The 'Giant' Yolk Sac - An *In Vitro* Model for Studying Early Placental Transport.

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for the Degree of Doctor of Philosophy.

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For Martin and Thomas.

Contents

Acknowledgements
Abstract

SECTION ONE - GENERAL INTRODUCTION Page
1.1 The placenta....1
1.2 Development of the fetal membranes in the rat....3
1.3 Endocytosis....6
1.4 The lysosomal system and endocytosis....8
1.5 Receptor mediated endocytosis....12
1.6 The pathway of receptor mediated endocytosis....13
1.7 Early embryonic nutrition....24
1.8 Localisation of hisiotrophic nutrition in the rat visceral yolk sac..26
1.9 Development of the embryo culture technique....32
1.10 Culture of the visceral yolk sac....38

SECTION TWO - GENERAL MATERIALS AND METHODS
2.1 Choice of animals....40
2.2 Mating proceedure....40
2.3 Serum preparation....40
2.4 Explantation of 9.5 day embryos....42
2.5 Culture of 'giant' yolk sacs....44
2.6 Harvesting of 'giant' yolk sacs....45
2.7 Collection of exocoelomic fluid and amniotic fluid....47
2.8 Culture of GYS after removal of embryonic tissue at 9.5 days....48
2.9 Explantation of 17.5 day in vivo yolk sacs....49

SECTION THREE - MORPHOLOGY OF THE 'GIANT' YOLK SAC
3.1 Introduction....50
3.2 Materials and Methods
1. Culture of 'giant' yolk sacs....52
2. Culture of GYS after removal of embryonic tissue....52
3. Explantation of in vivo yolk sacs....52
4. Quantification of yolk sac development....53
5. Preparation of tissue for transmission electron microscopy....53
6. Preparation of tissue for scanning electron microscopy....56
3.3 Results
1. External appearence of GYS during development....58
2. Quantification of yolk sac development....60
3. External appearence and ultrastructure of GYS....61
4. External appearence and ultrastructure of modified GYS....66
3.4 Discussion....68

SECTION FOUR - MORPHOMETRIC ANALYSIS OF YOLK SAC TISSUE
4.1 Introduction
1. Priciples of stereology....75
2. The importance of section thickness in stereology....75
3. Measurement of volume fraction....77
4.2 Materials and Methods
1. Explantation and culture of yolk sac tissue....78

2. Preparation of tissue for electron microscopy.....79
3. Orientation of tissue.....79
4. Microscopy.....80
5. Morphometric analysis.....80
6. The importance of sample size.....81
4.3 Results.....83
4.4 Discussion.....86

SECTION FIVE - THE UPTAKE OF ^{125}I-POLYVINYLPYRROLIDONE (PVP)
5.1 Introduction.....90
5.2 Materials and Methods
1. Rate of uptake of PVP by 17.5 day *in vivo* yolk sacs.....94
2. Rate of uptake of PVP by 17.5 day 'giant' yolk sacs.....96
3. Rate of uptake of PVP by GYS after removal of the embryo.....98
4. Calculating the rate of uptake of PVP as an Endocytic Index....99
5. Release of PVP from pre-loaded yolk sacs.....100
5.3 Experimentation and Results
1. Comparison of uptake in roller culture and shaking water bath.103
2. Comparison of uptake in different incubation conditions.....104
3. The physiological integrity of the 'giant' yolk sac.....107
4. Pre-incubation of *in vivo* yolk sacs in serum.....108
5. Pre-incubation of 'giant' yolk sacs in Medium 199.....110
6. Opening the 'giant' yolk sac.....112
7. Uptake by 'giant' yolk sacs at different gestational ages.....114
8. Uptake by 'giant' yolk sac after removal of the embryo.....115
9. Release of PVP by GYS and 17.5 day *in vivo* yolk sacs.....117
5.4 Discussion.....118

SECTION SIX - THE UPTAKE OF ^{125}I-BOVINE SERUM ALBUMIN (BSA)
6.1 Introduction.....126
6.2 Materials and Methods
1. Labelling of BSA with ^{125}iodine.....130
2. Efficiency of labelling.....130
3. Assessment of % TCA soluble radioactivity after dialysis.....131
4. Uptake of BSA by 17.5 day *in vivo* yolk sacs.....131
5. Uptake of BSA by 17.5 day 'giant' yolk sacs.....134
6. Uptake of BSA by GYS after removal of embryo at 9.5 days.....135
7. Calculating the rate of uptake of BSA as an Endocytic Index...136
Experimentation
6.3 Uptake of BSA by GYS and 17.5 day *in vivo* yolk sacs
1. Introduction.....140
2. Materials and Methods.....140
3. Results.....142
4. Discussion.....143
6.4 Release of BSA from GYS and 17.5 day *in vivo* yolk sac
1. Introduction.....147
2. Materials and Methods.....147
3. Results.....149
4. Discussion.....149
6.5 Inhibition of BSA uptake by EGTA
1. Introduction.....150
2. Materials and Methods.....150
3. Results.....151
4. Discussion.....152

Contents

SECTION SEVEN - THE UPTAKE OF ^{125}I-IMMUNOGLOBULIN G (IGG)
7.1 Introduction....154
7.2 Materials and Methods
1. Labelling of rat IgG....157
2. Efficiency of labelling....157
3. Assessment of % TCA soluble radioactivity after dialysis....157
4. Uptake of IgG by 17.5 day *in vivo* yolk sacs....158
5. Uptake of IgG by 'giant' yolk sacs....159
6. Uptake of IgG by GYS after removal of embryo at 9.5 days....160
7. Calculating the rate of uptake of IgG as an Endocytic Index...160
Experimentation
7.3 Uptake and processing of IgG by GYS and *in vivo* yolk sacs
1. Introduction....163
2. Materials and Methods....164
3. Results....165
7.4 Release of IgG and iodotyrosine after pre-loading yolk sacs
1. Introduction....168
2. Materials and Methods....168
3. Results....170
4. Discussion of uptake and release of IgG 170
7.5 Theoretical calculation of total IgG uptake....174
7.6 EM study of colloidal gold and colloidal gold-IgG uptake
1. Introduction....178
2. Materials and Methods....178
3. Results....180
4. Discussion....182

SECTION EIGHT - THE TRANSPORT OF AMINO ACIDS
8.1 Introduction....184
8.2 Materials and Methods
1. Culture of 'giant' yolk sacs....190
2. Preparation of incubation medium....190
3. Incubation of 'giant' yolk sacs....191
4. Liquid scintillation counting of samples....192
5. Correcting for quenching....193
6. Calculating rates of uptake and accumulation....195
8.3 Results....196
8.4 Discussion....198

SECTION NINE - THE GROWTH SUPPORTING PROPERTIES OF GYS EXOCOELOMIC FLUID
9.1 Introduction....200
9.2 Materials and Methods
1. Preparation of solutions....206
2. Preparation of culture media....207
3. Specific tests carried out on culture media....208
4. Explantation of 9.5 day embryos....208
5. Whole embryo culture....209
6. Harvesting embryos after 48 hours culture....209
7. Assessment of embryo growth and development....210
9.3 Results....213
9.4 Discussion....216

SECTION TEN - GENERAL CONCLUSIONS
10.1 General Conclusions.....220

SECTION ELEVEN - APPENDIX
A. Hank's Balanced Salt Solution (BSS).....228
B. Lowry technique for protein estimation.....229
C. Medium 199.....233
D. Radiolabelling of proteins.....235
E. Computer programme.....240
F. Eagle's Minimum Essential Medium:Vitamins.....241

SECTION TWELVE - REFERENCES
12.1 References.....242

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SECTION 1

GENERAL INTRODUCTION

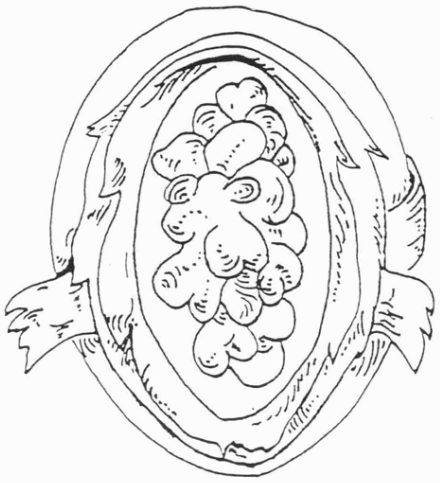
1.1 The Placenta.

The fertilised mammalian ovum is a large cell which contains food reserves within its cytoplasm. These food reserves support the preimplantation embryo for a variable length of time depending on species. However in all species, the ovum also depends on secretions of the oviduct and uterus for nutrition (Whittingham 1971).

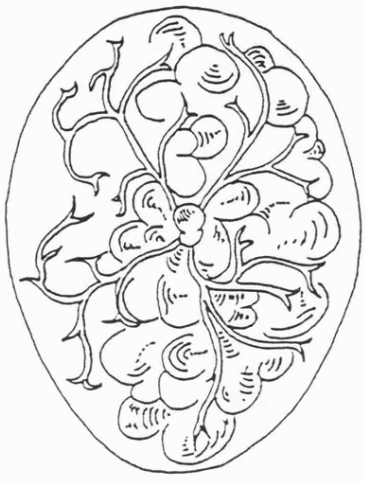
The ovum moves freely down the oviduct to the uterus but some contact between mother and embryo is essential for nutritional exchange throughout the rest of gestation. The organ responsible for nutritional exchange is the placenta. It was Mossman(1937) who defined the placenta as "any intimate apposition or fusion of the foetal organs to the maternal (or paternal) tissue for physiological exchange".

The placenta and fetal nutrition have long been of interest to man. In ancient Egypt the placenta was believed to contain the eternal soul and was preserved if possible throughout life. The ancient Greeks, in particular Aristotle(384-322 BC) taught the anatomy of the placenta and fetal membranes. Galen,in the second century AD also taught the anatomy of the pregnant uterus. Galen's theory of embryology,based on a series of four consequential stages (Figure 1.1) was accepted and not challenged until the Renaissance. The word placenta was first used by Realdus Columbus (1516-1559) to describe the human afterbirth as an "affusion of residual material in the form of a circular cake (placenta)", and it was not until the late seventeenth century that it acquired its present more general use, as quoted above (Mossman 1937).

Figure 1.1 Galen's four stages of embryonic life.

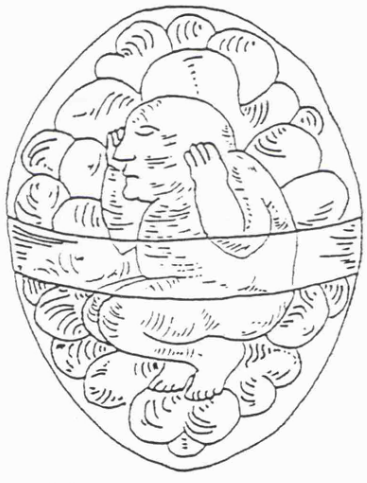


1."Seminal stage"



2."Tria principia"

3."Vascular map"



4."Embryonic stage"

The conventional placental mechanism involving the direct exchange of molecules between the maternal and embryonic blood, cannot be established until a large part of organogenesis has taken place and the embryo has a well developed beating heart and good circulation. Therefore all the nutritional requirements of the embryo during the complex process of organogenesis are met by specialised tissue or tissues. In the rat this is the the yolk sac placenta. Brunschwig (1927) first suggested that the yolk sac epithelium was a physiological

placenta. His conclusion was confirmed by Everett (1935) who described the yolk sac placenta of the rat as "an organ of exchange whose importance is not secondary to that of the allantoic placenta". New (1973) showed that the allantoic placenta, the major organ of nutrition during much of gestation, has no nutritive function during the early period of organogenesis,as rat embryos cultured *in vitro* to the 25 somite stage grew normally but did not develop an allantoic placenta. *In vivo* at this stage the allantoic placenta has started to develop.

1.2 Development of the fetal membranes in the rat.

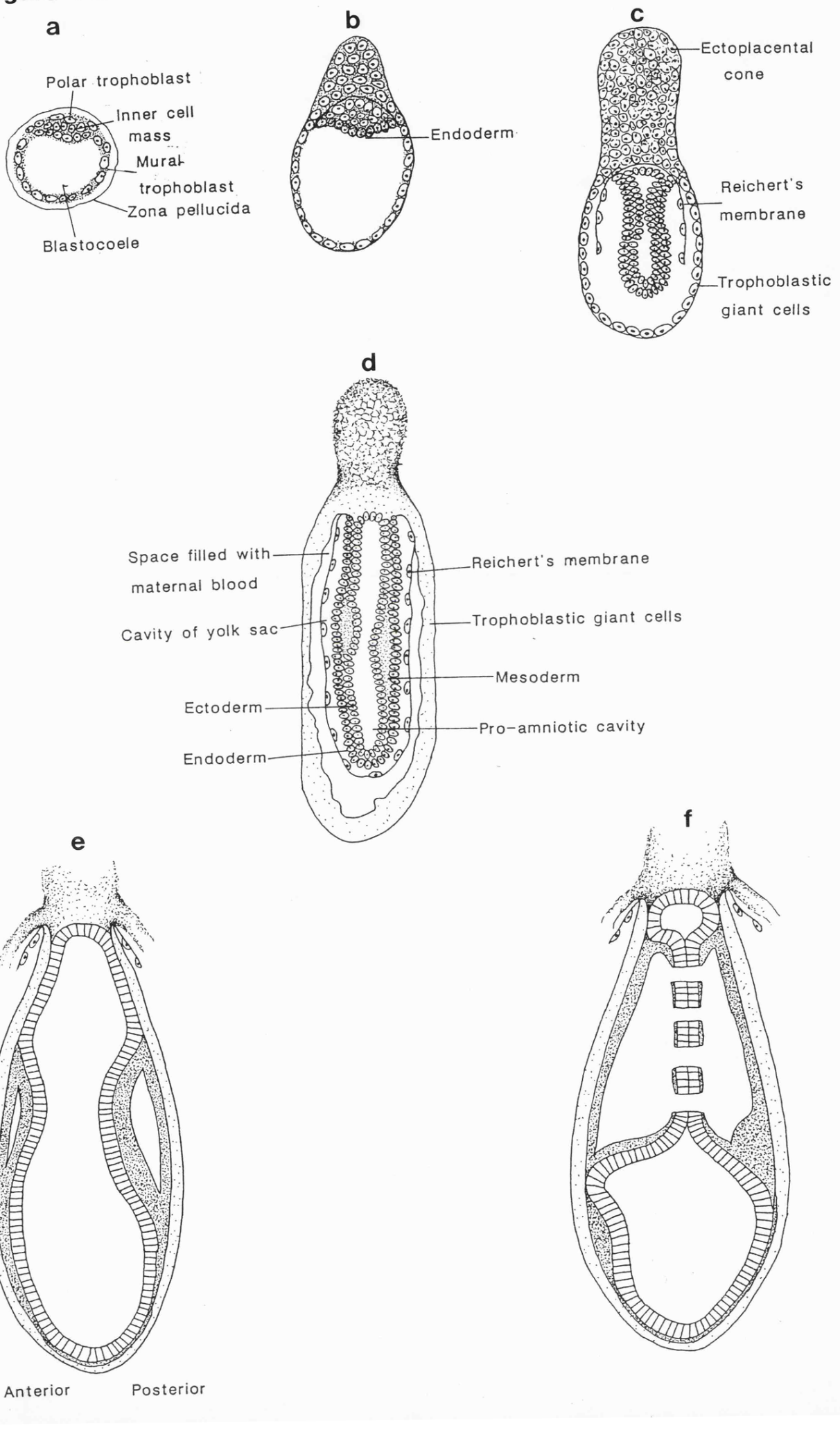
In order to discuss fully early embryonic nutrition in the rat, a brief description of the development of the fetal membranes is essential (see also Figure 1.2). The great diversity in mammalian fetal membrane development has been dicussed and illustrated at length by many authors (Mossman 1937, Amoroso 1952,)

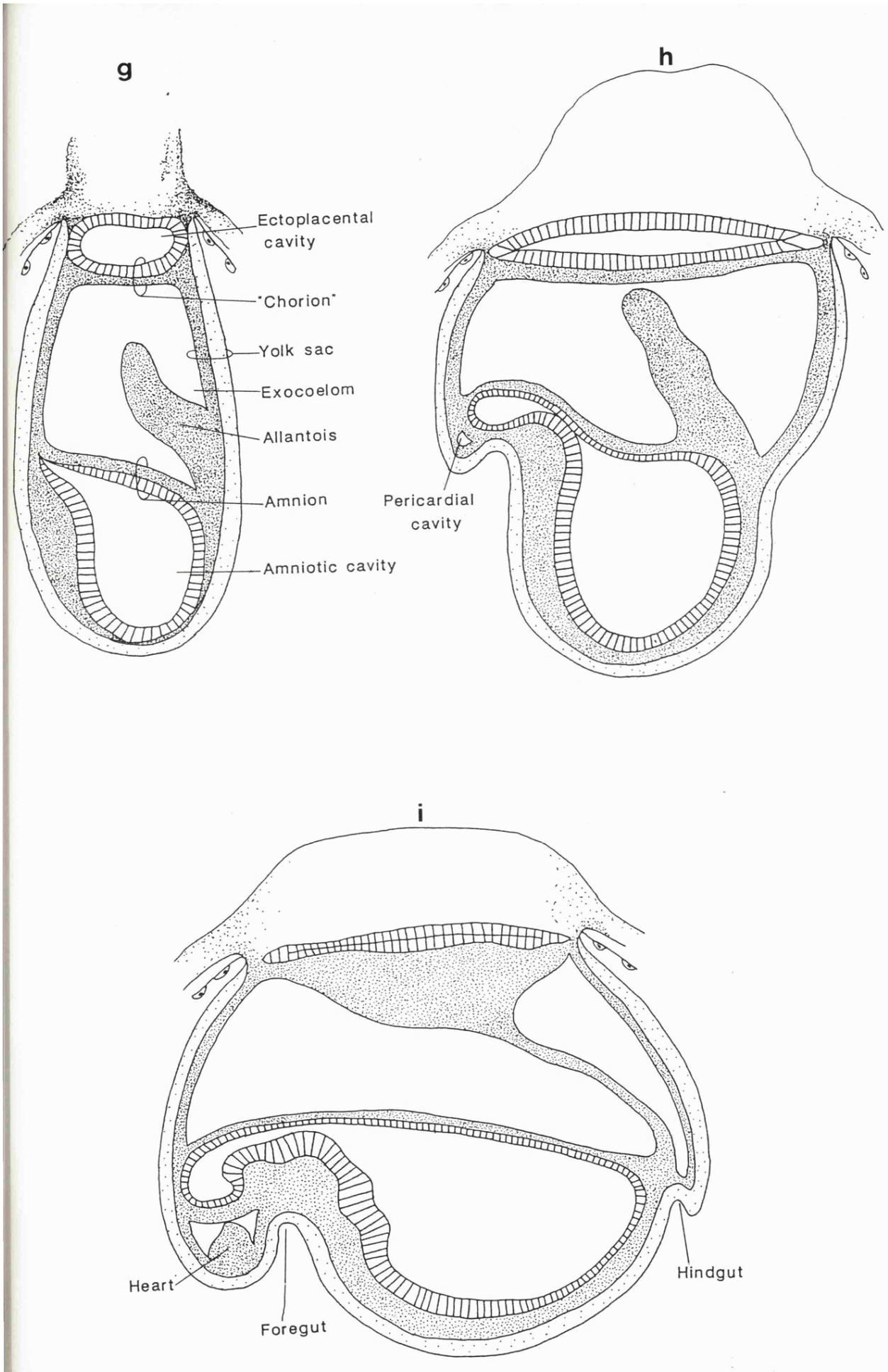
As the fertilised ovum passes down the oviduct all the cleavage divisions take place and as a result the embryo is at the late morula stage when it enters the uterus. A fluid filled cavity forms within the cell ball and gradually expands to form the blastocyst. This fluid is thought to have been secreted by the oviduct and the uterus and is transported into the blastocyst cavity. The blastocyst has an outer trophoblast cell layer which constitutes approximately two thirds of the total cells and eventually establishes contact with the maternal tissue, an inner cell mass which gives rise to the embryo, amnion and allantois, and a central cavity or blastocoele. The preimplantation blastocyst

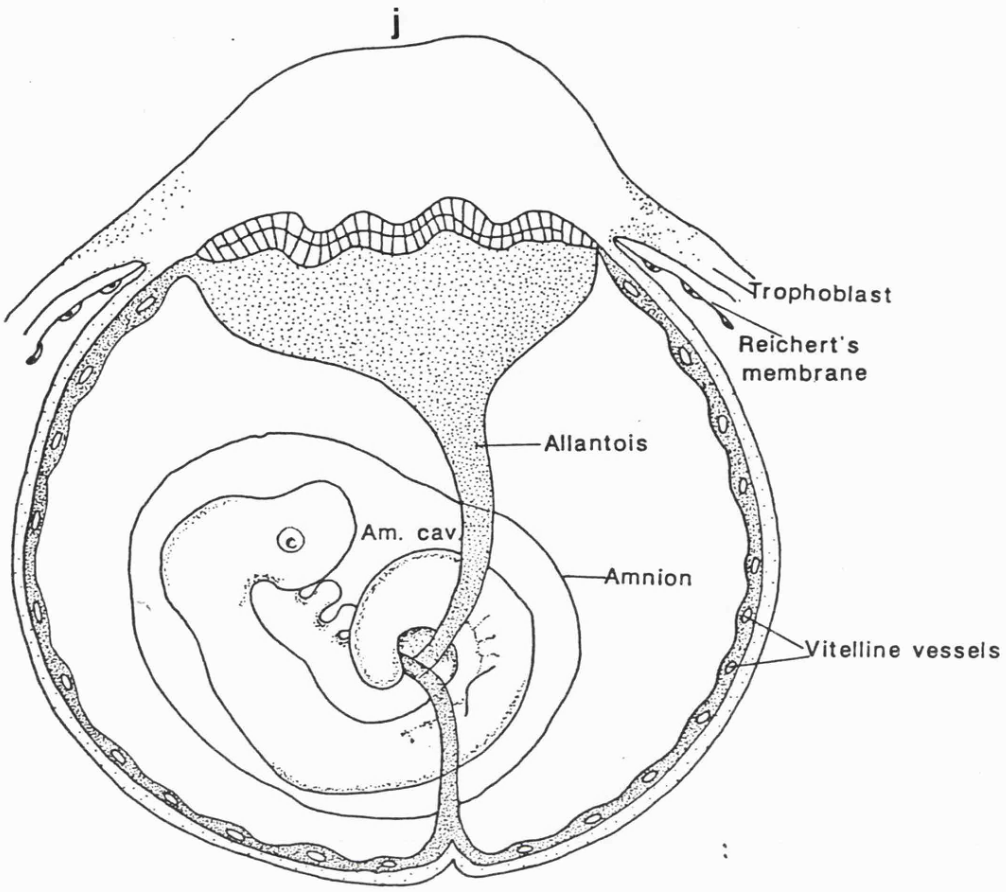
(Figure 1.2 (a)) is surrounded by a non-cellular translucent membrane, the zona pellucida, which appears to prevent implantation in the oviduct. Throughout the preimplantation period the ovum obtains some nutrition from the sparse metabolic reserves within its cytoplasm but mainly from the tubal and uterine fluids, the composition of which varies with the location within the genital tract and the hormonal condition of the mother (Hamner 1970). The loss of this membrane and the hormonal condition of the mother allows the blastocyst to attach eccentrically in a groove on the anti-mesometrial aspect of the uterine wall, on day 4-5. The trophoblast penetrates the uterine epithelium and erodes maternal tissue, glands and blood vessels (Figure 1.2 (b)).

