels.

In the cultured yolk sac, the administration of hydrocortisone had no detectable effect on IGF-II mRNA expression. The glucocorticoid-induced repression of growth must have been mediated through another mechanism of cell cycle suppression. Tomkins (1974) described glucocorticoid-mediated repression of growth, where cells at the G_1 phase were transformed to cells blocked in the G_0 phase. This was postulated to be due to glucocorticoid induction of molecules that interfere with normal gene transcription.

For glucocorticoids to exert effects on gene transcription, they must form an adduct with a cytoplasmic receptor, which can then cross into the nucleus and interact with specific nucleic acid sites (Begemann et al., 1988). The visceral yolk sac has glucocorticoid receptors, detectable at 14.5 days of gestation and present in increasing quantity until term (Carbone et al., 1985; Carbone et al., 1986), and the IGF-II gene sequence contains several sequences with strong homology to the consensus glucocorticoid responsive element, roughly equidistant between the two promoter sequences (Frunzio et al., 1986; Beck et al., 1989).

The lack of effect of glucocorticoids on cultured visceral yolk sac IGF-II was later backed up by preliminary data on litters from mothers treated in late gestation with glucocorticoids (Senior, unpublished observations). In early gestation growth factors such as epidermal growth factor, transforming growth factor-α and platelet-derived growth factor appear, and may play an important role in early embryological cell cycle control (O'Keefe and Pledger, 1983;

Nexo et al., 1980; Lee et al., 1985; Rappolee et al., 1988). IGF-II mRNA is switched on in the gastrulation stages of development and is expressed at high levels till term (Ohlsson et al., 1989; Beck et al., 1987) implicating a more important role in the later stages of embryogenesis. One can hypothesise that the natural down-regulation mechanism of IGF-II may be protected prenatally, by histone protection of the down-regulation site on the IGF-II gene.

Alphafetoprotein (AFP) mRNA expression in cultured yolk sacs was histologically localised in the endodermal cells. This finding correlates with the published accounts of yolk sac AFP production, where both the protein and mRNA have been localised to extraembryonic endoderm (Gitlin et al., 1967; Dziadek and Adamson, 1978; Adamson, 1986).

Only one transcript size of 2.2 kb was detected, corresponding to the normal pattern of expression of AFP mRNA (Chou et al., 1988; Wan et al., 1988). From the in situ hybridisation results, there appeared to be a significant rise in hybridised mRNA between 11.5 days and 14.5 days in culture, which was matched in vivo, and was in agreement with the published findings of yolk sac AFP mRNA synthesis (Muglia and Locker, 1984b; Sellem et al., 1984). The reported decline in yolk sac AFP mRNA transcription from 16.5 days was observed in the study of in vivo yolk sac transcription, with the dot blot hybridisation indicating a 10^3-fold decrease in detectable message after five days.

However, there was no detectable decline in AFP mRNA synthesis between 16.5 and 21.5 day cultured yolk sacs.

There have been no studies on the mechanism of repression in yolk sac AFP expression, possibly because a general pattern of biochemical and molecular repression of the yolk sac exists at this stage of gestation. The neonatal decline in liver transcription of AFP mRNA has been extensively studied (Nahon, 1987; Estanyol and Danan, 1988). Muglia and Rothman-Denes (1986) showed three functional regions 5' to the transcription initiation site - a promoter region, inactive in the absence of an upstream enhancer region, and a down-regulation site situated between the promoter and initiation sites. This down-regulation site lies between 250 and 800 base pairs upstream from the transcription initiation site, and its deletion results in high level transcription of the gene in both fetal and adult mouse liver (Vacher and Tilghman, 1989). This indicates that AFP gene repression is due to the imposition of dominant negative regulation, mediated by a silencer sequence.

The final experiment of this study was aimed at elucidating the source of the factors responsible for the production of the silencer sequence. Unfortunately this preliminary experiment was aborted at the emulsion autoradiography stage of hybridisation due to loss of emulsion efficiency. However, the X-ray autoradiograph indicated that yolk sacs cultured in normal medium, and nuclear and cytosolic fractions of 16.5 and 21.5 day fetal liver homogenate continued to express AFP mRNA between 16.5 and 22.5 days in

culture. Yolk sacs cultured from 16.5 days in maternal serum collected from pregnant animals of 17.5 days and 19.5 days gestation, switched off AFP mRNA expression, so that no detectable mRNA was present at 22.5 days. This result indicates that some factor present in late gestation maternal serum causes the visceral yolk sac to express the silencer sequence to the AFP gene.

CHAPTER SIX

General Discussion

6.1 GENERAL DISCUSSION

Before the experiments were carried out in these studies, the 'giant' yolk sac culture system had been used to study protein uptake as a model of visceral yolk sac function (Dunton *et al.*, 1986; Pratten *et al.*, 1987; Dunton, 1988). The results from the studies in this thesis indicated that the cultured yolk sac retains the same cellular differentiation between endoderm and mesoderm, and this was verified by a transmission electron microscopic study of cultured and *in vivo* yolk sac (Decatris, 1988). As well as the morphological similarities, there was comparable biochemical differentiation between cultured and *in vivo* yolk sac, with respect to erythropoiesis, change over from anaerobic to aerobic intermediary metabolism, and extra-embryonic transcription of alphafetoprotein and insulin-like growth factor II mRNA.

However, the question arises as to whether these qualitative morphological and biochemical findings provide sufficient evidence to use the culture model for the quantitative assessment of true visceral yolk sac placental function.

A parallel study by Decatris (1988) revealed that the cultured yolk sac exhibited basal levels of cathepsin B activity, whereas *in vivo* activity rose from 11.5 to 15.5 days. This indication that the cultured yolk sac may not process macromolecules through the lysosomal system, in the same quantity as is observed *in vivo*, indicates that a full

picture of the protein processing pathways in the cultured yolk sac is required to see whether the yolk sac culture model can be used to quantitatively assess protein turnover in the visceral yolk sac.

The major morphological difference between cultured and *in vivo* yolk sac, found in the light microscopic study, was that cultured yolk sac failed to deposit major accumulation of collagen fibres between the mesenchyme and mesothelium. Modification of the culture medium by increasing concentrations of sulphate, proline or glycosaminoglycans or adding exogenous growth factors may induce collagen biosynthesis and thereby improve the physical membrane structure, and increase the mimicry of normal yolk sac structure.

The results from the study of specific activities of hexokinase, pyruvate kinase and succinate dehydrogenase indicated that the initiation of aerobic glucose catabolism took place between 9.5 and 11.5 days both *in vivo* and in culture. However, as glycolytic and tricarboxylic acid cycle activity decreased between 15.5 and 17.5 days *in vivo*, there was no decrease in catabolic activity in culture. This finding supported the histological appearance of the yolk sacs, where the nuclear appearance of the cultured yolk sac indicated that the endodermal cells were still actively synthesising protein.

The *in situ* hybridisation experiments examining α-fetoprotein mRNA expression also indicated that cultured yolk sac continued to actively transcribe the AFP gene after 16.5

days, when the *in vivo* gene transcription was suppressed. This result, in combination with the carbohydrate catabolism results, indicates the lack of gene suppression in the cultured yolk sac. This was also the probable explanation for the constitutive expression of drug-metabolising P-450 isoenzymes in the endodermal cells of cultured yolk sac, in contrast to the inducible appearance of such enzymes in 15.5 to 18.5 day *in vivo* yolk sacs.

These morphological and biochemical differences mean that any conclusion about visceral yolk sac function drawn from the cultured yolk sac model must be made with strict reservation. However, the nature of the culture technique lends itself to the study of these specific differences, and other general developmental events in mid to late gestation.