Polar trophoblast cells continue to divide and produce the ectoplacental cone; these cells engulf and digest maternal erythrocytes (Morriss 1973). Mural trophoblast cells do not divide but their DNA content rises as they are transformed into primary giant cells (Figure 1.2 (c)). At the same time the inner cell mass is differentiating and an endodermal layer forms which is pushed into the cavity as the inner cell mass increases in size. The endoderm layer therefore covers the central ectoderm cells in which the primary embryonic cavity forms. Rodents and Insectivores have an additional membrane not found in other species, which was first described during the 19th century by Reichert, in the guinea pig, and which bears his name. In the rat, Reichert's membrane appears on day 6, secreted along the inner wall of the trophoblast (Tachi et al. 1970) and lies between the trophoblast giant cells and the parietal endoderm cells, both of which may contribute to its synthesis. It has been shown to be composed of almost parallel layers each resembling a basement membrane (Inoue et al 1983). Each

Figure 1·2


a
Polar trophoblast
Inner cell mass
Mural trophoblast
Zona pellucida
Blastocoele
b
Endoderm
c
Ectoplacental cone
Reichert's membrane
Trophoblastic giant cells
d
Space filled with maternal blood
Cavity of yolk sac
Ectoderm
Endoderm
Reichert's membrane
Trophoblastic giant cells
Mesoderm
Pro-amniotic cavity
e
Anterior
Posterior
f


g
Ectoplacental cavity
"Chorion"
Yolk sac
Exocoelom
Allantois
Amnion
Amniotic cavity
h
Pericardial cavity
i
Heart
Foregut
Hindgut



Legend for Figure 1.2. (Adapted from Steven and Morriss 1975)

(a) Blastocyst before implantation; Day 4.

(b) Implanting blastocyst, showing the proliferation of polar trophoblast cells and differentiation of the inner cell mass; Day 5.

(c) Inversion of germ layers. Origin of Reichert's membrane; Day 6.

(d) Early appearance of mesoderm; Day 6.

(e)-(g) Formation of the amnion, chorion and yolk sac; Days 8-9.

(h)-(i) Fusion of the allantois with "chorion" and obliteration of the ectoplacental cavity, thus forming the allantoic placenta. In the embryo "reversal" brings about the formation of the fore and hind gut. Blood islands (not shown) are beginning to develop in the yolk sac mesoderm; Day 10.

(j) Further development of the allantoic placenta. The embryo now turned so that it is dorsally convex; Day 11.

layer appears to be composed of cords (3-8 nm) arranged in a 3 dimensional network, between which are other tubular structures (basotubules) and a mesh of irregular felt like material. The Reichert's membrane persists until day 16 in the rat, when it ruptures and retracts to the margins of the chorioallantoic placenta. In early gestation Reichert's membrane is the only structure separating the maternal blood, which circulates into the maternal sinuses between trophoblast cells, from the yolk sac and embryonic endoderm (Figure 1.2 (d)). The membrane is particularly important as the inversion of the germ layers means that the chorion does not surround the embryo as in other species.

On day 8, the primitive streak forms and mesodermal cells migrate from it and lie between the endoderm and ectoderm. As the number of mesoderm cells increases a cavity appears, at what will become the posterior end of the embryo and spreads around the cylinder to form a complete annulus (Figure 1.2 (e)). The cavity increases in size pushing together the layers of mesoderm and ectoderm, which fuse and break down (Figure 1.2 (f)). This results in the formation of two horizontal membranes at 9.5 days - the amnion immediately above the embryo and the chorion near the ectoplacental cone (Figure 1.2 (g)). At day 10 the allantois develops at the posterior end of the embryo, grows across the exocoelom and fuses with the chorionic ectoderm (Figure 1.2 (h)-(i)). Blood vessels form within the allantois and subsequently invade the chorionic mesoderm so that only a thin layer of chorion separates the vessels from the maternal blood. Therefore on day 11-11.5 a haemochorial chorioallantoic placenta is formed and haemotrophic nutrition can begin (New 1978) (Figure 1.2 (j)). As haemotrophic nutrition becomes established there is a decrease in the relative importance of the

visceral yolk sac for supplying the nutritional requirements of the embryo (Steven 1975). However the yolk sac has other functions including the transport of passive immunity, protein synthesis and liver like functions, which remain important.

The visceral layer of the yolk sac which carries out nutritional exchange during early gastrulation, is composed of an outer layer of extra-embryonic endoderm and an inner layer of mesoderm. The endoderm actively takes up nutrients which have passed into the yolk cavity which surrounds the embryo, through Reichert's membrane. On day 11-12, blood islands form in the visceral yolk sac mesoderm and coalesce to form the vitelline circulation. A full description of the structure and functions of the yolk sac placenta are given in section 3.

1.3 Endocytosis

Endocytosis is a general term used to describe the uptake of extracellular materials within membrane limited vesicles,and it can be subdivided into two main categories - phagocytosis and pinocytosis. In this description I will concentrate mainly on the latter.

Phagocytosis

In the second half of the nineteenth century Metchnikoff (1893) observed the ingestion of particles by protozoan and metazoan cells. The phenomenon called phagocytosis has since been observed in many cell types and is thought to have both nutritive and defensive roles. It

involves the capture of particles larger than 1um in diameter. In this class of endocytosis there is a close adherence of the plasma membrane to the surface of the particle so that fluid is almost totally excluded (Stossel 1977). This close adherence is thought to be necessary to trigger the mechanism of phagocytosis.

Pinocytosis

Lewis(1931) observed a second type of plasma membrane activity in cultured rat macrophages. This activity occured in the absence of particles and resulted in the capture of droplets of extracellular fluid - this he called pinocytosis ("cell drinking"). Lewis also saw the same process in rat fibroblasts, sarcoma and other tumour cells. Using light microscopy many other workers have visualised this capture of liquid droplets which results in the formation of large fluid filled vacuoles and this has been termed macropinocytosis. This distinguishes it from pinocytosis observed in other cells, particularly those with a brush border, which results in the formation of submicroscopic vesicles - micropinocytosis. These small, membrane lined vesicles can be visualised immediately below the plasma membrane, with an electron microscope. The size of vesicles associated with these two subclasses of pinocytosis differs (Allison and Davies 1974) Micropinocytic vesicles being 70-100 nm in diameter and macropinocytic vesicles being 0.3-2.0 um in diameter. More recently, however it has been proposed that the larger vesicles may be derived from fusion of the much smaller ones. In the early 1950's when the first investigations into micropinocytosis were being carried out with the electron microscope (Palade 1953;Bennet 1956) the phenomenon was found to be prevalent in most mammalian cells.

Pinocytosis can be subdivided by considering the route of entry of the substance into the cell. Entry can be within a fluid droplet (fluid phase pinocytosis) or bound to the vesicle membrane (adsorptive pinocytosis) (Jacques, 1969). Also adsorptive pinocytosis can be further subdivided into the endocytosis of materials which bind in a non-specific manner to the cell surface (eg cationic ferritin binding to anionic sites on the plasma membrane) and the endocytosis of ligands mediated via binding to specific receptors on the cell membrane. The latter has been called specific receptor mediated pinocytosis.

1.4 The lysosomal system and endocytosis

Edelson and Cohn (1978) described endocytosis as "a sequence of distortions and reorganisations of the plasma membrane". The first step involves the invagination of an area of the plasma membrane to form a pit into the cytoplasm of the cell. The rim of this pit seals by the formation of a continuous plasma membrane and the closed vesicle so formed, which contains substances obtained from the extracellular environment, "pinches off" from the plasma membrane and becomes free within the cytoplasm. These small pinocytic vesicles move towards the centre of the cell. En route they can fuse with each other to form heterophagosomes and then form an intermediate compartment which has been given many names including the endosome (see section 1.6 p.19).

By definition lysosomes are membrane limited cytoplasmic organelles containing acid hydrolases (Cohn et al. 1966). When first

generated they are called primary lysosomes. There is some uncertainty about the origin of primary lysosomes. The acid hydrolases are manufactured by the rough endoplasmic reticulum (RER) and are then passed to the Golgi apparatus where they are concentrated and packaged. The Golgi apparatus-endoplasmic reticulum-lysosome (GERL) is an area of reticulum rich in acid phosphatase along the trans aspect of the Golgi complex. This may be a separate organelle specialised for the packaging or even the synthesis of some lysosomal enzymes.

Primary lysosomes fuse with either incoming endocytic vesicles or autophagosomes (vacuoles containing damaged cell organelles - described in more detail below) to form secondary lysosomes. Secondary lysosomes are therefore hybrid organelles with components from both the internal membrane system and the plasma membrane. The fusion of endocytic vacuoles with the lysosome results in the exposure of the endocytosed material to the lysosomal enzymes and results in the hydrolysis of any degradable macromolecules. After enzyme digestion is complete the small soluble products (of molecular weight approx. 300) can diffuse through the lysosomal membrane into the surrounding cytoplasm. Large non degraded material often pigmented and lipid-rich remains in the secondary lysosome still bounded by its membrane. This organelle is called a residual body or telolysosome and they accumulate within cells with age.

Autophagocytosis is the process by which worn out or damaged cytoplasmic organelles such as mitochondia, unwanted secretary granules or simply small areas of cytoplasm are isolated, enclosed by a membranous vesicle and digested. After fusion with a primary lysosome,

the secondary lysosomes of this kind are called autolysosomes, cytolysosomes or autophagic vacuoles. The method by which cytoplasmic organelles become isolated remains to some extent an hypothesis. As autophagosomes are often in close proximity to the Golgi apparatus it has been suggested that membrane of Golgi origin is responsible for the sequestration of cytoplasm. However the more favoured theory of Novikoff and Shin (1964) is that sequestration is by pre-existing cytomembrane (ER). They reported in liver cells the formation of autophagosomes by the wrapping of flattened cisternae of the ER around portions of cytoplasm. Indeed many autophagosomes are bounded by double membranes, very similar in ultra-structure to adjacent smooth or rough ER.

Historically, the concept of the lysosome as a distinct organelle began to appear (See review of De duve 1969). A series of experiments concluded that in liver homogenate, the hydrolytic enzymes are present in membrane limited organelles, associated with the mitochondrial fraction after differential centrifugation. De Duve proposed that the hydrolytic enzymes were enclosed to prevent hydrolysis of the cell. Gomori(1952) and Holt(1954) demonstrated histologically the presence of acid phosphatase in discrete droplets in the proximal tubule of kidney. Straus(1954) had separated granules from kidney which had a high concentration of acid phosphatase. These were soon found in many cells (see review Novikoff 1961). In 1956 Novikoff and colleagues identified lysosomes at the electron microscope level.

An elegant series of histochemical experiments (Straus 1964,1967) provided the vital evidence for fusion of the lysosome with the heterophagosome and proved the theory of intracellular digestion of

endocytosed material. In his experiments the vacuolar contents were stained blue for the marker protein horseradish peroxidase and red for the lysosomal marker enzyme acid phosphatase. Secondary lysosomes(heterophagosomes) which are the site of hydrolytic digestion appeared purple. Similar experiments were also carried out on the rat visceral yolk sac by Beck et al.(1967).

Having considered the general mechanisms of endocytosis, the highly efficient process of specific receptor mediated endocytosis will be discussed in greater detail - section 1.5.

1.5 Receptor Mediated Endocytosis.

Recently much work on endocytosis has been carried out by studying the highly efficient and specific process of receptor mediated pinocytosis, in which certain ligands are selectively internalised by cells after complexing to plasma membrane receptors. These ligands can be of nutritional value after lysosomal digestion, or are transported back to the plasma membrane or targeted to certain membrane domains (eg transferrin and IgG).

The uptake of many physiologically important macromolecules by receptor mediated endocytosis, has been studied in many systems. I shall therefore not attempt to give a detailed description of each, but outline generally the main pathway involved in receptor mediated endocytosis and discuss a few examples. Particular emphasis will be placed on the two new 'organelles' now believed to play an important role in receptor mediated endocytosis. Firstly the coated vesicle, observed initially in erythroblasts (Bessis and Breton Gorius 1957), and now known to be important in the selective movement of receptors and the uptake of ligands. Secondly a pre-lysosomal compartment thought to be responsible for the sorting of receptors and ligands, which has been described in many cell types and given a variety of names including receptosome (Willingham and Pastan 1980), endosome (Helenius et al. 1983) and CURL (Geuze et al. 1983).

1.6 The pathway of receptor mediated endocytosis.

Receptors.

The concept of the receptor was introduced approximately 100 years ago by Langley (1878). He worked on the response of muscle cells to nicotine and postulated the idea of a 'receptive substance' at the site of nicotine application. This idea was later applied more generally by Ehrlich and a receptor was thought of as a recognition site for a specific region of a drug or ligand. The definition of a receptor has changed little since but can now be described in terms of structural, kinetic and physiological parameters and therefore interactions can be quantified.

Anderson et al.(1977) used ^{125}I-labelled and ferritin-labelled low density lipoprotein (LDL) as visual probes to study the surface distribution of LDL receptors and the subsequent endocytosis of LDL in cultured human fibroblasts. Receptors bind LDL with great affinity and carry LDL into the cell where the protein and cholesterol components are separated within lysosomes.Experiments using LDL-ferritin showed that even at 4^{o}C (when lateral movement of receptors would be restricted, (Frye and Edidin 1970) approximately 70% of the LDL receptors were concentrated at coated regions of the plasma membrane which occupied less than 2% of the cell surface. In fact, LDL-ferritin binding sites are located in the coated regions even when cells are fixed in formaldehyde prior to incubation with LDL (Anderson et al. 1976). It seems in this system that receptors are already concentrated at coated regions and that ligand induced 'patching' or 'capping' (de Petris and

Raff 1972) is not occurring. However in other systems, eg in mouse 3T3 cells, fluorescent labelled Epidermal Growth Factor (EGF) has been shown to be distributed randomly at $4^{o}C$ and only cluster in the coated regions after warming to $37^{o}C$ in the presence of EGF. Similar results have been obtained using insulin and α-2 macroglobulin with 3T3 cells.

Like other transmembrane proteins receptors are thought to be synthesised in the Golgi apparatus and then randomly inserted into the plasma membrane. The receptors then migrate laterally in the plane of the membrane to coated pits. Using various mutations causing an effect on the functioning of the LDL receptor Goldstein et al. (1979) described the existence of two active sites on the receptor. The first, a binding site on the external surface, and the second, an internalisation site, postulated to be on the cytoplasmic surface. Once at a coated pit, there is an interaction between the internalisation site on the receptor and the clathrin coat (see section on coated vesicles p15), which fixes the receptor in the coated pit. There is some debate as to whether the receptors reach coated pits by diffusion or if they are actively propelled (Bretscher 1976).

Studies on the biochemical properties of the plasma membrane have been carried out to try to elucidate how endocytic vesicles are formed and techniques used involve selectively labelling the plasma membrane using a variety of markers including lectins,receptor bound ligands, antisera, ^{125}I-lactoperoxidase and radioactive amino acids.

The fate of receptors and the processes by which they may be recycled is discussed on page 21.

Coated vesicle.

Coated vesicles were first observed in erythroblasts (Bessis and Breton Gorius 1957) and later observed in many different cells and tissue from both animals and plants. In 1964 Roth and Porter first proposed that coated vesicles were important in the specific uptake of extracellular protein based on their work on the uptake of yolk protein in mosquito oocytes.

In 1969 Kanaseki and Kadota isolated a partially purified preparation of coated vesicles from guinea pig brain and demonstrated that the coat previously described as 'fuzzy' was constructed of a regular lattice of pentagons and hexagons arranged in a basket. Pearse isolated coated vesicles from pig brain by differential centrifugation and demonstrated that the coat contained a major 180,000 KD polypeptide called clathrin. In 1981 two groups (Ungewickell and Branton, Kirchhausen and Harrison) independently demonstrated that the subunit comprised of clathrin, consisted of a trimeric structure containing three 180,000 KD chains and three light chains of molecular weight 33,000 KD and 36,000 KD (in the ratio 2:1). They demonstrated that each light chain was bound to a heavy chain. Ungewickell and Branton also demonstrated that the clathrin subunit when dried had a very distinctive 'triskelion' structure. Since this initial work many complex models have been proposed for coated vesicle structure, based on the molecular weight of empty 'baskets' and more recently, using negatively stained preparations, Crowther and Pearse (1981) have proposed a detailed model of the 'cage' structure. In their early study Kanaseki and Kadota (1969) first proposed that the coated vesicle was formed by the hexagon lattice

distorting to form pentagons, which induced a curvature of both the lattice and the under lying membrane. This eventually led to the formation of a closed lattice enclosing membrane to form a closed coated vesicle. It appears that energy is required for some part of this process, as the formation of coated vesicles is greatly decreased at $0^{o}C$ and in the presence of metabolic inhibitors.

Coated vesicles appear to be relatively short lived organelles and very soon after internalisation they undergo fusion with each other and loose their clathrin coat. Recent work has shown the uncoating probably involves an enzyme and an energy source. Patzer et al. (1982) have shown both ATP and cytosol extract are necessary to uncoat the vesicles in vitro. A polypeptide of 70,000 KD has been found with ATP dependent uncoating activity in a preparation of purified bovine brain cytosol. This enzyme has been called 'Uncoating ATPase' (Schlossman et al . 1984). It binds to the intact clathrin coat, hydrolyses ATP and the triskelions are released complexed to the enzyme. In this complexed form the triskelions are unable to reform into cages. The mechanism by which the complexes are ultimately broken down for coat reassembly, are unknown.

Some authors have postulated that different categories of coated vesicles exist which carry either selected bound macromolecules for transport across the cell or non-selected macromolecules for digestion in the lysosomes ('Wild' Theory). Moxon et al. (1976) showed that in the rabbit yolk sac there exist different classes of endocytic vacuoles in IgG transport and fluid phase transport. Also Haigler et al. (1979) localised ferritin conjugated to EGF and HRP in different endocytic

vacuoles in A-431 carcinoma cells.

However Abrahamson and Rodewald (1981) showed it possible for different receptor bound proteins, possibly with different destinations, and also some non-bound fluid phase proteins to be present in one coated vesicle. They carried out this work in neonatal rat intestine and injected the proximal intestinal epithelium with IgG conjugated to ferritin (IgG-ft) and unconjugated horseradish peroxidase (HRP). Using electrom microscopy they studied the distribution of markers and found:-

(i) Virtually all the apical endocytic vesicles contained both markers.

(ii) Only IgG-ft was bound to the apical plasma membrane.

(iii) HRP did not bind and was not transferred but remained in the apical lysosomes, identified by a positive acid phosphatase reaction.

(iv) HRP used to measure fluid phase pinocytosis, was taken up at the same rate in the presence and absence of IgG, indicating that endocytosis is constitutive and not stimulated by IgG binding.