The final series of *in situ* experiments gave results which tend to point towards the maternal serum constituent of the culture medium, as the controlling factor in the yolk sac expression of gene suppression. Collection of serum from pregnant dams at the appropriate stages of gestation may provide evidence for the nature of these factors, using the culture system in conjunction with techniques such as differential dialysis and two-dimensional gel electrophoresis, to isolate essential maternal-borne factors required for gene suppression in visceral yolk sac.

Voutilainen and Miller (1987) published an account of coordinate tropic hormone regulation of mRNAs for IGF-II and P-450 cholesterol side chain cleavage enzyme. These results obtained in human steroidogenic tissues of ovary and adrenal

gland, open up the potential to link the research into P-450 and IGF-II expression in the rat visceral yolk sac, carried out in this thesis, and to use the culture technique to examine the possible regulatory effects of prolactin and dibutyryl cAMP on the expression of IGF-II and P-450.

A final avenue of research to be explored using the cultured yolk sac may involve the incorporation of genetic information into the cells of the cultured yolk sac, by electrocorporation. To facilitate this approach, attempts could be made to reproduce the culture technique using mice, which provide a sounder background data bank of genetic information.

Whatever the choice of direction for future research, experiments utilising the cultured yolk sac model to study cellular developmental events, will benefit from the continuous differentiation of two extra-embryonic cell lineages with different characteristics, developing as a closed vesicle, in an easily accessible serum-based culture medium.

APPENDICES

APPENDIX A

Hank's balanced salt solution was used as an explantation medium, and was prepared using the following recipe:

Salt	mg/l
KCl	400.0
KH_2PO_4	600.0
$MgSO_4.7H_2O$	200.0
NaCl	8000.0
$NaHCO_3$	700.0
Na_2HPO_4	47.5
Glucose	1000.0
Sodium phenol red	17.0

These salts were autoclaved in 950mls of 'Q' water, along with 185.5mg $CaCl_2$ in 50mls of Q. $CaCl_2$ was autoclaved separately to prevent calcium salt precipitation. On cooling, the two solutions were mixed, and stored at $4^{o}C$.

APPENDIX B

Medium 199 was used in the culture medium, and was purchased as powder from Gibco, Paisley, UK (formulation overleaf). 2.2g $NaHCO_3$ was added to each litre prepared, the solution was vacuum-filtered through a 500ml filter, and staored at $-4^{o}C$. Penicillin and streptomycin were added to give final concentrations of 100IU/ml and 100µg/ml respectively.

Medium 199

	Hanks' Salts Cat. No. 071-1200	Earle's Unmodified Salts Cat. No. 071-1100
Inorganic Salts	mg/L	mg/L
$CaCl_2$ (anhyd.)	140.00	200.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.72	0.72
KCl	400.00	400.00
KH_2PO_4	60.00	
$MgSO_4$ (anhyd.)		97.67
$MgSO_4 \cdot 7H_2O$	200.00	
NaCl	8000.00	6800.00
$NaH_2PO_4 \cdot H_2O$		140.00
Na_2HPO_4 (anhyd.)	47.70	
Other components		
Adenine sulphate		10.000
Adenosinetriphosphate (Disodium salt)		1.000
Adenylic acid		0.200
Cholesterol		0.200
Deoxyribose		0.500
D-Glucose		1000.000
Glutathione		0.050
Guanine HCl (free base)		0.300
Hypoxanthine (Na salt)		0.354
Phenol red		20.000
Ribose		0.500
Sodium acetate		50.000
Thymine		0.300
Tween 80™ *		20.000
Uracil		0.300
Xanthine (Na salt)		0.344
Amino Acids		
DL-Alpha-Alanine		50.000
L-Arginine HCl		70.000
DL-Aspartic acid		60.000
L-Cysteine HCl $\cdot H_2O$		0.110
L-Cystine (2 HCl)		26.000
DL-Glutamic acid $\cdot H_2O$		150.000
L-Glutamine		100.000
Glycine		50.000
L-Histidine HCl $\cdot H_2O$		21.880
L-Hydroxyproline		10.000
DL-Isoleucine		40.000
DL-Leucine		120.000
L-Lysine HCl		70.000
DL-Methionine		30.000
DL-Phenylalanine		50.000
L-Proline		40.000
DL-Serine		50.000
DL-Threonine		60.000
DL-Tryptophan		20.000
L-Tyrosine Disodium Salt		57.660
DL-Valine		50.000
Vitamins		
Ascorbic acid		0.050
alpha Tocopherol phosphate (disodium salt)		0.010
d-Biotin		0.010
Calciferol		0.100
D-Ca pantothenate		0.010
Choline chloride		0.500
Folic acid		0.010
i-Inositol		0.050
Menadione		0.010
Niacin		0.025
Niacinamide		0.025
Para-aminobenzoic acid		0.050
Pyridoxal HCl		0.025
Pyridoxine HCl		0.025
Riboflavin		0.010
Thiamine HCl		0.010
Vitamin A (acetate) [a]		0.140