In summary the principal selection for IgG transfer in this system, is its binding to the surface receptors and its continued binding throughout transport, thus avoiding lysosomal digestion. There must therefore be a site in the cell where 'sorting' takes place.

The apical cytoplasm of the neonatal rat intestine , and that of other absorptive cells, is very complex containing endocytic vacuoles, small vesicles, multivesicular bodies and lysosomes. Abrahamson and Rodewald (1981) postulated that the endocytic vacuoles may fuse with each other and the small apical vacuoles, which have been shown to lack lysosomal enzymes. The structures thus formed may be similar to the

receptosomes described by Willingham and Pastan (1980) in fibroblasts, and the endosomes observed by Helenius et al.(1983) as well as the original heterophagosome defined by de Duve (see review 1969).

The Endosome.

More recently endosomes, which have been generally defined as vesicles formed during endocytosis, have been classified by various workers according to their morphology eg. endosomes I, II and III (Helenius et al. 1983) or according to their position in the cell eg peripheral or juxtanuclear endosomes (Hopkins 1983, 1985). Geuze et al. (1983) used the term CURL (compartment of uncoupling of receptor and ligand) to describe endocytic vesicles with curled 'arm-like' extentions which are probably the same as the endosomes II and III of Helenius et al. (1983).

Due to their complex structure, endosomes have been found difficult to isolate. However if the structures are specifically labelled with a radioactive ligand, they can be more sucessfully identified after fractionation. From such isolation studies, work has been carried out to analyse endosomal membrane and it has been shown to be similar to plasma membrane, with a high cholesterol/phospholipid ratio. However more detailed analysis using gel electrophoresis revealed a protein pattern different to both plasma membrane and lysosomal membrane (Dickson et al. 1983).

Endosome function.

Having discussed the basic terms involved, endosome function can best be described by following the path of a ligand through the complex endosome structure.

The ligands are bound to plasma membrane receptors and internalised in coated vesicles. These almost immediately lose their coats, and fuse with each other and peripheral endosomes (type I). There then follows an intraluminal acidification which causes some receptor-ligand complexes to dissociate (Harford et al. 1983; Wileman et al. 1985). The acidification may be brought about by the action of an ATP driven proton pump (Galloway et al. 1983). During this period the endosomes become gradually larger and some develop 'arm-like' extentions. These structures are often referred to as CURL. It has been suggested that the 'arms' are rich in receptors, whereas the lumen contains free ligands. This may imply that they are important in 'sorting'.

Ligands (eg. IgG) remaining bound to their receptors at an acid pH, would be expected to also concentrate in the 'arms', in a similar manner to receptors, and either recycle to the apical surface or in polarised cells be transported to the basolateral surface. The mechanism by which receptors concentrate in the 'arm' processes is not fully understood but it may be similar to the clustering of receptors in the coated region of the plasma membrane described on page 13. However the clustering of receptors in coated pits relies on an interaction between the receptor and the 'coat' protein clathrin. There is no evidence of

clathrin in CURL.

Two models have been proposed by Helenius et al. (1983) for the method by which endosomes function to recycle receptors and direct ligands to the lysosomes.

(i) Endosomes could be considered a stable organelle. Vesicles derived from coated vesicles would fuse with the endosome and deliver receptor-ligand complexes and any free ligands. After acidification of the lumen and any dissociation of ligand-receptor complexes, the receptors now clustered in the tubular 'arm' processes would be transferred to the surface in small vesicles. Other small vesicles would transfer receptor-ligand complexes to their destination and any free ligands to the lysosomes.

(ii) In the second model the endosome and its contents move together through the cell. Endosomes form through the fusion of incoming endocytic vacuoles derived from coated vesicles and they develop in size and structural formation. Receptors are again located mainly in the tubular 'arm' processes and are transferred to their destination in small vesicles. The main body of the endosome however, itself fuses with the lysosomal system and its contents are degraded.

The use of small vesicles for the transport of receptors and receptor-ligand complexes to their destination seems efficient. However a similar use of small vesicles to transfer the relatively large quantity of luminal contents to the lysosomes seems unlikely, in view of the large surface-volume ratio of such structures. The second model therefore seems more favourable and fits in with the available data.

Receptor recycling.

It is clear that the process of endocytosis involves the internalisation of large amounts of plasma membrane, which must be recycled in order to maintain the efficiency of endocytosis. Receptor fate varies and can be divided into four groups:-

(i) Receptor recycled; Ligand directed to lysosomes (eg LDL (Goldstein et al. 1979), mannose-6-phosphate (Fischer et al. 1980)).
(ii) Receptor recycled; Ligand not targeted to lysosomes but remains attached to the receptor until its destination is reached (eg IgG (Abrahamson and Rodewald 1981)).
(iii) Receptor not recycled; Ligand targeted to lysosomes (eg EGF (Carpenter and Cohen 1976), insulin (Kasuja et al. 1981)).
(iv) Receptor not recycled; Ligand not transported to lysosomes (eg IgA (Mostov and Blobel 1982)).

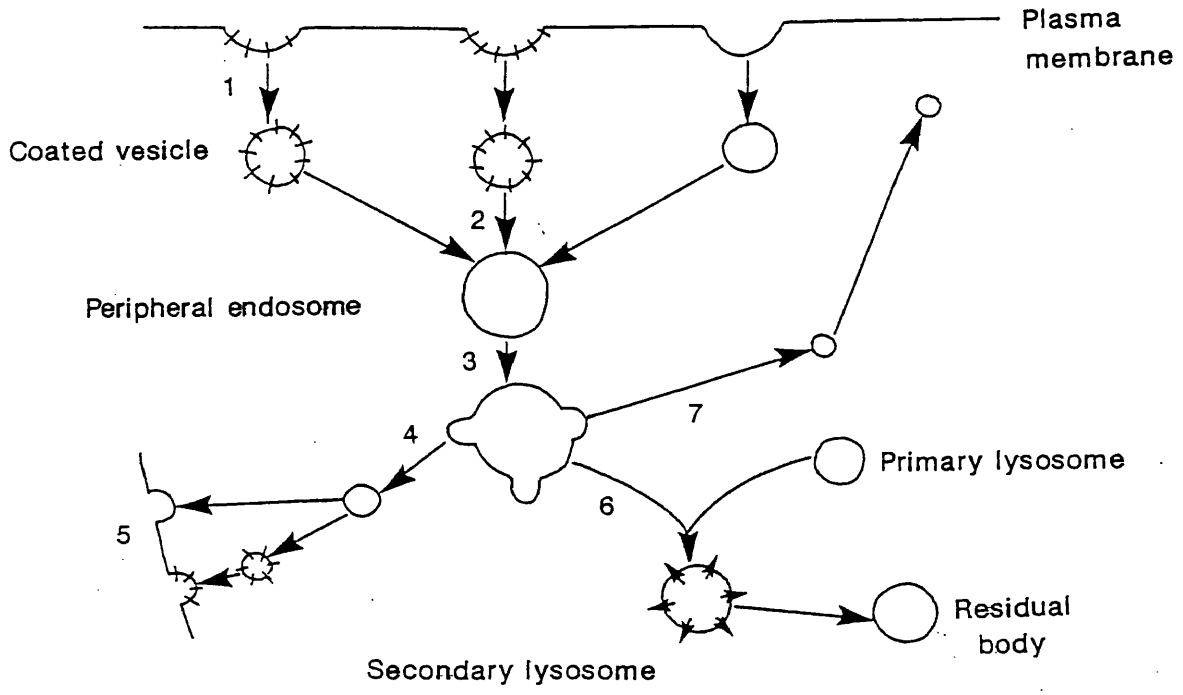
Therefore some receptors are degraded and others are recycled to the apical cell surface. It has been shown that the uptake of some ligands is linear with time and continues even in the presence of an inhibitor of protein synthesis. As the number of ligands internalised far exceeds the binding capacity of surface receptors and any initial receptor pool, in these cases, there must be some form of recycling. (eg Anderson et al (1977) using LDL).

Under normal circumstances receptors will also be synthesised and inserted into the plasma membrane as described on p 14.

Some receptors however do not recycle, but are degraded in the lysosomes. The exact process by which this occurs is uncertain but it may be that receptors are extruded from the endosome membrane into the membrane of an inclusion vesicle, free in the endosome lumen. Hence the receptors are degraded in the lysosome with the other luminal contents (Harding et al. 1985). EGF receptors in fibroblasts have been shown to be degraded because after the initial phase of receptor mediated endocytosis there is a depletion of approximately 80% of surface receptors and a new lower steady state of endocytosis.

It is perhaps easiest to summarise diagramatically (Figure 1.3) this overall view of the likely general mechanism for receptor mediated endocytosis, from the evidence reviewed and discussed here.

Figure 1.3 Pathway of Receptor Mediated Endocytosis.



Legend for Figure 1.3

1. Membrane invagination to form coated and uncoated vesicles.
2. Loss of coat and fusion of endocytic vesicles to form peripheral endosome.
3. Development of endosome into complex CURL. Acidification and sorting of contents.
4. Transport of functional proteins to basolateral surface.
5. Exocytosis in coated and uncoated vesicles.
6. Fusion of CURL with primary lysosomes to form secondary lysosomes. Digestion of contents.
7. Recycling of receptors to apical surface.

1.7 Early embryonic nutrition

Meyer (1925) used the term embryotroph to describe all the nutritional material available to the embryo throughout gestation the sources of which vary. Early embryonic nutrition can be subdivided into two distinct types - histiotrophic and haemotrophic nutrition.

Histiotrophic nutrition

Immediately after implantation until the formation of the chorioallantoic placenta, the embryo relies on so called histiotroph for nutrition. Histiotrophic nutrition can be defined as the intracellular breakdown of maternally supplied macromolecules by the fetal membranes. Histiotroph is generally made up of three types of material, although the relative importance of each varies in different species (Amoroso 1952).

(i) Material derived from the digestion of endometrial cells,

(ii) Macromolecules secreted by the endometrial glands,

(iii) Macromolecules taken up directly from the maternal blood/serum.

The description of early embryonic histiotrophic nutrition will concentrate on its utilisation by the inverted visceral yolk sac found in the rat. The majority of work described in the literature has been carried out using the rat because the yolk sac is easily dissected free from the other fetal membranes. Also in Rodents as well as Lagomorphs the yolk sac has reached a high level of functional development, being the main tissue in these species capable of the histiotrophic breakdown

of macromolecules. Also as the yolk sac remains functionally active until term it can be studied over a prolonged period.

However histiotrophic nutrition is carried out by all mammalian embryos, the tissue involved varying in different species. For example, in Carnivores the chorionic lining of the haemophagous organ has been shown to be capable of endocytosis and to contain some hydrolytic enzymes (Creed and Biggers 1964). In the Rhesus monkey the syncytiotrophoblast has been shown to take up colloidal carbon particles during early gestation (Wislocki and Bennett 1943). In man too the syncytiotrophoblast is strongly acid phosphatase positive and electron microscopic examination of the tissue has shown pinocytic activity.

In rodents the histiotroph is taken up by pinocytosis into the endoderm cells of the visceral yolk sac and is broken down in the vacuolar system by the action of hydrolytic enzymes (Beck et al. 1967). The soluble products are then passed to the embryo via the yolk sac blood circulation and by diffusion before the circulation develops. However not all proteins are digested ; it was shown that antibodies are selectively transported across the yolk sac in the rabbit and in the rat (Brambell and Halliday 1956).

Haemotrophic nutrition

In contrast to histiotrophic nutrition, haemotrophic nutrition involves the exchange of solutes across the placental barrier between the maternal and fetal circulations, which are in close association.

Haemotrophic nutrition begins when the embryo has a well developed beating heart and when the chorioallantoic placenta has been well perfused by the fetal and maternal circulation. The passage of solutes between the two circulations occurs by a number of methods:-

(i). Most solutes cross the placental barrier by simple diffusion along a chemical or electro-chemical gradient eg respiratory gases, urea.

(ii). Some substances, such as glucose cross by "facilitated" diffusion, again along a diffusion gradient but at a much greater rate than would be expected for the size, charge and solubility of the molecule.

(iii). Some metabolites cross the placental barrier by active transport involving a membrane carrier and the expenditure of energy.

(iv). A final group of materials are "dragged" across the placental barrier by the bulk flow of other solutes.

Grosser (1927) suggested that the efficiency of the placental barrier could be directly equated to the thickness of the placental barrier (ie the number of cell layers between the maternal and fetal circulation). This has since been disproved but can still be used as a method of classifying placental types (Beck and Lloyd 1977).

1.8 Localisation of histiotrophic nutrition in the rat visceral yolk sac

Much work has been carried out on the rodent visceral yolk sac since its importance as the sole organ involved in early embryonic nutrition, was first proposed (Brunschwig 1927; Everett 1935); substantial evidence exists indicating that the endodermal cells are actively involved in histiotrophic nutrition.

Light microscopy of the visceral yolk sac shows the endodermal cells to be columnar with a microvillous border at the apical surface and beneath this a well developed vacuolar system (Wislocki et al. 1946). These features are characteristic of active cells possibly indicative that the apical plasma membrane is undergoing pinocytosis.

Electron microscopic studies on yolk sac endoderm cells have confirmed observations made at the light microscope level, and small pinocytic vacuoles have been observed to form by the invagination of the plasma membrane (Padykula et al. 1966; Jollie and Triche 1971). Although there are some morphological changes in the visceral yolk sac endoderm throughout gestation characteristic of its maturation (Lambson 1966), the general features of the cells remain unchanged, which indicates their continued function.

Much work has been carried out to demonstrate the functional activity of the visceral yolk sac endoderm cells, by studying their ability to pinocytose material at the apical cell surface. This has been visualised at the light microscope level using a variety of markers most of which are not digested by the cells and accumulate within the lysosomes. The markers used include trypan blue (Goldman 1909; Wislocki 1921; Beck et al. 1967; Krzyzowska-Gruca and Schiebler 1967; Lloyd et al. 1968),ferritin (Krzyzowska-Gruca and Schiebler 1967; Lambson 1966),fluorescent labelled antibodies (Mayersbach 1958; Larsen and Davies 1962; Wild 1970),colloidal gold (Luse 1957) and ^{58}Co-labelled vitamin B_{12}. Many markers visible under the electron microscope have also been

used to study endocytosis, eg. Lambson (1966) used ferritin. In the above and in the more recent work carried out by Gupta et al.(1979) and Gupta and Beck(1981), markers were found to be distributed throughout the vacuolar system.

The work relating generally to the histochemical and biochemical demonstration of lysosomal enzymes has been discussed already and many lysosomal enzymes have been located in the visceral yolk sac. Histochemical techniques have been used to locate acid phosphatase (Wislocki et al. 1940; Bulmer 1965;Johnson and Spinuzzi 1966; Beck et al. 1967) and many other lysosomal enzymes are also believed to be present (Beck et al. 1967; Schultz 1969). Electron microscopic studies have further located acid phosphatase. It has been shown to be membrane bounded within the primary lysosomes and heterophagosomes but not within the endocytic vacuoles or the cannalicular system (Beck et al. 1967; Beck and Lloyd 1968). Although these results confirm that the endoderm cells of the visceral yolk sac can pinocytose protein from the external environment and that these cells contain lysosomal enzymes, they do not show the link between the two observations indicative of histiotrophic nutrition.

By injecting horseradish peroxidase(HRP) into pregnant rats (Beck et al. 1967) it has been shown that a positive staining reaction is found in the yolk sac endoderm which is membrane bounded. Also using the double staining method of Straus(1964) in which tissue is stained for acid phosphatase (red) and HRP (blue), the fusion of the two stains (purple) can be seen. This is consistant with the theory that heterophagosomes and lysosomes fuse to form heterolysosomes in which

hydrolysis of the endocytosed material (in this case HRP) takes place. The same phenomenon was studied biochemically (Beck and Lloyd 1968) by intravenously injecting rats at 17.5 days of gestation with HRP. The conceptuses were removed serially over a 30 hour period and the concentration of the enzyme in the yolk sacs assessed. The concentration of HRP increased to a maximum value at 6 hours and then gradually decreased over the next 24 hours. A later experiment eliminated the possibility that the HRP was being released intact.

A further series of experiments (Williams et al. 1971) which shows dramatically that the visceral yolk sac is capable of the endocytosis and subsequent breakdown of protein was carried out using ^{125}I-labelled bovine serum albumin(BSA). Yolk sacs removed from rats previously injected with ^{125}I-labelled BSA were maintained in organ culture using a raft method. Analysis of the culture medium throughout the experiment showed that very little of the BSA was released into the culture medium intact but ^{125}I-labelled iodotyrosine (the main degradation product) was released. Also differential centrifugation of yolk sac homogenate located ^{125}I-BSA in a fraction very similar to lysosomal enzymes.

Furthermore it has been shown (Williams et al. 1975a,b) that explanted 17.5 day yolk sac can be maintained at 37^{o}C in shaker culture in tissue culture Medium 199 containing 10% calf serum. Over a seven hour period they have been shown to retain their abilty to pinocytose and degrade macromolecules. The details of experiments carried out using this system are fundamental to this thesis and will therefore be discussed at length in subsequent chapters (see sections 5-7).

The final step in histiotrophic nutrition must be the transmission of the products of digestion to the developing embryo. This has in the past proved more difficult and until recently much of the evidence was of an indirect nature. Payne and Deuchar (1972) showed that the visceral yolk sac was essential for the growth and development of 10.5 day rat embryos. After the removal of this membrane the embryos failed to grow. Other studies involved the effect of various teratogens thought to cause their effect on embryonic development by direct action on the yolk sac. Trypan blue becomes concentrated in the lysosomes after maternal injection (Lloyd et al. 1968). The dye is known to inhibit the action of various lysosomal enzymes and it was postulated that this action may interupt histiotrophic nutrition and hence prevent normal embryonic development. More recently, however it has been shown that although trypan blue does inhibit the action of lysosomal enzymes in 17.5 day yolk sacs (Davies et al. 1969), it has a greater effect by inhibiting pinocytosis (Lloyd et al. 1971; Williams et al. 1973).

The work of New and Brent(1972) on the effect of sheep anti-rat yolk sac antibody on rat embryos in culture, showed a profound effect if the antibody was added to the culture medium but no effect if it was injected into the amniotic cavity or the cavity between the amnion and the yolk sac. The presence of the antibody at the surface of the yolk sac must therefore in some way inhibit normal yolk sac function. Further work on the effects of anti-sera on rat embryos and visceral yolk sac grown in organ culture has been carried out by Webber (1985). Recently it has been shown likely that the effects are caused by the inhibition of pinocytosis.

Direct evidence for the uptake and degradation of proteins and the subsequent rebuilding of the breakdown products into new yolk sac and embryonic proteins has been shown by Freeman et al.(1981) using ^{125}I-labelled BSA and ^{3}H-labelled rat serum proteins.

Many workers have shown that the visceral yolk sac can absorb certain vital dyes which are not absorbed by the chorioallantoic placenta (Goldman 1909; Everett 1933; Gillman et al. 1948). This indicates that the yolk sac tissue has a specificity for absorbing certain macromolecules and it was Noer and Mossman(1947) who suggested that the two organs complement each other, in the uptake of these specific macromolecules. The rat visceral yolk sac has been shown to be important in the transport, as well as digestion of protein. It has been demonstrated that the passage of specific antibodies to the embryo occurs via the yolk sac and not via the chorioallantoic placenta (Brambell and Halliday 1956). This same phenomenon has also been observed in cultured yolk sacs at 11.5 days (Huxham and Beck 1981) and at 17.5 days (Weisbecker et al. 1983). Other maternal macromolecules including transferrin, have also been shown to pass to the embryo via the yolk sac (Huxham and Beck 1985).

The yolk sac can also synthesise protein which is transferred to the embryo. This function is particularly important before the establishment of the chorioallantoic placenta. The proteins synthesised in a variety of species include alphafetoprotein, transferrin, albumin, prealbumin, alpha-antitrypsin, embryo-specific alpha globulin and conalbumin (Gitlin and Kitzes 1967; Gitlin and Perricelli 1970; Dziadek

and Adamson 1978; Janzen et al. 1982; Huxham 1982; Huxham and Beck 1984).

1.9 Development of the embryo culture technique

The inaccessibility of the embryo during pregnancy has long been a problem for embryologists, and therefore a system for culturing the embryo and its surrounding membranes has many advantages for the study of embryonic development and nutrition.

The culture of post implantation embryos was first investigated in the 1930's with little success and was developed to a greater extent in the 1960's. Waddington and Waterman (1933) first cultured rabbit blastodiscs to the 6-9 somite stage on plasma clots. Jolly and Lieure (1938) obtained limited development of rat and guinea pig embryos in homologous serum. After culturing from the primitive streak stage 37% of the rat embryos had embryonic axes and a beating heart, only 9% had a functioning blood circulation, none had limb buds or a circulation in the allantois. Similar results were obtained with rat embryos cultured in heparinised rat plasma and embryonic extract by Nicholas and Rudnick (1934,1938).

Little further development was made until the 1960's; Smith (1964) cultured 4-8 somite mouse embryos for 18 to 20 hours in 5% CO_2 on a nutrient agar clot, having first opened the yolk sac and amnion. The resulting embryos had 16 somites and no circulation. New and Stein (1964) refined this method by culturing mouse and rat embryos on plasma clots. They obtained good embryonic development. In 1966 New found that

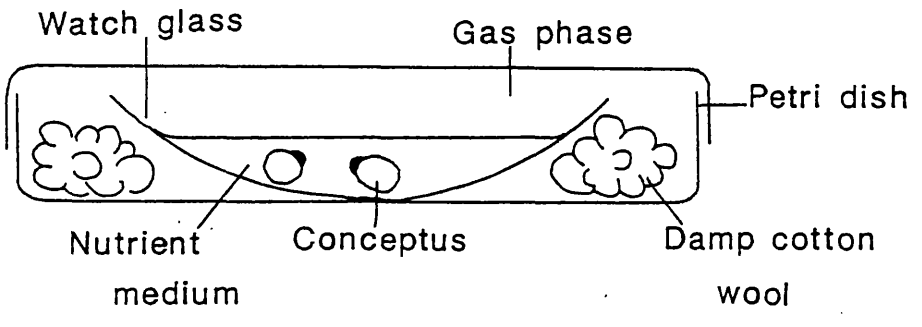
rat embryos grew as well in rat serum as on a plasma clot. The work of New and his colleagues following on from this observation has greatly improved techniques for the culture of the post implantation embryo.

A variety of culture methods have been employed in the above experiments and the efficiency of each varies with the nature and aims of the experiment.

(i)Watch glass culture

Watch glass cultures and other methods involving the use of a static medium give good growth and differentiation of rat embryos explanted on or before day 11. In such cultures the embryos are placed on a watch glass containing nutrient medium, inside a petri dish lined with damp cotton wool to provide humidity. An O_2;CO_2;N_2 gas mixture can be supplied to the culture as required (Figure 1.4). Nutrient media used include plasma clots (New and Stein 1964), homologous serum (New 1966) and mixtures of serum and chemically defined medium (Clarkson et al. 1969).

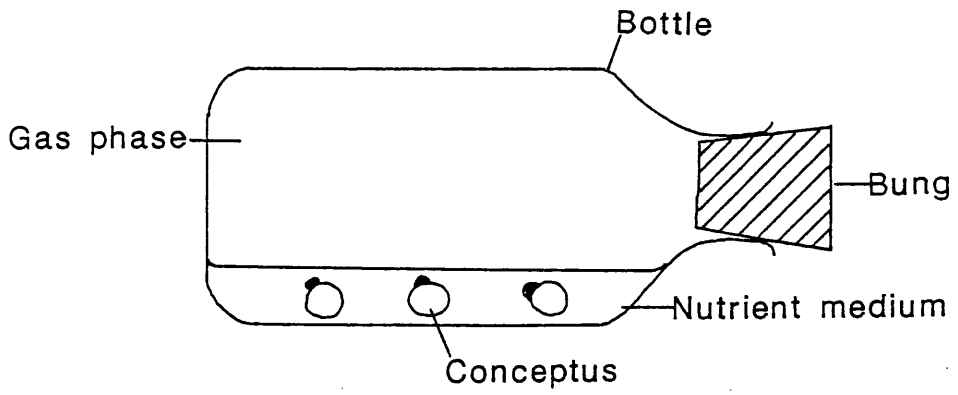
Figure 1.4. Watch glass culture of embryos.



(ii) Roller tubes

This system involves the use of glass bottles or tubes which are placed horizontally and continuously rotated by rollers or a rotating disc. An appropriate amount of culture medium is placed in each bottle and the remaining volume is filled with a suitable $O_2;CO_2;N_2$ mixture, which is either fed continuously into the chamber or replaced at intervals throughout the culture period (Figure 1.5). Improved development of rat embryos was achieved using this method (New 1978) because the efficiency of oxygenation, nutritional exchange and the removal of any toxic metabolites from the immediate vicinity of the embryo are all greatly improved by the swirling of the medium.

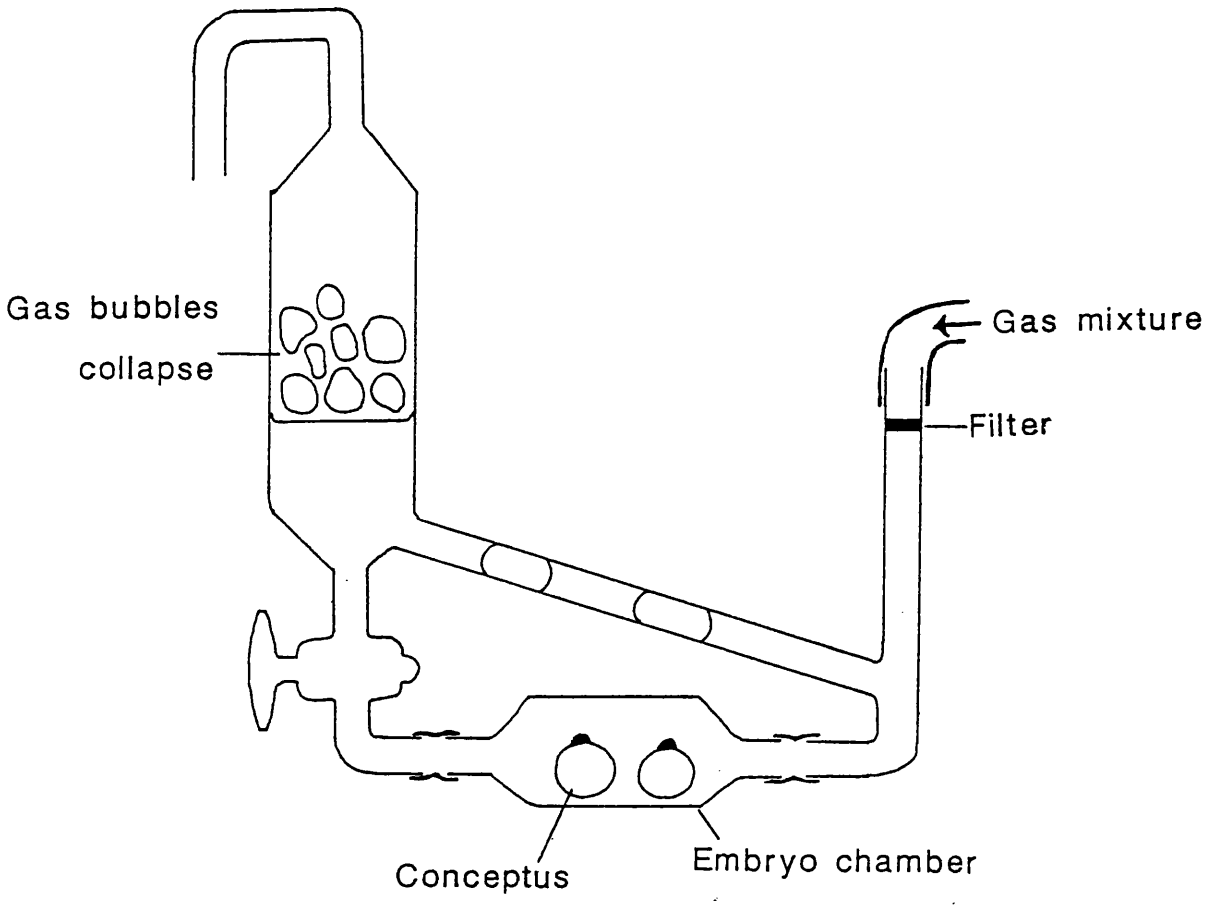
Figure 1.5. Culture of embryos in roller tubes.



(iii) Circulator

A further modification of the culture technique is the use of a "circulator" (New 1971) through which medium is continuously circulated. Embryos can be explanted into the apparatus at any stage between the early egg cylinder and the 50-58 somite stage. The advantages of this system are that the embryos can be continually observed *in situ* (Figure 1.6) and more importantly the gas phase and the nutrient medium can be continuously replenished. More complex systems requiring a pump have also been used (Tamarin and Jones 1968).

Figure 1.6. Culture of embryos in a 'circulator'.



As the techniques outlined make use of the yolk sac placenta found in rodents and marsupials, the choice of animals for such experiments is immediately reduced. Taking into account other variables such as litter size, the ability to time pregnancy accurately, the availability of the animal and the efficiency of growth in culture, the list of suitable species is further curtailed.

For these reasons the species most often used in whole embryo culture has been the rat. Mice are used less extensively (New and Stein 1964; Clarkson et al. 1969). Also a little work has been carried out using the hamster (Givelber and di Paulo 1968) and the opossum (New and Mizell 1972).

The whole embryo culture technique has been widely used for the study of normal embryonic development during organogenesis (see review by New 1978), and is also used as a system for observing the effect of various teratogens.

The whole embryo culture technique has also been used in some investigations into yolk sac development and function. Gupta et al. (1982) compared the structure of the visceral yolk sac over the 48 hour culture period with *in vivo* yolk sacs of the same age. They found the morphology to be very similar *in vivo* and *in vitro* and as the vacuolar system was found to be of a similar size, when compared morphometrically, at 9.5 days and 11.5 days in the two systems, they concluded that the yolk sac appeared to be functioning normally during culture. Freeman et al. (1981) used the whole embryo

culture system to demonstrate directly that the yolk sac has the capacity to take up and digest protein, to transport the breakdown products to the developing embryo and to synthesise new protein.

However for the specific study of yolk sac function the culture method of New is not ideal as the yolk sacs are relatively small and hence not much tissue is available for morphological, histological and biochemical investigation. As the yolk sac has been shown to retain its ability to transport and digest protein throughout gestation (Beck et al. 1967; Davies et al. 1969) culture methods spanning a later and more prolonged period of gestation have been used extensively for studying yolk sac function. Such cultures of yolk sac tissue alone are of great value in simplifying the complexities of yolk sac function in isolation from the rapidly developing embryo.

1.10 Culture of the visceral yolk sac.

The work of Sorokin and Padykula (1964) showed that the visceral yolk sac could indeed be maintained in culture for a long period of time. Yolk sacs were explanted on day 13 and cultured on solid media for up to fourteen days. The morphology of the cultured yolk sac was found to be very similar to that of *in vivo* yolk sacs at the same gestational age. The tissue appeared to be functionally active as endocytosis was occurring.

Also much valuable work into yolk sac function has been carried out using the technique developed by Williams et al. (1975a,b), in which 17.5 day rat yolk sacs explanted directly from the mother, can be maintained in organ culture for up to 12 hours. Although the length of incubation is much shorter than that used by Sorokin and Padykula, the technique is very reliable and has enabled direct measurement of the uptake, transport and digestion of various radiolabelled macromolecules to be extensively studied. This work will be referred to in detail in the later chapters.

Recently a further method of culturing the rat visceral yolk sac has been developed (Al-Alousi PhD Thesis 1983). The technique involves an extension of New's whole embryo culture by six or seven days and although the embryo soon dies and is partially autolysed the yolk sac continues to grow and can reach a diameter in excess of 2 cm. The vesicle formed after this extended culture period has been called the 'giant' yolk sac. Preliminary studies have shown that the morphology of the 'giant' yolk sac is similar to that of *in vivo* yolk sacs of the same

age. It has also been demonstrated that the system possesses a continuous epithelium impermeable to lanthanum nitrate. The yolk sacs are functionally active in the uptake of colloidal gold and have been shown to possess similar amounts of the lysosomal enzyme acid phosphatase to *in vivo* yolk sacs of the same age. The major advantages of this system are:-

(i). The large amount of tissue for morphological, histochemical and biochemical analysis.

(ii). The prolonged nature of the culture period.

(iii). The physiological integrity of the yolk sac vesicle, which separates the exocoelomic fluid from the culture medium and thus makes the system ideal for the study of the vectorial nature of transport mechanisms.

(iv). The large quantity of exocoelomic fluid which may have embryonic growth supporting properties.

This preliminary work on the 'giant' yolk sac leaves many questions about the system unanswered. I aim, in this thesis to describe further studies carried out to assess the viability of the 'giant' yolk sac as an *in vitro* model for studying early placental transport.

The work includes, further detailed studies of the ultra-structure of the 'giant' yolk sac; extensive investigations into the functional activity of 'giant' yolk sac tissue in comparison with *in vivo* yolk sac tissue at the same gestational age, by directly measuring the uptake, digestion and transport of a variety of radiolabelled macromolecules, and also a study of the growth supporting properties of the fluid within the exocoelomic cavity of the 'giant' yolk sac.

SECTION 2

GENERAL MATERIALS AND METHODS

2.1 Choice of animals.

An outbred colony of Wistar rats maintained at the University of Leicester animal house were used. Breeding females of 200 g were caged in groups of five with a cycle of 12 hours of artificial light and 12 hours darkness. Breeding males were caged individually. Animals were fed rat chow and water ad libitum.

2.2 Mating proceedure.

Male and female rats were paired in individual mating cages in the late afternoon. The following morning the litter tray beneath each cage was checked for the presence of vaginal plugs, which were taken as an indication that mating had taken place. As mating has been shown to generally occur around midnight (Long and Evans 1922), the females were assumed to be 0.5 days pregnant at midday on the morning that plugs were found. After mating, pregnant females were caged in groups of five with other females at the same stage of pregnancy until they were required for experimentation.

2.3 Serum preparation.

Whole rat serum, which was used throughout as a culture medium, was obtained from both male and non-pregnant and pregnant female Wistar rats from the University colony and external breeders. A supply of prepared serum was maintained at -20^{o}C for immediate use. The age, sex and strain of rat from which serum is obtained has been shown to have no effect on embryos subsequently cultured in the serum (New,1967).

To provide serum, both male and female rats were anaesthetised with diethyl ether, and the aneasthesia was maintained by placing an ether soaked pad in a small jar over the nose of the animal. The ventral surface of the abdomen was soaked with 70% methanol to prevent loose fur entering the abdominal cavity. A midline incision was made in the skin from the supra-pubic region to the xiphoid process. A second incision was made through the underlying muscle layer, along the linea alba to avoid major blood vessels. Two further incisions were made towards the left and right iliac fossae to improve access to the abdominal cavity. The abdominal contents were moved to the left and any fatty tissue cleared from the posterior abdominal wall to expose the bifurcation of the aorta into the common iliac arteries. The right common iliac artery was steadied with a pair of sterile forceps and a 1.5 mm needle and 10ml syringe was used to remove as much blood as possible from the artery. It was important to control the level of anaesthesia so the animal did not die before the maximum amount of blood had been collected.

The blood was immediately centrifuged at 3000rpm for 10 minutes before clotting took place (Steele 1972;Steele and New 1974). After centrifugation the tubes were allowed to stand until a cell-free fibrin clot had formed. This clot was squeezed using sterile forceps to remove the contained serum and the tube recentrifuged at 3000rpm for 5 minutes. The clear serum (approximately 50% of whole blood) was removed from the centrifuge tube using a sterile needle and syringe. Care was taken not to disturb the red cells and any serum containing haemolysed red blood cells was discarded as it is known not to support optimal embryonic growth. The serum obtained from a number of rats was pooled in a 20ml

"universal" tube. Antibiotics (100 IU/ml penicillin; 100µg/ml streptomycin) were added to reduce the risk of infection in this highly nutritious medium.

Prior to culture the serum was heat inactivated at 57°C for 30 minutes, this has been shown to destroy complement in the serum and hence improve its capacity to support normal embryonic development (New 1978). The serum was then either maintained at 37°C for immediate use or stored at -20°C until required.

2.4 Explantation of 9.5 day embryos.

The female judged to be 9.5 days pregnant following the presence of vaginal plugs after mating, was anaesthetised as described in the previous section and the abdominal cavity opened using sterile instruments. Approximately 10ml of blood was usually obtained from a female rat and this was prepared as described in section 2.3.

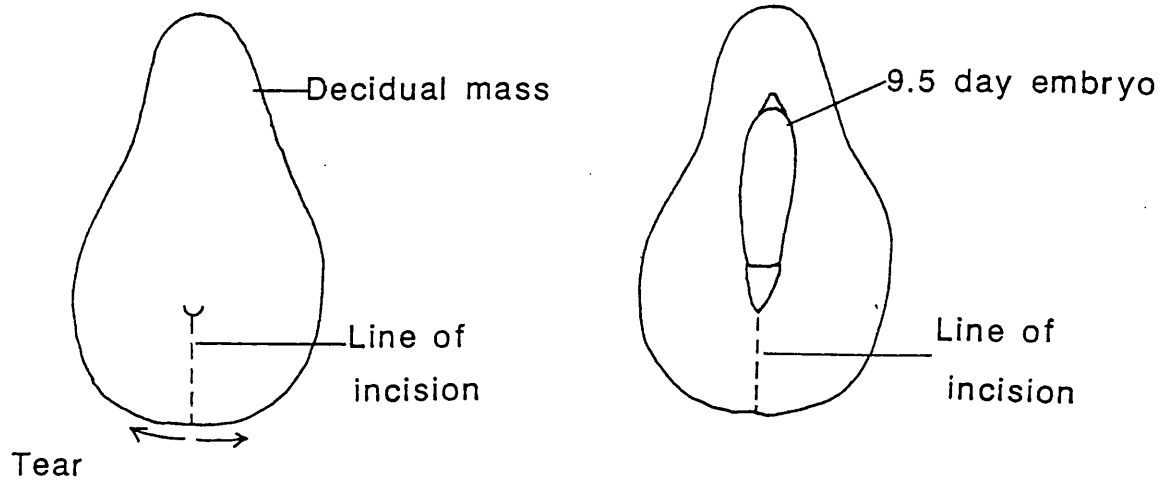
The uterus containing decidual swellings was dissected out, care being taken to remove any adipose tissue from the mesometrial border. The uterus was placed in a petri dish containing warm sterile Hank's balanced salt solution(BSS) (see Appendix A). The remainder of the procedure was carried out with the aid of a dissecting microscope, in the sterile conditions of a laminar flow cabinet.

The uterine wall was cut between each conceptus and the muscle layer torn along the mesometrial border with watch makers forceps to reveal the pear shaped decidual masses. These were gently teased out and

transferred to fresh sterile Hank's BSS.

With the aid of a disecting microscope each decidual mass was steadied with a pair of watch makers forceps and an incision made along the centre of the decidual tissue (Figure 2.1). This allowed the two halves to be gently pulled apart to expose the embryo in one half. A further midline incision in this half loosened the embryo sufficiently for it to be teased from the decidua without damage. Reichert's membrane was torn apart at the lower end of the conceptus and displaced towards the ectoplacental cone, where it was gently cut free. This allows the growing embryo to expand fully and develop normally (New and Stein 1964). Plate 2.1 shows the 9.5 day conceptus after removal of Reichert's membrane. When all the conceptuses had been explanted in this way they were checked to ensure none were damaged. They were then randomly allocated to culture bottles.

Figure 2.1 Disection of pear shaped decidual mass.