References:

[1]Proc. Soc. Exp. Biol. Med., 73.1 (1950). Morgan, Morton and Parker.

Remarks:

a Values established by the Tissue Culture Standards Committee.

*Trademark ICI Americas, Inc.

APPENDIX C

To estimate protein content of visceral yolk sac material, the assay of Lowry et al. (1959) was employed. The two stock Folin reagents were prepared as follows:

Folin A

100ml 2% (w/v) Na_2CO_3

1ml 1% (w/v) $CuSO_4.5H_2O$

1ml 2% (w/v) $(CH(OH)COONa)_2.2H_2O$

Folin B

25% (w/v) Folin Ciocalteu reagent diluted with distilled water.

APPENDIX D

Recommended nomenclature of the P450 gene superfamily

(Nebert et al., 1987)

Family, subfamily and gene designator	Some of the previous literature names
P450IA1	rat c, mouse P_1, rabbit form 6, human P_1
P450IA2	rat d, mouse P_3, rabbit form 4, human P_3
P450IIA1	rat a
P450IIA2	human P450 (1)
P450IIB1	rat b, rabbit form 2
P450IIB2	rat e
P450IIC1	rabbit PBc1
P450IIC2	rabbit PBc2, K
P450IIC3	rabbit PBc3, 36
P450IIC4	rabbit PBc4, 1-8
P450IIC5	rabbit form 1
P450IIC6	rat PB1
P450IIC7	rat f
P450IIC8	human form 1
P450IIC9	human mp
P450IIC10	chicken PB15
P450IID1	rat db1, human db1
P450IID2	rat db2
P450IIE1	rat j, rabbit form 3a, human j
P450IIIA1	rat pcn 1
P450IIIA2	rat pcn 2
P450IIIA3	human HLP, nf

Family, subfamily and gene designator	Some of the previous literature names
P450IVA1	rat LAω
P450XVIIA1	bovine 17α, human 17α
P450XXIA1	bovine, mouse and human C21A
P450XXIA2	bovine, mouse and human C21B
P450XIA1	bovine 11β, human 11β
P450XXIIA1	bovine scc, human scc
P450LIA1	yeast lan
P450CIA1	pseudomonas putida cam.

Gene families are designated by Roman numerals, subfamilies by capital letters, and individual genes by Arabic numbers.

APPENDIX E

A single attempt was made to localise the subcellular distribution of P-450 isoenzymes in the rat visceral yolk sac, using immunocytochemistry on cryo-electron microscopic sections. The method used was a modification of the Tokuyashu method described in Griffiths *et al*. (1984).

17.5 day *in vivo* yolk sac (BNF-treated) and 17.5 day cultured yolk sac were placed, unfixed, in 2.3M sucrose for 15 minutes, then immediately frozen in liquid nitrogen. The remaining procedure was then carried out at Central Toxicology Laboratory, ICI plc, Alderley Park.