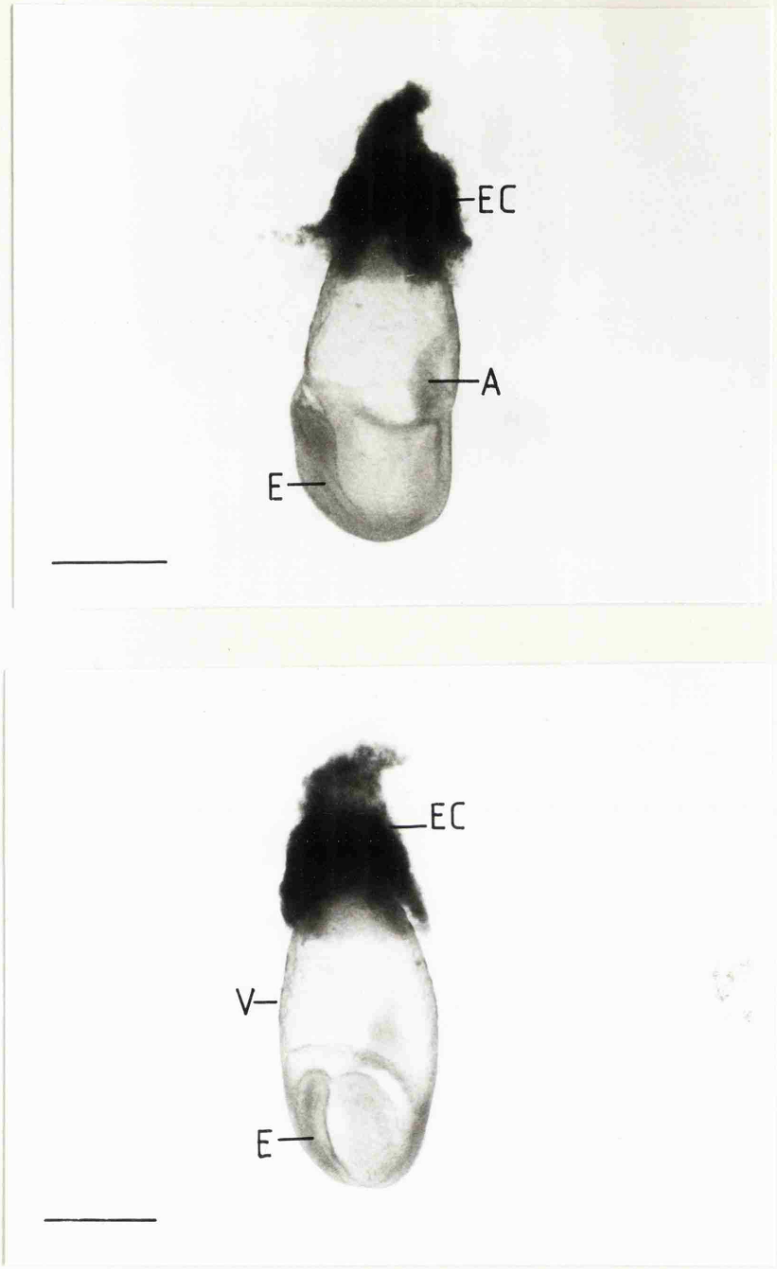


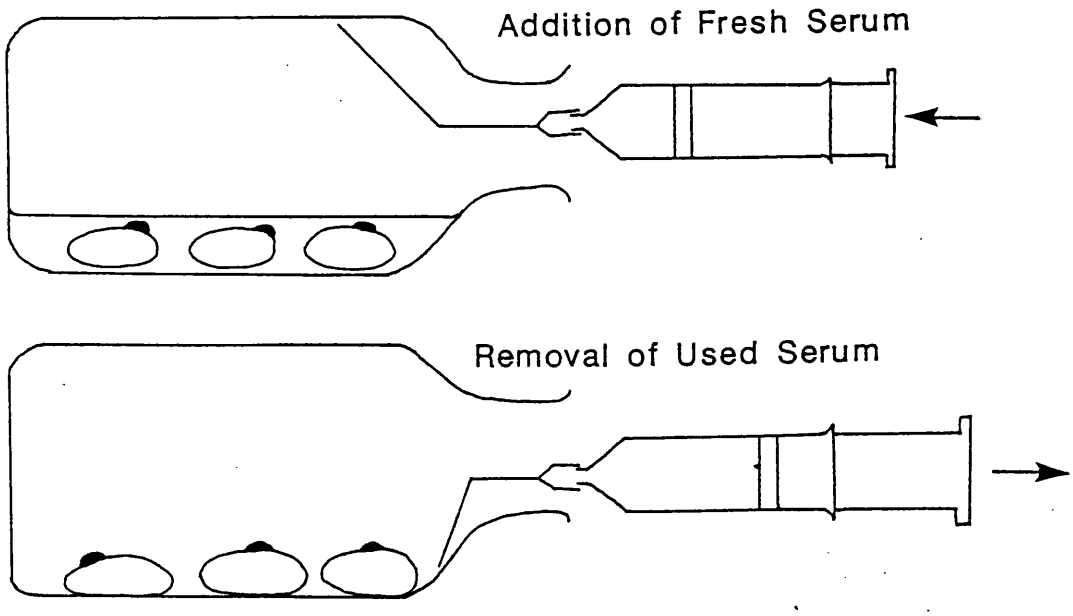
Plate 2.1 9.5 day egg cylinders. Note the ectoplacental cone (EC), visceral yolk sac (V), allantois (A) and embryonic tissue (E). Bar = 0.5mm.

2.5 Culture of 'giant' yolk sacs.

Following explantation the 9.5 day conceptuses were cultured in 60ml glass bottles, rotating horizontally in a roller incubator as described by New (1973). Each conceptus was cultured in 1ml of immediately centrifuged, heat inactivated rat serum and a maximum of seven conceptuses were placed in each bottle. The serum was carefully introduced to each bottle using a syringe and bent needle. This avoided the formation of bubbles which can trap the conceptuses and strand them partially out of the culture serum. The egg cylinders 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, the yolk sacs being 17.5 or 18.5 days, in terms of conceptional dates, at the end of the culture period.

After 2 days of culture the conceptuses were transferred to bottles containing fresh serum. A maximum of 3 conceptuses were placed in each 60ml bottle, to ensure the yolk sacs had sufficient room for expansion during the remainder of the culture period. The culture serum was changed every 2-3 days thereafter old serum was carefully removed from the culture bottle using a syringe and bent needle, care being taken not to damage the yolk sac membranes (Figure 2.2). Throughout the procedure the culture bottles were maintained in the horizontal position. Fresh serum was immediately placed in the bottles again using a syringe and bent needle, the flow of serum being aimed at the side of the bottle (Figure 2.2). After each change of serum the cultures were regassed with a mixture of $20\%O_2;5\%CO_2;75\%N_2$.

Figure 2.2 Addition and removal of serum.



2.6 Harvesting of 'giant' yolk sacs.

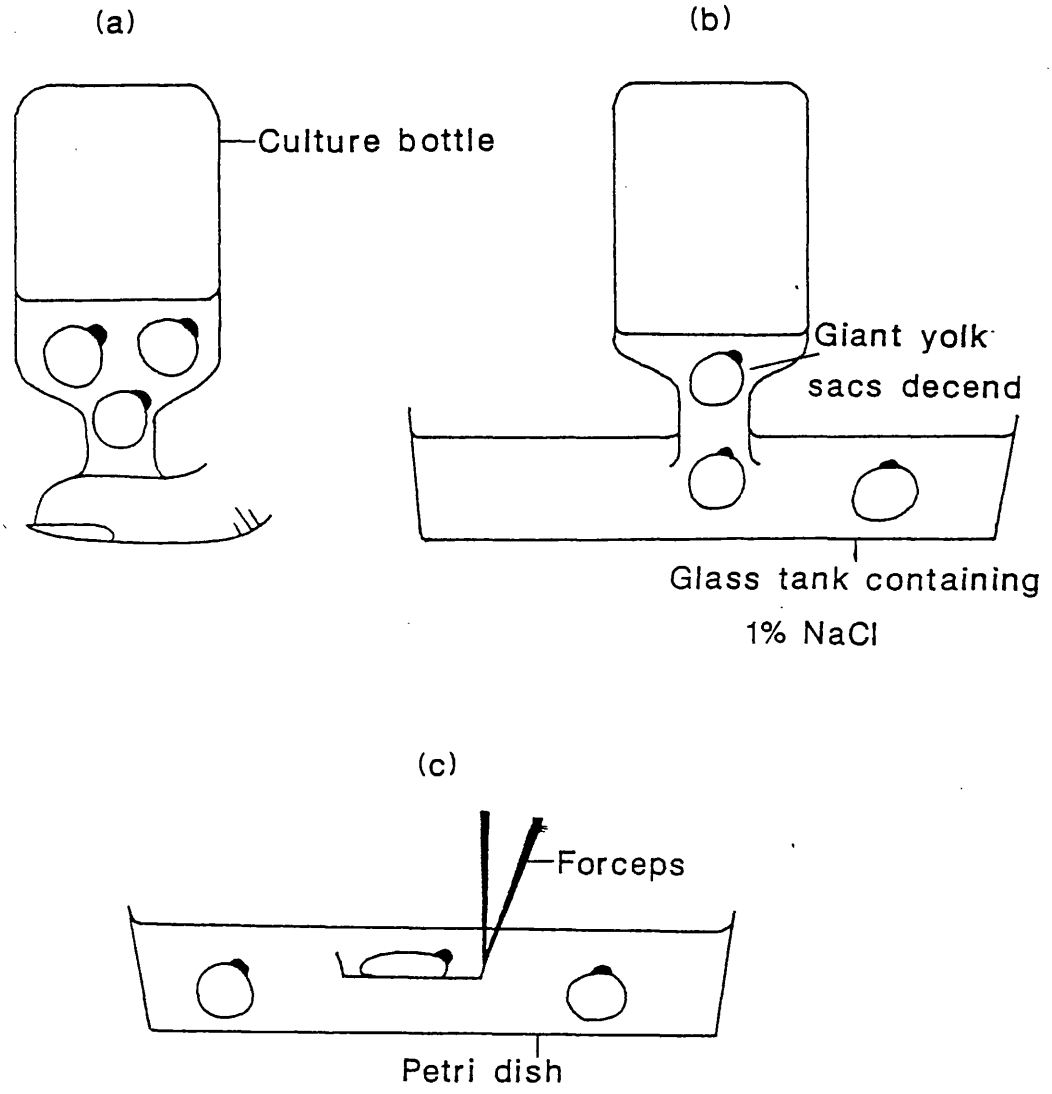
The large and relatively delicate nature of the 'giant' yolk sacs after the prolonged culture period, necessitates a special technique for handling the yolk sacs and removing them from the culture bottle. This method avoids damaging the yolk sacs and was used in all the experiments involving 'giant' yolk sacs.

The culture serum was removed carefully using a bent needle and 2ml syringe. The yolk sacs were then washed twice in warmed sterile Hank's BSS which was introduced and removed from the culture bottles again using a bent needle and syringe.

The yolk sacs were covered once again with sterile Hank's BSS and the bottles inverted so that the neck was below the surface of Hank's

BSS contained in a tank (15cm/15cm). The 'giant' yolk sacs decended into the tank under gravity and could be removed for observation using a small petri dish held in a pair of forceps (Figure 2.3).

Figure 2.3 Harvesting of 'giant' yolk sacs.



2.7 Collection of extraembryonic coelomic fluid and amniotic fluid.

'Giant' yolk sacs removed from the "harvesting" tank in a small petri dish, were placed under a dissecting microscope and the yolk sac diameter measured using an eyepiece graticule. Plate 2.2 shows a typical 17.5 day 'giant' yolk sac. The majority of the Hank's BSS was removed carefully from the petri dish so that on aspiration there was no contamination of the fluids contained in the 'giant' yolk sac by Hank's BSS.

The extraembryonic coelomic fluid from within 'giant' yolk sacs was collected using a fine needle and a 1ml syringe and pooled in 10ml centrifuge tubes. Care was taken not to tear the yolk sac membranes during this process, the amnion always remained intact around the partially autolysed embryo. Amniotic fluid was also collected in this way using a fresh sterile needle and 1ml syringe and again amniotic fluid from the 'giant' yolk sacs was pooled. The fluids were centrifuged at 3000rpm to remove cell debris, antibiotics (100 IU/ml penicillin; 100µg/ml streptomycin) were added and the fluids stored at -20°C if not immediately required.

The whole yolk sac after removal of the ectoplacental cone and the amnion containing any embryonic tissue, was routinely assessed for protein content as an estimation of size, using a modification of the technique of Lowry et al. (1951) (See Appendix B).

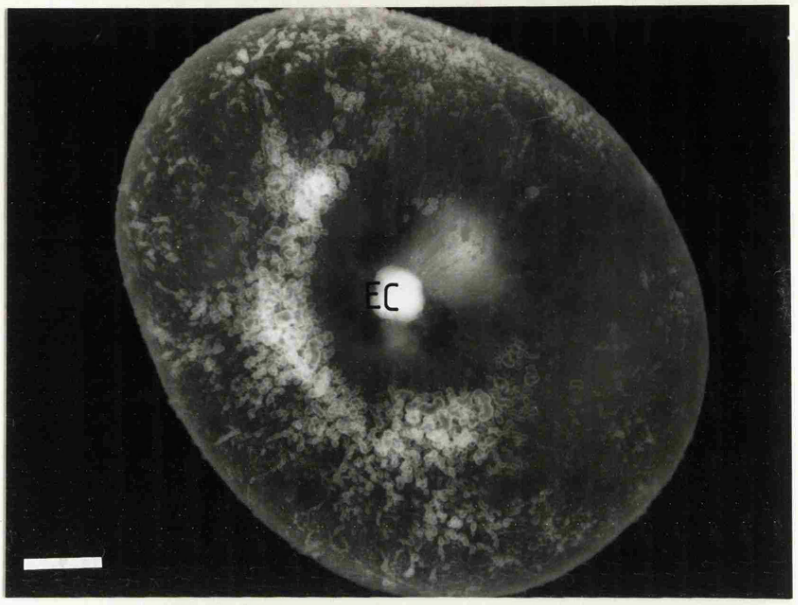


Plate 2.2 A typical 17.5 day 'giant' yolk sac. Note the ectoplacental cone (EC). Bar = 2mm.

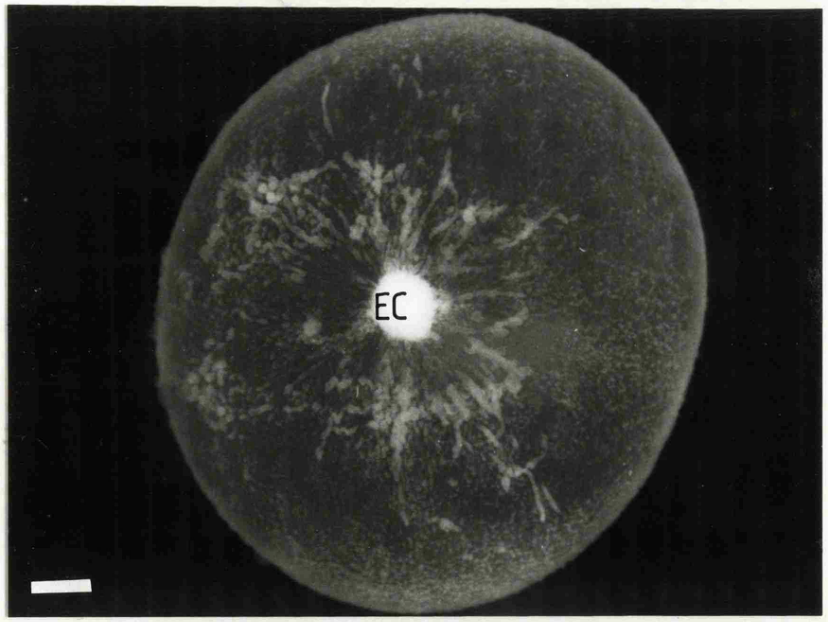
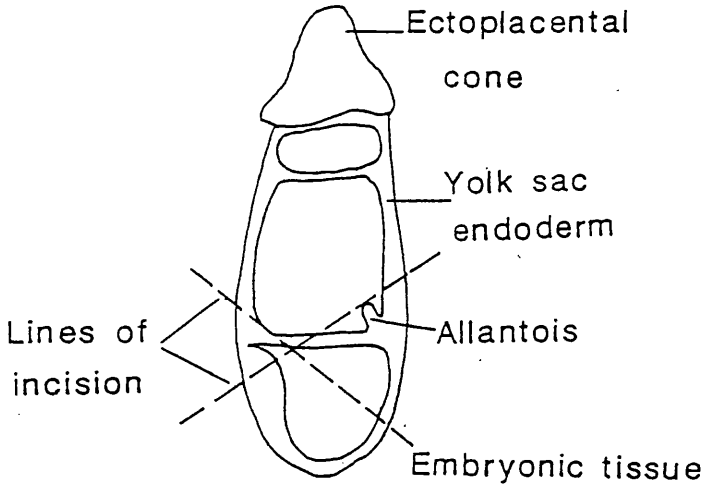


Plate 2.3 A typical 17.5 day 'giant' yolk sac after removal of the embryonic tissue at 9.5 days. Bar = 1.5 mm.

2.8 Culture of GYS's after removal of the embryonic tissue at 9.5 days.

Females judged to be 9.5 days pregnant were anaesthetised and the embryos explanted as described in section 2.4. Reichert's membrane was again torn apart and gently removed from each conceptus. The egg cylinders were then steadied at the ectoplacental cone with a pair of watch makers forceps and the embryonic tissue at the lower end of the egg cylinder removed using a fine scalpel. Care was taken to remove all the embryonic tissue but as little as possible of the yolk sac endoderm (Figure 2.4).

Figure 2.4 Removal of embryonic tissue from 9.5 day egg cylinder.

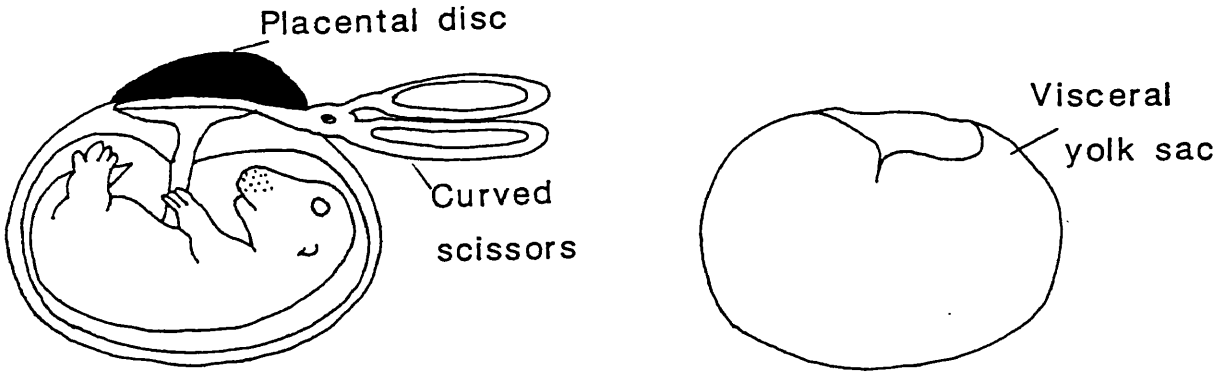


After the removal of the embryonic tissue the remainder of the conceptus, made up predominantly of the visceral yolk sac, was cultured as described in section 2.5; being gassed initially with a mixture of 5%O_2; 5%CO_2; 90%N_2 and every 24 hours thereafter with a mixture containing 20%O_2;5%CO_2;75%N_2. The culture serum was changed every 2-3 days and the culture period was again 8 or 9 days. Plate 2.3 shows a 17.5 day 'giant' yolk sac cultured after prior removal of the embryo.

2.9 Explantation of 17.5/18.5 day in vivo yolk sacs.

Female rats judged to be 17.5 or 18.5 days (as required) were anaesthetised and the abdominal cavity opened to expose the uterus, now swollen by the developing embryos. The uterus was dissected out and placed in warmed sterile Hank's BSS. An incision was made along the mesometrial border of the uterine musculature with curved scissors and the placental discs coaxed free from the uterus. The whole conceptuses were placed in fresh Hank's BSS. At this point the gestational age of the conceptus was assessed by observing the size and external development of the embryo. The placental discs were removed using a pair of curved scissors (Figure 2.5) and the yolk sacs separated from the embryo and amnion. The yolk sacs were washed well in fresh sterile Hank's BSS to remove any remaining blood. The yolk sacs were then used for morphological, morphometric and biochemical analysis. When yolk sacs were being prepared for electron microscopy the explantation was carried out as quickly as possible, the yolk sacs being exposed only briefly to the Hank's BSS and were placed immediately in fixative.

Figure 2.5 Isolation of visceral yolk sac tissue.



SECTION 3

MORPHOLOGY OF THE 'GIANT' YOLK SAC

3.1 Introduction.

The 'giant' yolk sac system has been developed as a technique for the prolonged culture of the rat viseral yolk sac in isolation, in which the yolk sac membranes remain intact. The functions of the yolk sac in the uptake, transport and digestion of macromolecules, so important for early embryonic nutrition, can be studied using this system. However before undertaking experiments into the functioning of the 'giant' yolk sac systems, it is important to first assess the growth and development of the tissue under culture conditions in comparison with *in vivo* tissue removed immediately from the pregnant female (section 2.9); Thus giving an insight into the potential of the 'giant' yolk sac as a model for studying visceral yolk sac function.

Many authors have described the structure of the rat visceral yolk sac at various stages of gestation using both the light microscope and the electron microscope (Padykula et al. 1966; Lambson 1966; Jollie and Triche 1971). Studies have also been carried out using the guinea pig visceral yolk sac (King and Enders 1970). In addition the morphology of yolk sac tissue has been described after being maintained in a variety of culture conditions over varying periods of time (Sorokin and Padykula 1964; Gupta et al. 1982;).

The work presented here, however, is the first detailed description of the morphology of the 'giant' yolk sac. The ultrastructure of the cultured tissue has been compared to 17.5 day *in vivo* yolk sac tissue removed directly from the pregnant female. Also the growth and development of 'giant' yolk sacs cultured by both methods outlined in

this thesis (see sections 2.5 and 2.8) has been quantitatively assessed.

3.2 Materials and Methods.

3.2.1 Culture of 'giant' yolk sacs by the original method.

'Giant' yolk sacs were cultured as outlined in section 2.5, conceptuses being explanted at 9.5 days from the pregnant female and grown in whole, heat inactivated rat serum in a roller incubator. 'Giant' yolk sacs for use in the functional studies of the uptake of radiolabelled substrates were routinely cultured to 17.5 days (ie. 8 days in culture), however in this morphological investigation cultures were terminated at various stages throughout gestation and observed under a dissecting microscope before preparation for electron microscopy.

3.2.2 Culture of 'giant' yolk sacs after removal of embryonic tissue.

The culture of 'giant' yolk sacs after removal of the embryonic tissue is outlined in section 2.8. It involves the removal, with a fine scalpel, of the embryonic tissue situated at the pole opposite to the ectoplacental cone of the 9.5 day egg cylinder. The remainder of the conceptus, made up predominantly of the visceral yolk sac can then be successfully cultured to 17.5 days; the system being free from any adverse effects the dead embryo may produce.

3.2.3 Explantation of control yolk sacs.

Control yolk sacs were explanted directly from the pregnant female at various stages of gestation for comparison with 'giant' yolk sac

tissue. The method followed at all stages was identical to that outlined for the removal of 17.5/18.5 day yolk sacs (section 2.9).

After culture to the appropriate stage 'giant' yolk sacs were harvested as described in section 2.6 and observed using a dissecting microscope and either assessed developmentally (see section 3.2.4) and photographed or immediately prepared for electron microscopy (outlined in section 3.2.5).

3.2.4 Quantification of yolk sac development.

The growth and development of 'giant' yolk sacs cultured following both the original and modified culture techniques was assessed by terminating cultures at various stages (11.5, 13.5, 15.5 and 17.5 days of gestation) and measuring the yolk sac diameter. At each gestational age a mean value +/- SD was calculated and a plot drawn to illustrate the rates of development.

3.2.5 Preparation of tissue for Electron Microscopy.

The preparation of both 'giant' yolk sac tissue and *in vivo* yolk sac tissue was identical. The different stages of the process are outlined here.

1. Initial preparation of yolk sac tissue.

Control yolk sac tissue was removed quickly from the pregnant female as outlined in section 2.9 and the placental disk, embryo and amnion removed.

'Giant' yolk sacs were removed from culture (see section 2.6) and the exocoelomic fluid collected (section 2.7). The embryonic tissue and amnion (if present) and the ectoplacental cone were removed and the yolk sac tissue washed in Hank's BSS.

The above procedures were carried out as quickly as possible to prevent any deterioration of the tissue before primary fixation.

2. Primary fixation.

The whole yolk sacs were fixed for 2 hours at $4^{o}C$ in 3% gluteraldehyde in sodium/phosphate buffer pH 7.3.

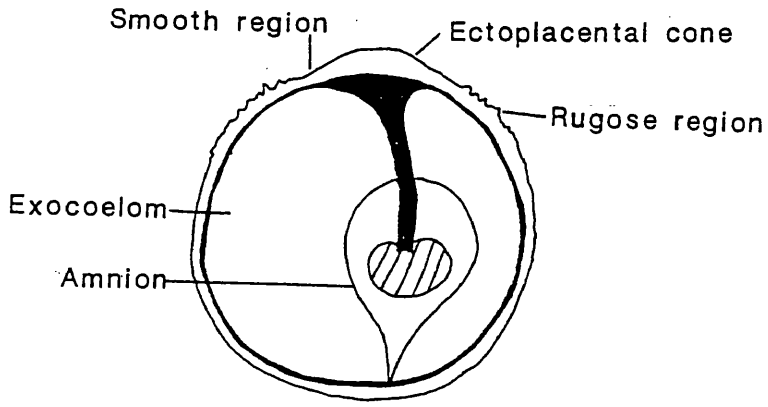
3. Sucrose buffer wash.

Whole yolk sacs were washed thoroughly for 2 x 1hour and then over night in fresh sucrose buffer at $4^{o}C$ (6.84g sucrose made up to 100ml with sodium/phoshate buffer pH 7.3).

4. Selection of tissue.

Identical areas of yolk sac were carefully selected and routinely the area chosen was the rugose area found in a wide band below the ectoplacental cone (figure 3.1) much more prominent in the 'giant' yolk sac. Other areas were also studied for comparison. Small pieces of tissue approximately 2mm / 4mm were cut at random from comparable areas of the yolk sacs.

Figure 3.1. Selection of 'giant' yolk sac tissue.



5. Secondary fixation.

Tissue pieces were post-fixed at 4°C with 2% osmium tetroxide for 1 hour.

6. Dehydration and embedding.

The fixed tissue pieces were washed in distilled water and dehydrated at room temperature through a series of alcohols and embedded in araldite blocks.

7. Sectioning the tissue.

The araldite blocks were trimmed and thick sections (approximately 1um) cut. These were stained with toluidine blue for preliminary observation of the tissue. The blocks were then retrimmed once the orientation of the tissue had been noted and ribbons of pale gold/silver ultra thin sections cut using an Reichert-Jung OMU4. The sections were mounted on clean copper grids.

8. Staining.

The ribbons of sections were stained for 1 minute with uranyl acetate and 6 minutes with lead citrate using the technique of Reynolds (1963).

The sections were then observed with a Jeol 100S or Jeol 100 CX electron microscope with an acceleration voltage of 80 KV. Photographs were taken at various magnifications.

3.2.6 Preparation of tissue for Scanning Electron Microscopy (SEM).

All tissue was prepared for SEM observation using the following schedule.

1. Fixation.

Whole yolk sacs either removed immediately from the pregnant female or from culture bottles were prepared for fixation as outlined in section 3.2.5(1). The whole yolk sacs were immediately fixed for 2 hours in 3% glutaraldehyde in sodium/phosphate buffer at $4^{o}C$, washed well in sucrose buffer (6.84g/100ml) and post fixed in 2% osmium tetroxide for 1 hour at $4^{o}C$.

2. Selection of tissue.

In the case of small, early gestation yolk sacs the whole structure was processed, but with the larger yolk sacs of a greater gestational age, the appropriate piece of tissue was selected for further processing and SEM observation.

3. Dehydration and critical point drying.

The tissue was dehydrated through an alcohol series and finally

left for 30 minutes in 100% acetone. It was then placed in gelatine capsules pierced at either end, in acetone and critical point dried.

4. Mounting and gold coating.

The dried yolk sac tissue, which appeared white and was extremely brittle was carefully mounted onto SEM stubs using quick drying silver paint. The tissue was then gold coated.

3.3 Results.

3.3.1 External appearance of 'giant' yolk sac during development.

Plate 3.1 shows pictorially three stages in the development of 'giant' yolk sacs cultured by the original technique (right) and the modified technique (left) after prior removal of the embryonic tissue. The following account of the development of the two systems outlines clearly some of their similarities and differences which will be discussed in detail in later sections (3.3.3 and 3.3.4).

At 9.5 days (Plate 3.1a) after the removal of the embryonic tissue the egg cylinder is reduced to approximately half its original size. However as much as possible of the yolk sac has been retained.

At 11.5 days (Plate 3.1b) after 2 days of culture the incision to remove the embryonic tissue has healed fully (see section 3.3.4) and the remaining yolk sac vesicle is spherical and almost the same size as the 11.5 day yolk sac cultured by the original method and containing a well developed 25 somite embryo.

At approximately 12.5 days the embryo in the original culture system dies, it is partially autolysed over the remainder of the culture period and remains surrounded by the amnion. The visceral yolk sac continues to grow and develops a characteristic structure which is similar to both culture systems (Plate 3.1c) and which is described in detail in section 3.3.3. At 17.5 days although the general external appearence of yolk sacs cultured by the two systems is very similar,

those cultured after removal of the embryonic tissue are significantly smaller.

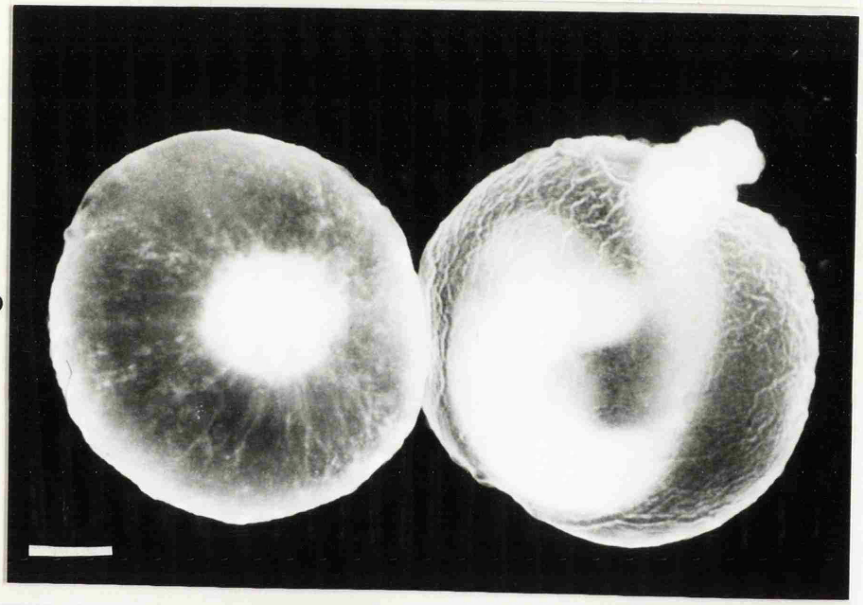
Plate 3.1 Three stages in the development of 'giant' yolk sacs cultured with and without the embryo.

(a) R - 9.5 day egg cylinder.
L - 9.5 day egg cylinder after removal of embryo.
Bar = 0.3 mm.

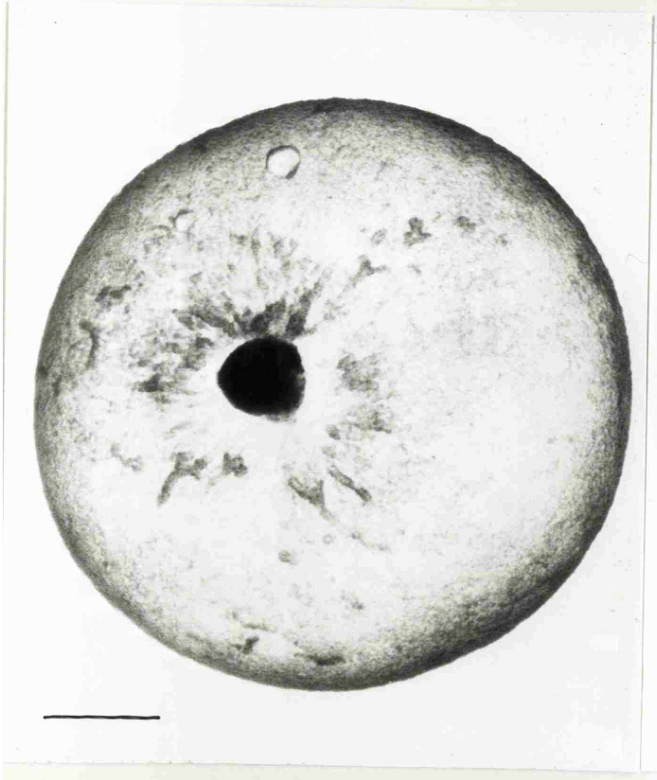
(b) R - 11.5 day conceptus with well vascularised YS.
L - 11.5 day yolk sac without embryo.
Bar = 1 mm.

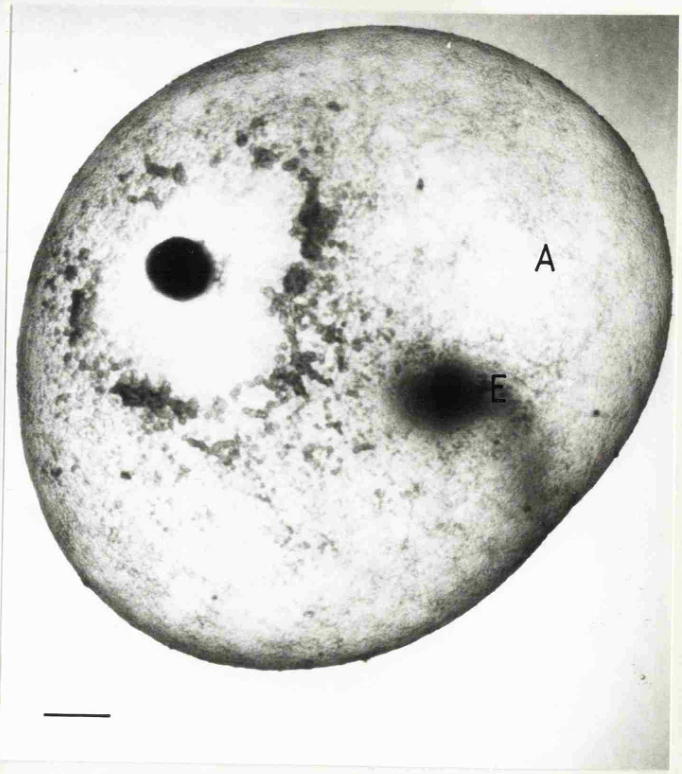
(c) R - 17.5 day 'giant' yolk sac. The remains of the embryo (E) are present within the amnion (A).
L - 17.5 day 'giant' yolk sac without embryo.
Bar = 2 mm.

a



b





3.3.2 Quantifying the growth and development of 'giant' yolk sacs by measuring the yolk sac diameter throughout culture.

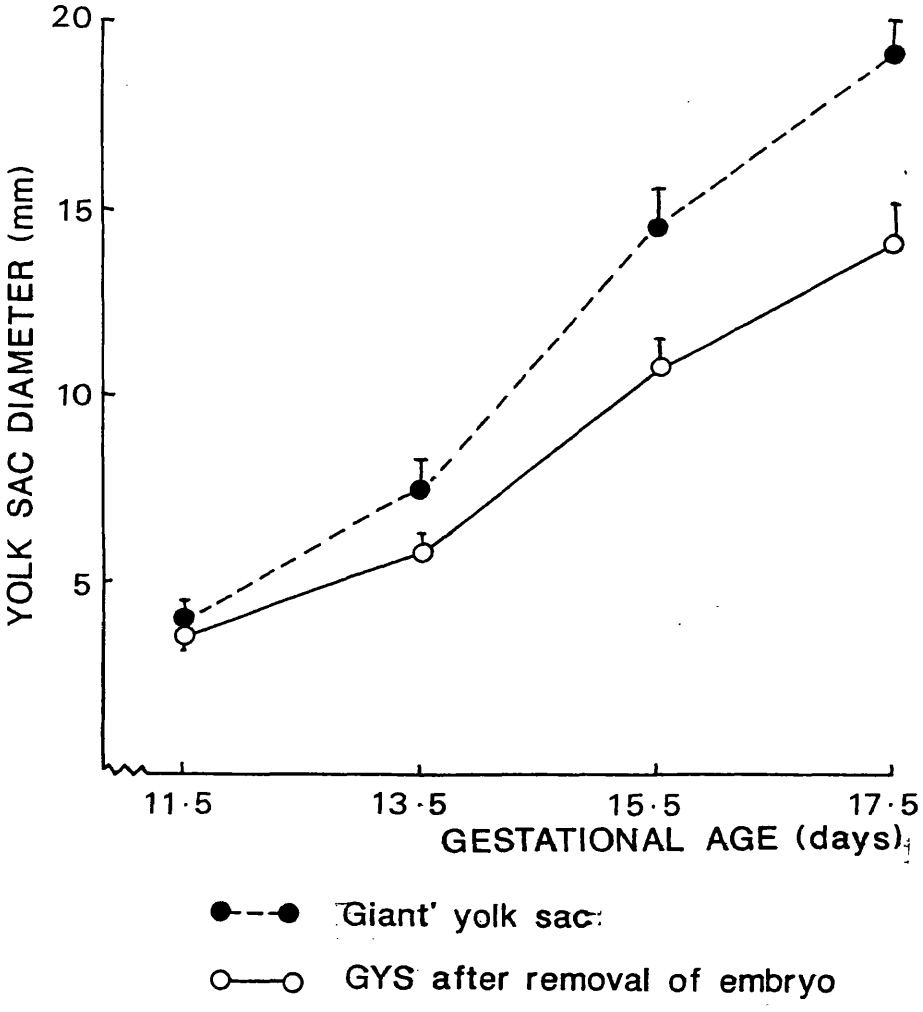
The growth and development of 'giant' yolk sacs throughout the eight days of culture was plotted to reveal the relative rates of growth of the yolk sacs using the two culture techniques (Figure 3.2). The results are expressed in terms of the diameter (mm) of the yolk sacs at different stages of gestation. The results are also presented in Table 3.1 below.

Table 3.1. Assessmment of yolk sac diameter throughout culture of GYS.

Method of Culture	Yolk sac diameter (mm)			
	11.5 days	13.5 days	15.5 days	17.5 days
GYS - Original Culture	4.11+/-0.22 (n = 34)	7.48+/-0.61 (n = 35)	14.5+/-0.74 (n = 33)	19.07+/-0.81 (n = 35)
GYS - Revised Culture	3.64+/-0.20 (n = 22)	5.84+/-0.45 (n = 24)	10.8+/-0.61 (n = 26)	14.10+/-0.94 (n = 24)

The results show 'giant' yolk sacs grown by both culture techniques increase in size throughout the culture periods but the rate

Figure 3.2 **Assessment of yolk sac diameter throughout culture of 'giant' yolk sacs.**



of growth and the final size reached after 8 days in culture is less in the case of 'giant' yolk sacs grown after prior removal of the embryonic tissue.

3.3.3 Detailed observations of the external appearance and ultrastructure of 'giant' yolk sacs grown by the original method.

External structure of 'giant' yolk sac.

After 2 days in culture the 11.5 day embryo is at the 25 somite stage, has a well developed circulatory system described in detail by Mensah-Brown (PhD Thesis 1987) and a beating heart. The rudiments of many systems are present including the brain, branchial bars, optic and otic vesicles and limb buds. The embryo lies ventrally convex surrounded by the amnion, within the yolk sac.

After approximately 12.5 days this previously actively developing embryo dies and the embryonic tissue within its amnion begins to deteriorate. The yolk sac tissue however continues to grow and the yolk sac vesicle as was shown above can reach a diameter of 2cm at 17.5 days.

The 'giant' yolk sac, as mentioned in section 3.3.1 has a characteristic structure which becomes more apparent as the culture progresses. Plate 3.1 (c) shows a typical 17.5 day 'giant' yolk sac. The ectoplacental cone is surrounded by a small zone of smooth tissue and then a wider region of rugose tissue which is thrown into complex folds, the convoluted nature of which decreases towards the pole opposite to

the ectoplacental cone. Plate 3.2 shows the remains of the embryonic tissue contained within the amnion. The allantoic connection to the ectoplacental cone may or may not be severed. The actual size of the amnion varies and can either closely surround the remains of the embryo and contain very little amniotic fluid or can be almost as large in diameter as the yolk sac. Generally however as is the case in Plate 3.2 the size of the amnion is mid-way between these two extremes.

Plate 3.3 shows the rugose area of the 'giant' yolk sac at higher magnification revealing its highly convoluted structure. Using scanning electron microscopy the surface structure can be further investigated and the microvillous border to each cell was revealed (Plate 3.4).

Electron microscopic examination of 'giant' yolk sac structure.

At the electron microscope level the structure of the 'giant' yolk sac was found to be generally very similar to the *in vivo* yolk sac tissue, however a few differences were observed and will be discussed. Plate 3.5 (a) and (b) show the overall structure of 'giant' yolk sac tissue at 17.5 days.

It can be seen that the yolk sac is made up of three layers of cells separated by two basement membranes.

(i) The outer endodermal layer made up of columnar cells having a microvillous border at the apical surface and a basally situated nucleus. These cells contain numerous endocytic vacuoles varying in size and electron density, in the apical cytoplasm. The cytoplasm also contains many well developed

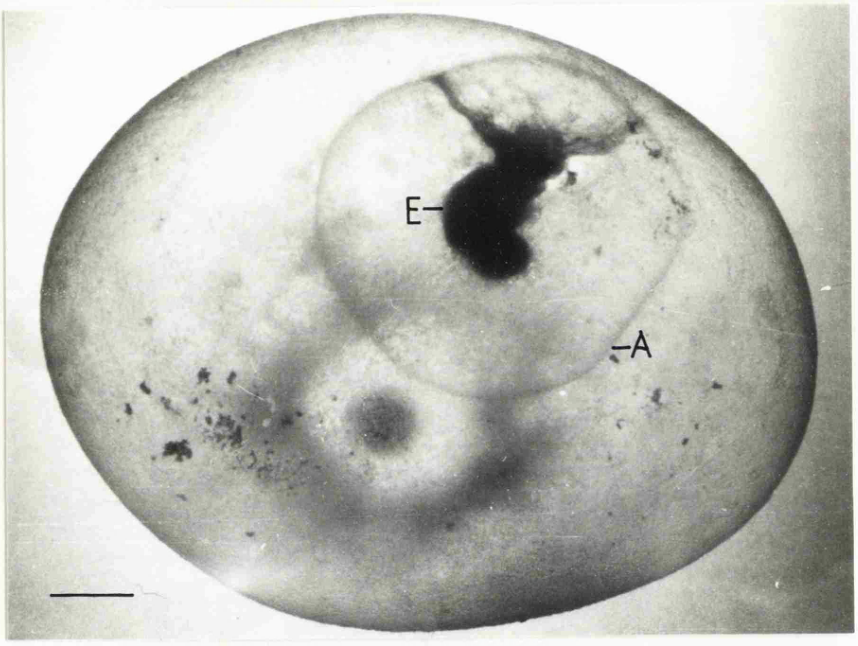


Plate 3.2 A view of the embryonic remains (E) within the intact amnion (A) of a 17.5 day 'giant' yolk sac. Bar = 2 mm.