1) Tissue blocks were thin sectioned on a Reichert ultra-microtome with cryochamber attachment set at -110^{o}C, and using a glass knife.
2) Sections were retrieved and thawed on a loop of 2.3M sucrose, and transferred to grids.
3) Grids were incubated with 10% calf serum in PBS for 10 minutes, then incubated with anti-rat P-450c (see Chapter 4.2.3).
4) Binding of primary antibody was visualised using Protein-A-Gold second antibody, contrasted with 0.2% (w/v) uranyl acetate.

APPENDIX F

Frozen sections used for <u>in situ</u> hybridisation were pre-hybridised for 20 minutes at 42°C, in hybridisation buffer.

Hybridisation buffer was prepared using the following recipe:

	g/5 litres
NaCl	175.32
NaH_2PO_4	13.00
Na_2HPO_4	23.66
EDTA	7.31
Ficoll	1.00
BSA	1.00
Polyvinyl pyrrolidone	1.00
Herring sperm DNA	5.00

All ingredients were dissolved separately in 3 litres of Q. To this was added 2 litres of deionised formamide. The resulting solution was adjusted to pH 7.4.

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ABSTRACT

By culturing 9.5 day rat egg cylinders as closed yolk sac vesicles, after surgical removal of the embryonic disc, it was shown that the cultured yolk sac retained the essential morphological characteristics of the *in vivo* yolk sac over an eight day culture period, and significant improvements were made in the original culture methodology, with regard to survival rate and serum requirement, by culturing in Medium 199-supplemented whole rat serum, and increasing oxygen tension over the last half of the culture period.

Yolk sacs cultured in this manner exhibited the same cellular morphology as *in vivo* yolk sacs removed after 17.5 days of gestation, but did not show the same degree of haemopoietic development or basement membrane synthesis. However, the cultured yolk sac endodermal cells appeared to be proliferative at 17.5 days, whereas 17.5 day *in vivo* yolk sacs did not have the same nuclear appearance.

The culture system was used to study aspects of carbohydrate metabolism and xenobiotic metabolising capability, and was used to study the expression and control of two genes - alphafetoprotein and insulin-like growth factor II, in rat visceral yolk sac.

Study of hexokinase, pyruvate kinase and succinate dehydrogenase activity in cultured and *in vivo* yolk sacs from 9.5 days to 17.5 days of gestation, indicated a rise in the level of aerobic tricarboxylic acid cycle catabolism from 9.5 days to 13.5 days of gestation *in vivo* and in culture, as measured by an increase in succinate dehydrogenase activity. Concommitant with this rise in aerobic carbohydrate catabolism, there was a slight decrease in glycolytic activity as measured by hexokinase and pyruvate kinase activities *in vivo* and in culture. Activities of all three enzymes significantly decreased from 15.5 to 17.5 days *in vivo* ($p < 0.05$), but there was no observed decrease in carbohydrate catabolism in culture.

Expression of cytochrome P-450IA1 gene, measured by substrate-turnover enzyme assay and immunocytochemistry, was localised to the endodermal cells, and found to be inducible in *in vivo* yolk sacs from 15.5 to 18.5 days, but expressed as constitutive activity from 9.5 to 17.5 days in culture, postulated to be due to the absence of negative regulatory factors of either maternal or fetal derivation.

Using *in situ* hybridisation techniques to qualitatively and quantitatively determine AFP and IGF-II mRNA, expression of AFP mRNA was localised to endodermal cells, whilst IGF-II mRNA was expressed in both cell lineages.

Down-regulation of IGF-II mRNA was shown in neonatal liver by glucocorticoids, but these compounds did not surpress expression in the cultured yolk sac.

A thousand-fold decrease in detected AFP mRNA between 16.5 days and 21.5 days of gestation *in vivo* was not paralleled in culture, and preliminary data indicated that this was due to primary or secondary effects of a maternally-derived transcription factor.