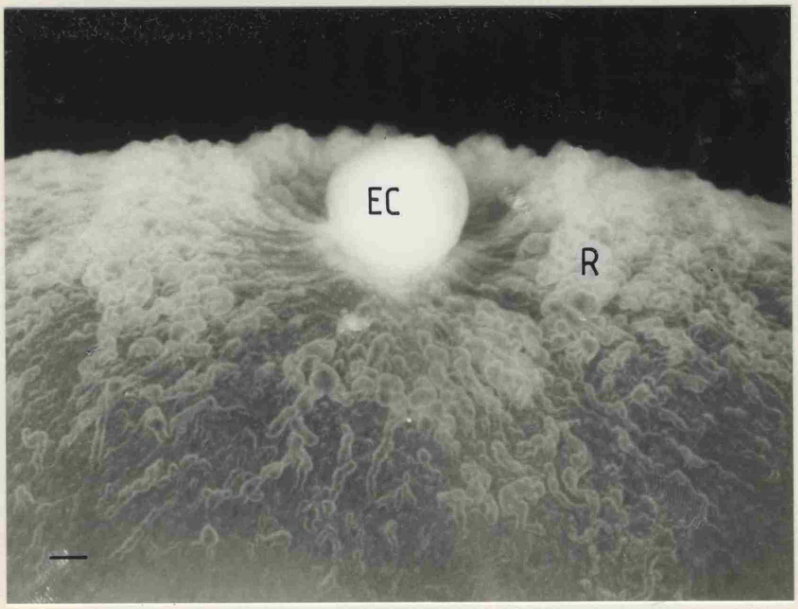
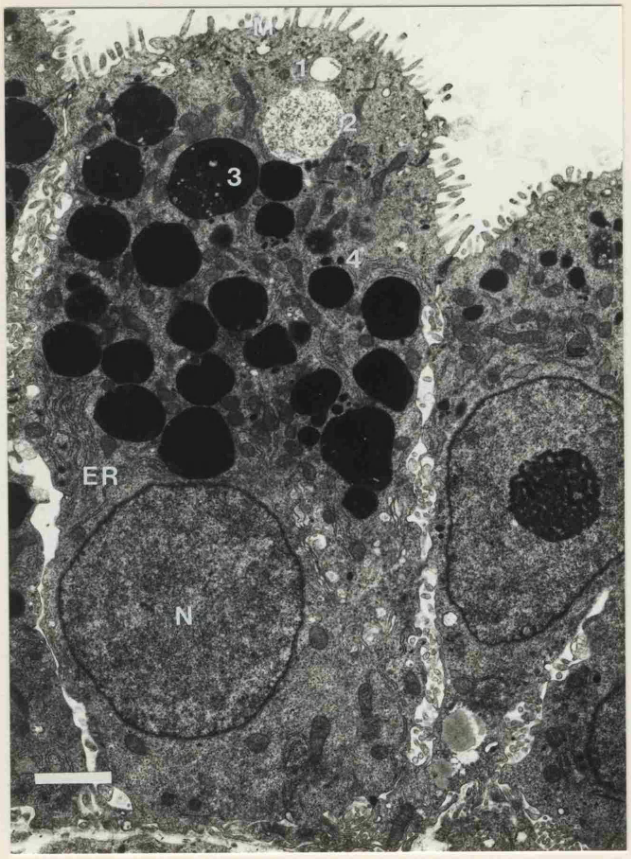


Plate 3.3 High magnification of the rugose area (R) of the 'giant' yolk sac, around the ectoplacental cone (EC). The surface of the yolk sac is shown to be thrown into complex folds. Bar = 0.5 mm.

Plate 3.4 SEM showing surface of 'giant' yolk sac endodermal cells. Note the well developed microvillous border.

Plate 3.5a Typical example of 'giant' yolk sac endoderm cells. Note the microvillous border (M) and numerous coated invaginations and coated vesicles. The lysosomal system exhibits large electron lucent vacuoles (1), large electron lucent vacuoles containing flocculent material (2), large electron dence vacuoles (3) and small electron dence vacuoles (4). There is a well developed endoplasmic reticulum (ER) and a basally situated nucleus (N). Bar = 2 μm.

Plate 3.5b High magnification of the lysosomal system in the 'giant' yolk sac endoderm cell. Bar = 2 μm.


1
2
3
4
ER
N

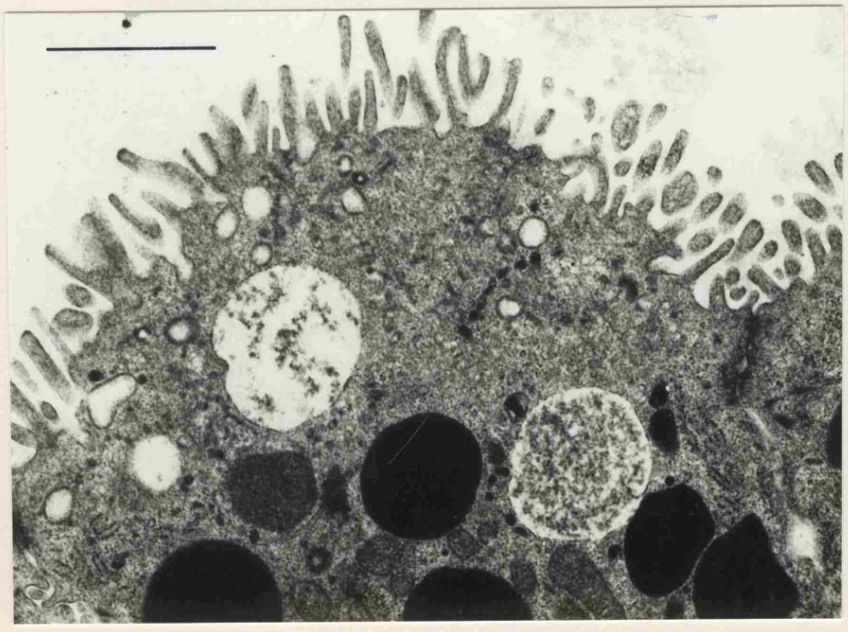


Plate 3.6 Typical 17.5 day *in vivo* yolk sac endoderm cell. Note a well developed canalicular system in the apical cytoplasm (*), extensive rough endoplasmic reticulum (ER), glycogen deposits (G) and a significantly smaller lysosomal compartment than in plate 3.5. Bar = 2 μm.

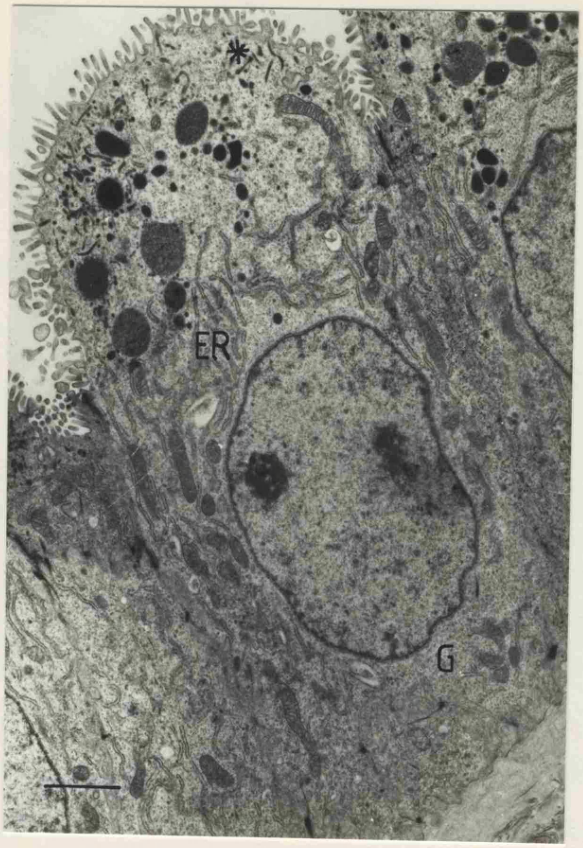
Plate 3.7 The three cell layers, separated by two basement membranes, which make up the visceral yolk sac.

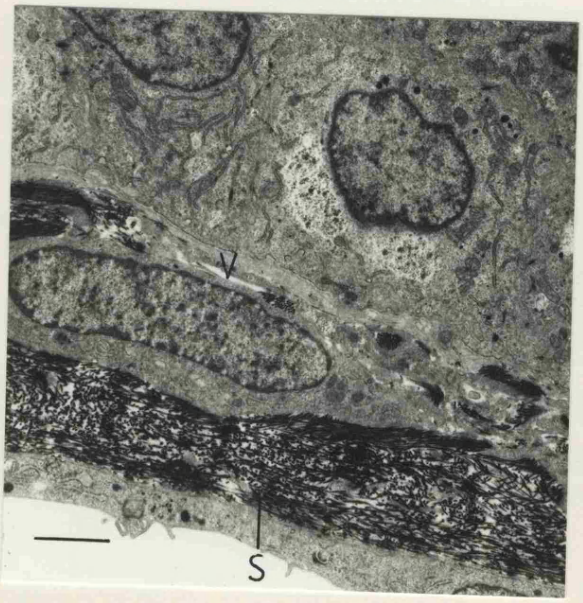
(1) Endoderm cells with basally situated nuclei.
The visceral basement membrane (V).

(2) Mesenchymal cells.
The serosal basement membrane (S).

(3) Flattened mesothelial cells.

Bar = 2 μm.


ER
G


V
S

mitochondia, an extensive Golgi apparatus and granular endoplasmic reticulum characteristic of an active protein producing cell. Typical junctional complexes are present composed of zona occludens, zona adherens amd macula adherens. This layer rests on the visceral basement membrane.

(ii) The second layer of cells consists of scattered mesenchymal cells and collapsed fetal capillaries.

(iii) The third cell layer, which lines the exocoelom, consists of flattened mesothelial cells and is separated from the second cell layer by the serosal basement membrane.

In vivo yolk sacs explanted directly from the pregnant female at 17.5 days showed a generally similar morphology to the 'giant' yolk sacs as can be seen from Plate 3.6. Plate 3.7 shows the three cell layers mentioned above which make up the visceral yolk sac. However some differences were apparent:

(a) In the endoderm cells of in vivo yolk sacs glycogen deposits were present in varying amounts. No similar large deposits were seen in the 'giant' yolk sac endoderm cells, although occasionally a small amount of glycogen was observed scattered in the cytoplasm. Plate 3.8 shows such aggregates - the glycogen being in both the α and β configuration and distributed both apically between the endocytic vacuoles and also in the basal cytoplasm. The amount present in the cells was very variable.

(b) The serosal basement membrane was thicker in the in vivo yolk sac tissue and contained more collagen fibrils. Plate 3.9 illustrates well the presence of collagen fibrils in the

basement membranes of control yolk sac tissue. The relatively delicate nature of the 'giant' yolk sac in comparison to the *in vivo* yolk sac tissue also indicates a difference in the amount of collagen fibres.

(c) The vacuolar compartment of the 'giant' yolk sac endoderm cells and the individual vacuoles appeared larger than in the *in vivo* yolk sac endoderm cells. This can be seen from Plate 3.5a and was considered an important difference, possibly indicating a modification of yolk sac function. A quantitive comparison of vacuolar volume was consequently carried out using morphometric techniques (see section 4). It has been suggested that the size of the vacuolar volume may be indicative of the endocytic activity of the cells. Plate 3.5b shows part of the lysosomal system in 'giant' yolk sac tissue. The differing vacuoles are illustrated well:

(i) coated vesicles,
(ii) larger electron lucent uncoated vesicles,
(iii) large electron lucent vesicles containing varying amounts of flocculent material,
(iv) large electron dense vacuoles,
(v) small electron dense vacuoles.

A more detailed study of yolk sac structure revealed several other features of interest, which in general were observed in both 'giant' yolk sac and *in vivo* yolk sac tissue. Any differences observed between the two tissue types will be commented on.

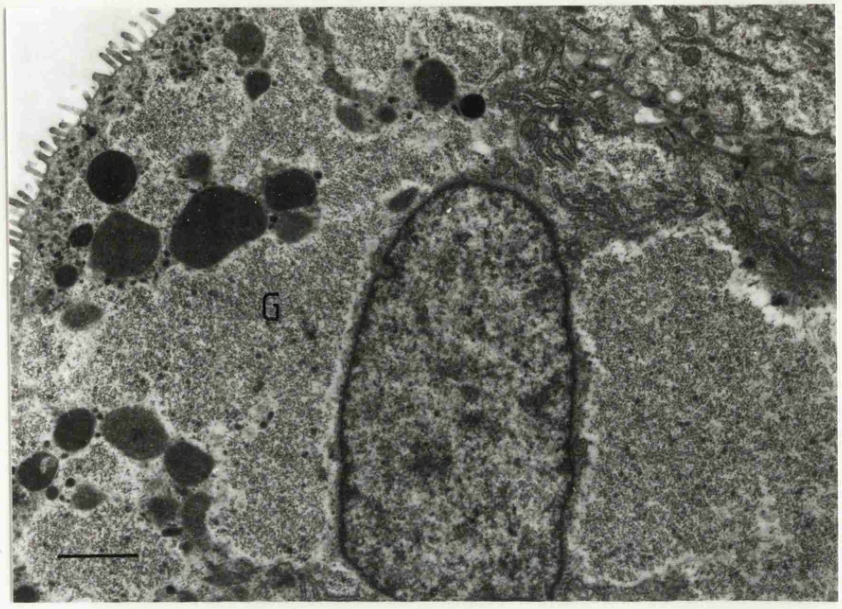


Plate 3.8 Glycogen deposits (G) in a 17.5 day *in vivo* yolk sac endoderm cell. Note the two configurations of glycogen (α and β). Bar = 2 μm.

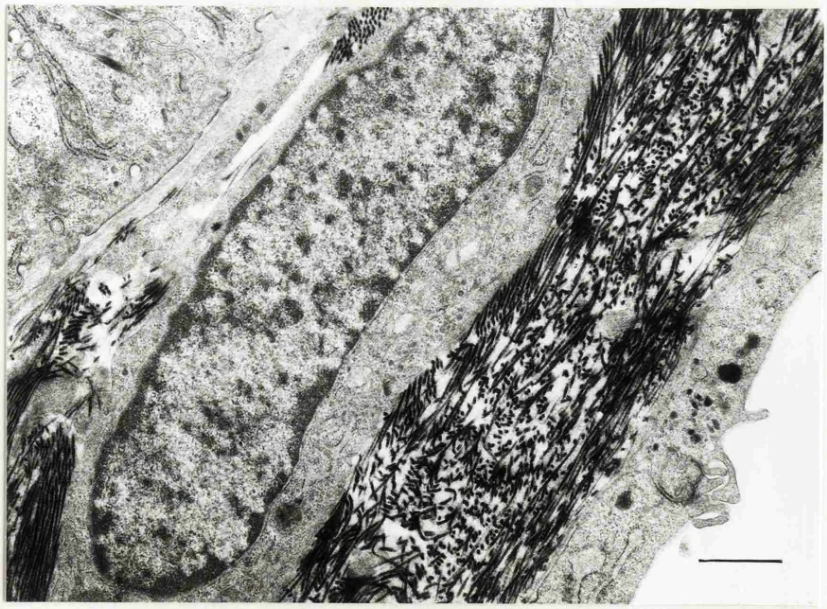


Plate 3.9 Large amounts of collagen fibres in the serosal basement membrane of 17.5 day *in vivo* visceral yolk sac. Bar = 1 μm.

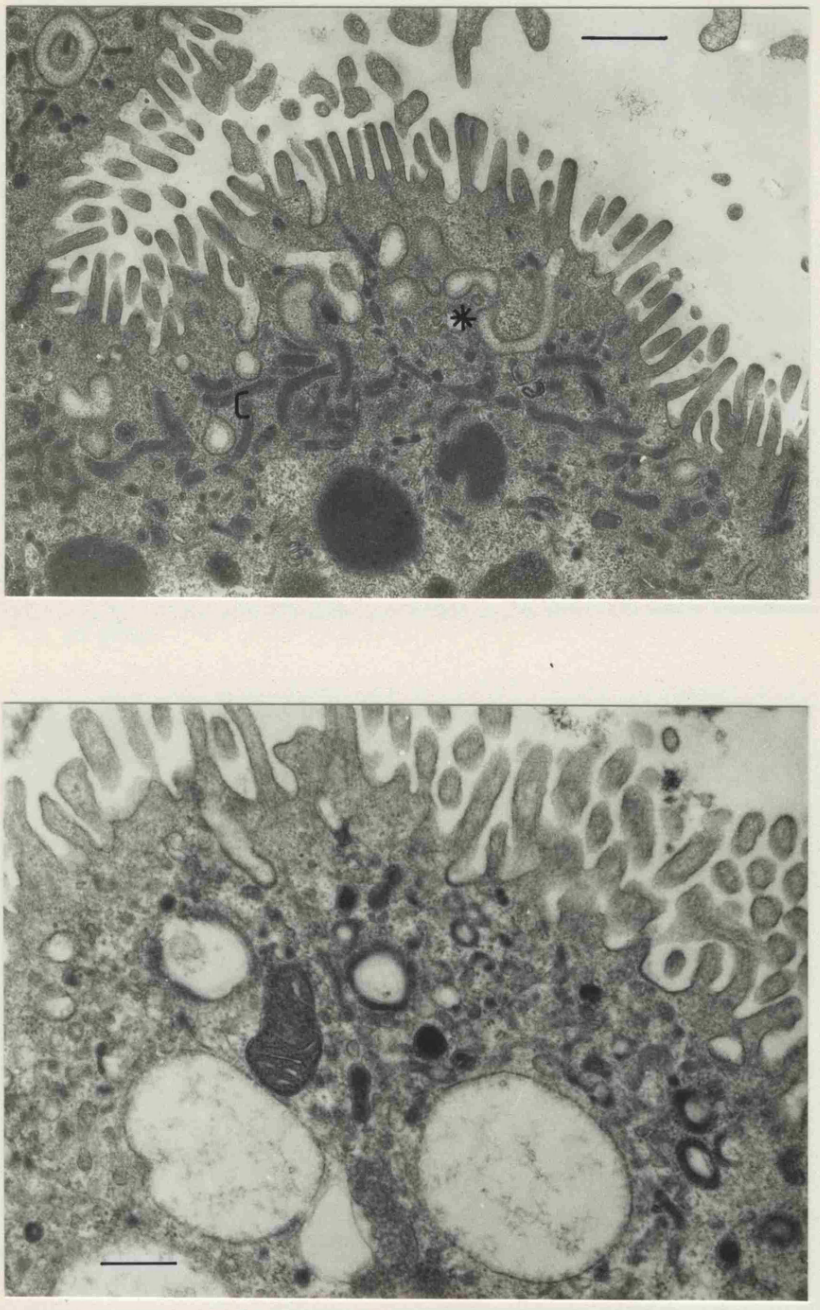


Plate 3.10 The apical cytoplasm in

(a) 17.5 day *in vivo* yolk sac tissue. Note the complex canalicular network (*) and numerous coated vesicles (C). Bar = 0.8 μm.

(b) 17.5 day 'giant' yolk sac tissue. The cannalicular system is much less complex. Bar = 0.5 μm.

1. Plate 3.10 (a) and (b) show the microvillous border and apical cytoplasm of both 17.5 day *in vivo* yolk sac and 'giant' yolk sac endoderm cells. The cannalicular system can be clearly seen in the *in vivo* tissue (b) but such a complex network is not present in the 'giant' yolk sac tissue (a). Plate 3.11 shows a coated invagination and vesicle at high magnification.

2. Plate 3.12 shows the junctional complex between the 'giant' yolk sac endoderm cells. There is a dilation of the intercellular space below the junctional complex and the lateral cell margins are convoluted in places.

3. Plate 3.13 (a) and (b) show membrane bounded structures containing numerous membrane profiles apparently free above the microvillous border, although (b) does appear to have a 'microvillous' like projection. Plate 3.13 (c) shows many similar structures appearing as the swollen tips of microvilli. These structures were also seen some distance from the microvillous border and not connected to it. Similar structures were seen in the intercellular spaces.

4. Plate 3.14 shows the autophagic sequestration of a portion of cytoplasm containing an electron dense vesicle. This process was not commonly seen in either 17.5 day *in vivo* yolk sac tissue or 'giant' yolk sac tissue.

5. Plate 3.15 shows two myelin figures situated in the region of a large deposit of glycogen. Myelin figures were more usually observed within the cytoplasm in both tissue types.

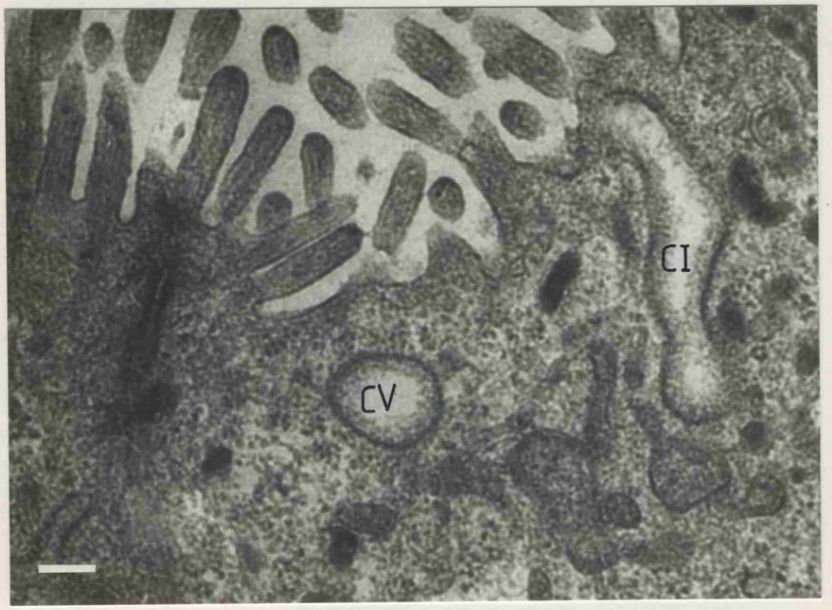


Plate 3.11 A coated invagination (CI) and coated vesicle (CV) in 'giant' yolk sac tissue. Bar = 0.2 μm.

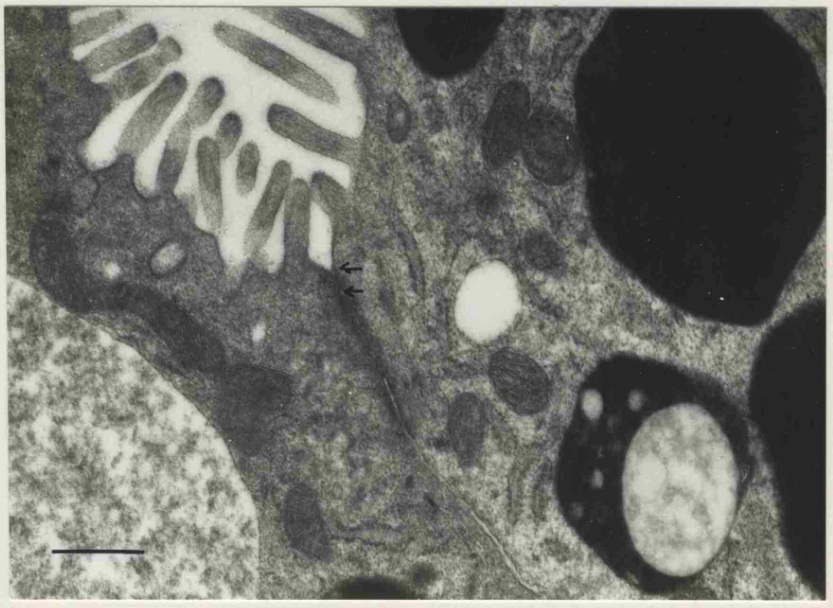
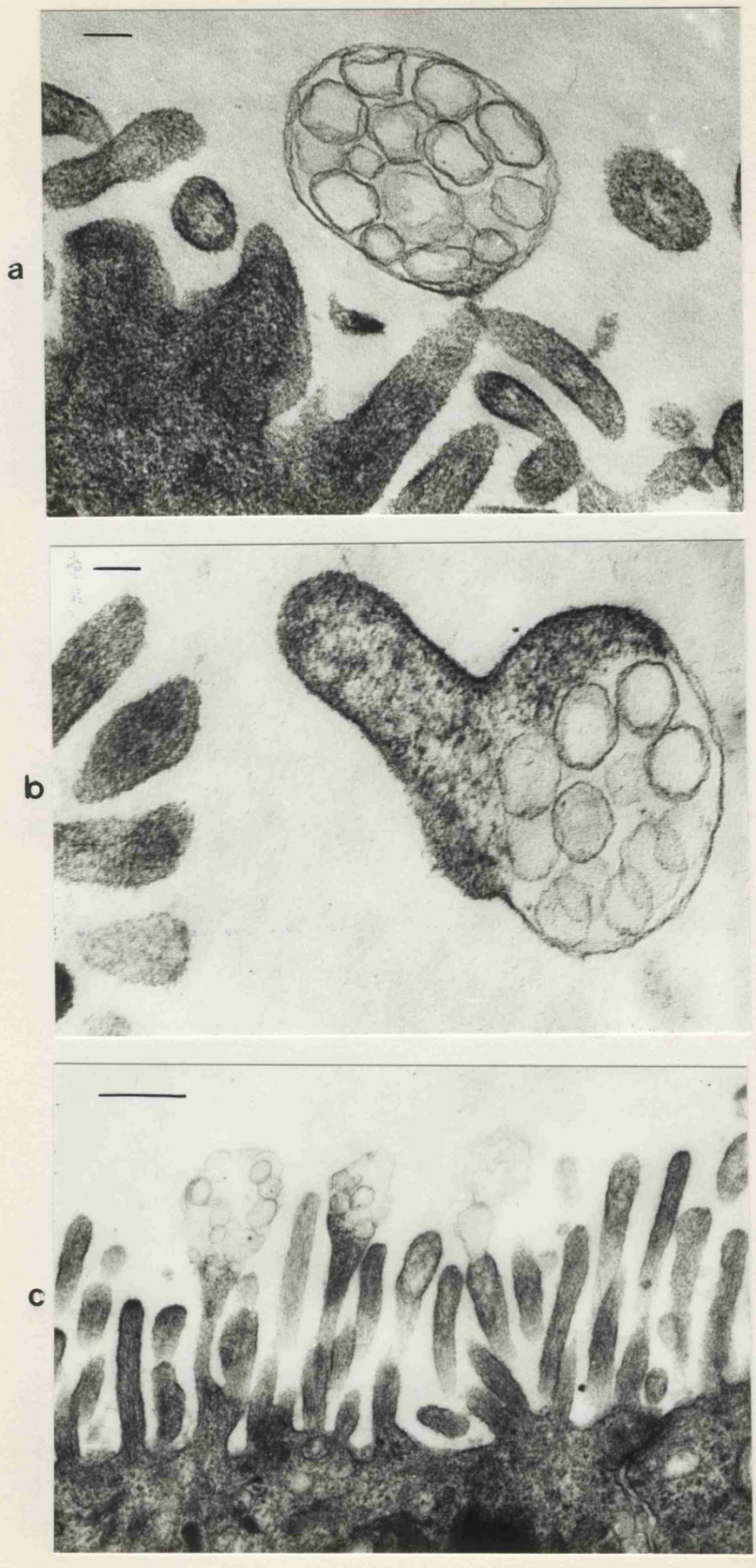


Plate 3.12 A junctional complex. Note particularly the region of the tight junction (between arrows). Bar = 0.5 μm.

Plate 3.13 Membrane bounded structures containing numerous membrane profiles,

(a) apparently free in the cytoplasm. Bar = 0.1 μm.
(b) with 'microvillous' like projection. Bar = 0.1 μm.
(c) appearing as the swollen tips of microvilli. Bar = 0.5 μm.


a
b
c

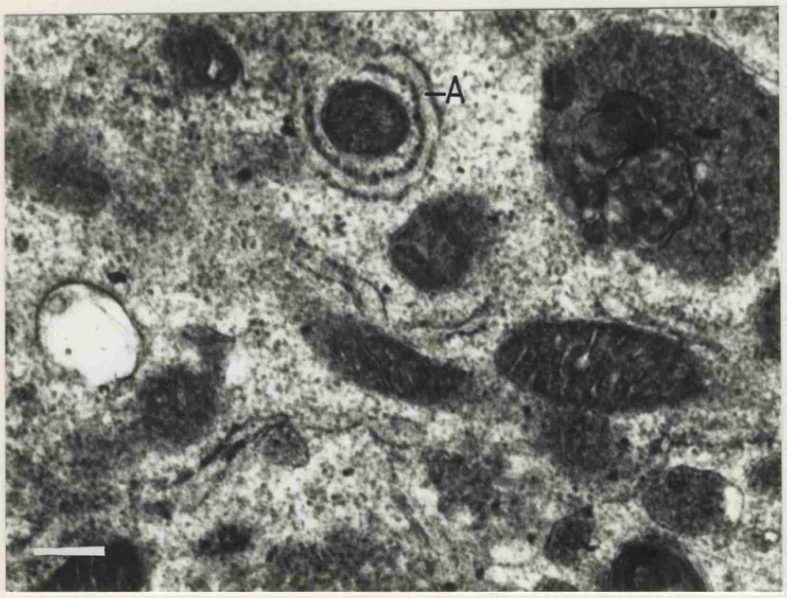


Plate 3.14 The autophagic sequestration of a portion of cytoplasm. The autophagic vesicle (A) is almost closed around an electron dense vesicle. Bar = 0.3 μm.

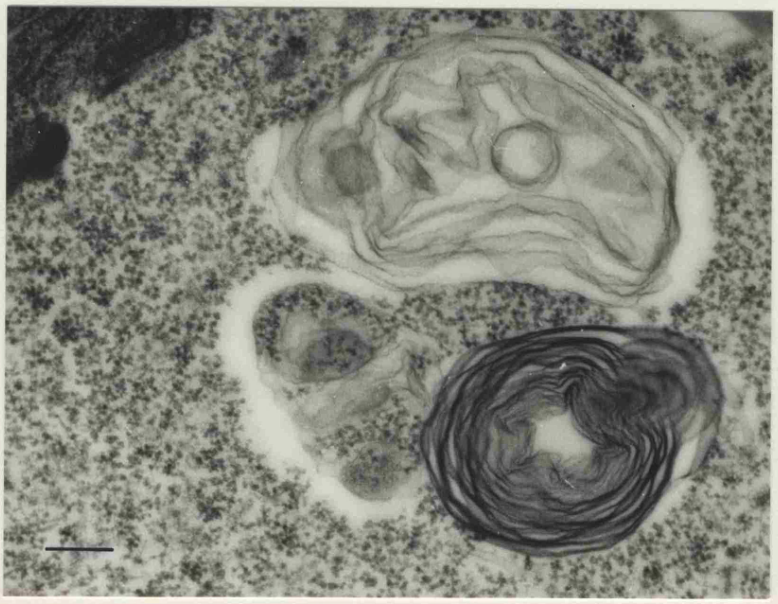


Plate 3.15 Myelin figures within a large glycogen deposit. Bar = 0.3 μm.

6. Plate 3.16 shows coated vesicles and coated pits at the basal cell surface of a 'giant' yolk sac endoderm cell. This may indicate that exocytosis is occuring.

3.3.4 Detailed observations of the external appearence and ultra-structure of GYS grown after removal of the embryonic tissue.

External structure of GYS after removal of embryonic tissue.

Plate 3.1a in section 3.3.1 shows a 9.5 day egg cylinder before and after removal of the embryonic tissue.

The edges of the yolk sac which adhere temporarily at the scalpel incision line, quickly grow together when incubated in the normal incubation conditions of whole rat serum with an atmosphere of 20% O_2;5%CO_2;75%N_2. Such healing will also occur in serum free Medium 199. SEM examination of the area of the incision after varying periods of incubation showed that the incision had healed well after 3 hours. Plate 3.17 shows the healed wound after 3 hours incubation in whole rat serum. Note the cells elongating along the margins of the wound and the collected cell debris along the line of the wound - features typically found in wound healing.

Once the tissue is healed the yolk sac quickly swells and becomes spherical, containing exocoelomic fluid. Plate 3.1b shows that at 11.5 days the yolk sac cultured by this modified method (right) was only slightly smaller than that cultured by the original method which at this

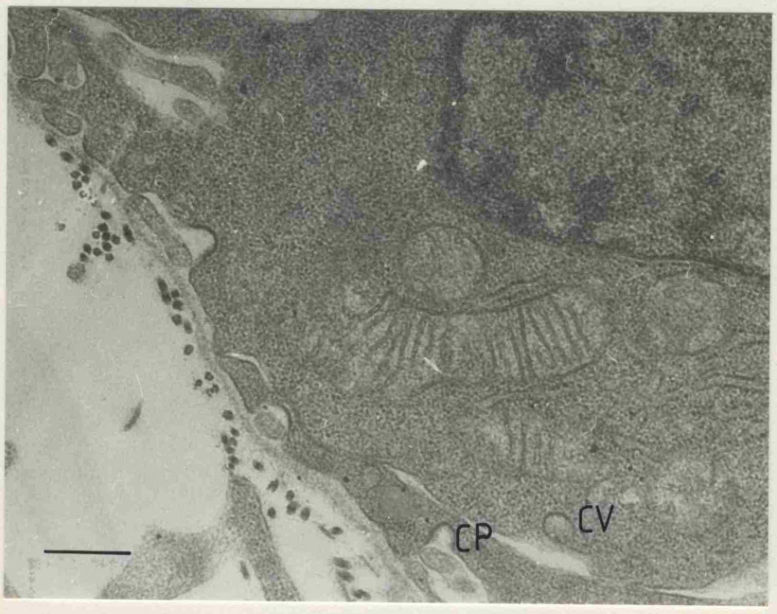


Plate 3.16 (?)Exocytosis occuring at the basal surface of 'giant' yolk sac endoderm cells. Note a coated vesicle (CV) and coated pits (P). Bar = 0.5 μm.

stage contained a well developed 25 somite embryo.

Plate 3.18 shows the pole opposite to the ectoplacental cone of a 17.5 day yolk sac.

It was observed that at approximately 11 days blood islands form in a circle around the ectoplacental cone. They reach their maximum size at around 11.5 days and then become gradually more faint and disappear at approximately 12.5 days. The blood islands are a deep red in colour. Plate 3.19 shows a typical corona of blood islands in a yolk sac at 11.5 days of gestation.

Electron microscopic examinaton of 'giant' yolk sac structure grown after removal of the embryonic tissue at 9.5 days.

Plate 3.20 shows the overall structure of 'giant' yolk sac tissue cultured by the modified method after prior removal of the embryonic tissue. It can be seen that the cell layers of the yolk sac do not differ significantly from the 'giant' yolk sacs cultured by the original method and described in detail in section 3.3.3. The only obvious difference in the morphology of the two systems is that 'giant' yolk sacs cultured without an embryo show no development of yolk sac vessels within the mesenchymal layer.

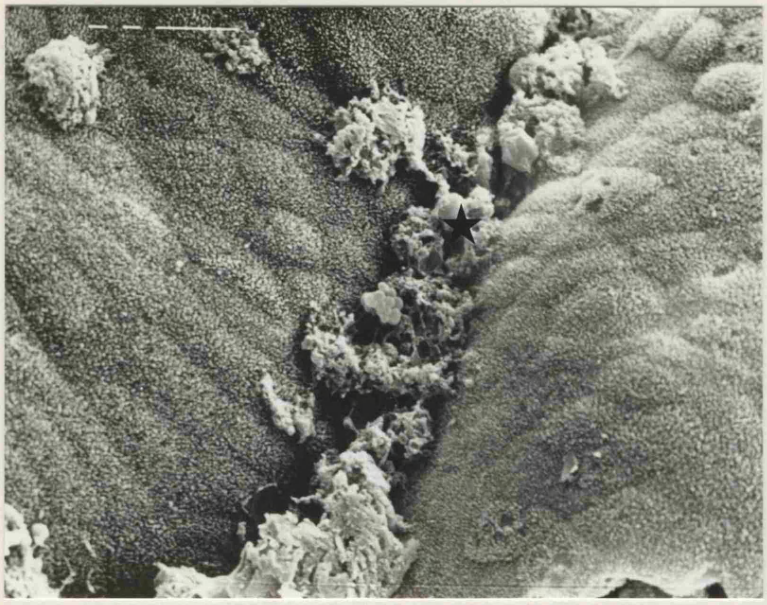


Plate 3.17 SEM of wound formed 3 hours after removal of the embryonic tissue from a 9.5 day egg cylinder. Note the line of cell debris (*) and the elongated cells along the margins of the wound. Bar = 10 μm.

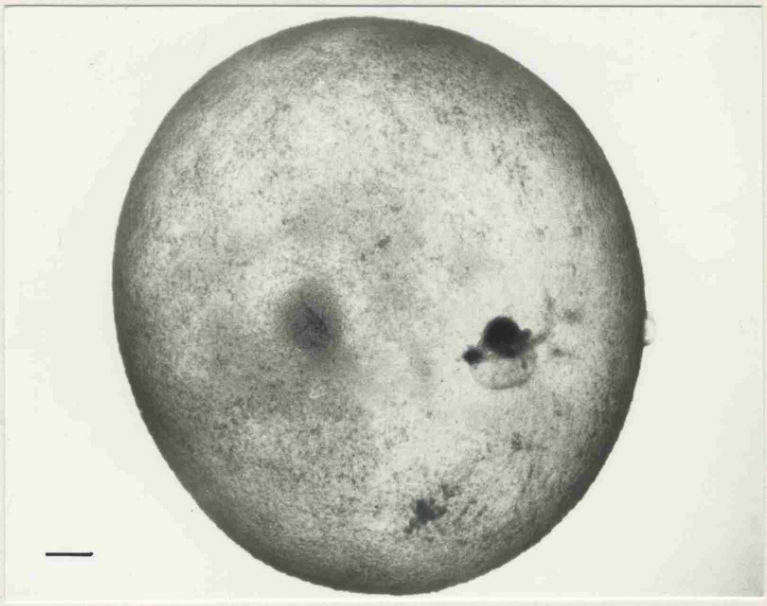


Plate 3.18 The pole opposite the ectoplacental cone in a 17.5 day 'giant' yolk sac, after the removal of the embryonic tissue at 9.5 days. Bar = 1.5 mm.

Plate 3.19 Typical corona of blood islands in an 11.5 day yolk sac. Bar = 1.5 mm.

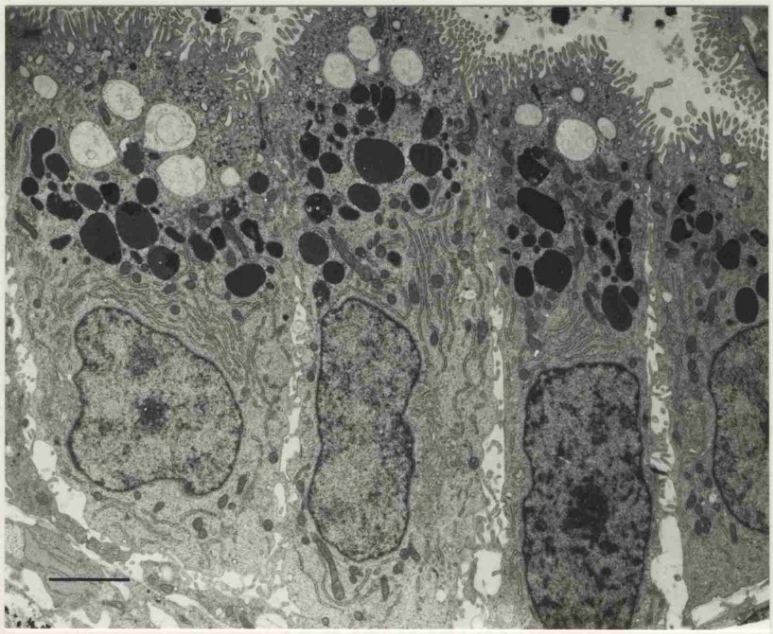


Plate 3.20 Electron micrograph of 'giant' yolk sac endoderm cells after removal of the embryo at 9.5 days. Bar = 3 μm.

3.4 Discussion.

The purpose of this comparative morphological study is to assess the potential of the 'giant' yolk sac culture techniques described in sections 2.5 - 2.8. It is essential that the integrity of the yolk sac tissue is maintained throughout culture and that prior to their utilisation in quantitative uptake experiments, the cells of the yolk sac appear healthy and functionally active. Comparison of cultured tissue with _in vivo_ tissue explanted immediately from the mother at 17.5 days emphasises that the fundamental differences between the systems, do lead to corresponding small differences in the cell morphology. This is inevitable but what is remarkable is that the cultured tissue (grown with or without the embryo) was shown overall to be very similar to _in vivo_ tissue, despite the prolonged period in culture and the lack of an actively developing embryo and a chorioallantoic placenta. As outlined in sections 3.3.3 and 3.3.4 the main difference in the morphology of 'giant' yolk sacs and 17.5 day _in vivo_ yolk sacs is the greatly enlarged vacuolar volume observed in the 'giant' yolk sac endoderm cells. Previous authors (eg Gupta et al. 1982) have suggested that the size of the vacuolar volume may be indicative of the rate of endocytosis. Although this is an oversimplificaton and the size of the vacuolar volume also depends on numerous other factors including the rate of lysosomal digestion and the rate of exocytosis, it does give some indication of the functional state of the cells. For this reason the difference in the vacuolar volume of endoderm cells in the 'giant' yolk sac and control yolk sac was quantitatively assessed using morphometric analysis (section 4) and will be discussed in greater detail in section 4.4.

In general terms the cultured cells appeared healthy, and dead or dying cells were rarely seen. Also there were many features present, typical of an active cell in which endocytosis and protein production were taking place.

Firstly the cells all possessed a well developed microvillous border and between the microvilli were frequent complex coated invaginations which formed a network in the apical cytoplasm. This so called cannalicular system was present in the cultured tissue although not always as well developed as in the in vivo tissue, and increased the surface area available for endocytosis. The presence of many small endocytic vesicles (both coated vesicles and uncoated vesicles) indicates that endocytosis is actively occurring. It has been shown that many coated vesicles lose their coats very soon and fuse to form larger electron lucent vacuoles. The large electron dense vacuoles are probably secondary lysosomes, although identification of lysosomes purely by morphology is difficult. The small electron dense vacuoles may be primary lysosomes. A histochemical study similar to that carried out by Beck et al.(1967) would be useful to clearly identify the various lysosomal components. At the end of lysosomal digestion all that remains within the lysosomal membrane are the undigestible lipid-rich residues which are often pigmented. These structures remaining are called residual bodies or telolysosomes and their appearance is often an indication of cell aging. Lipid droplets were also observed in both tissue types usually situated in the basal cytoplasm.

Other features typical of protein production are a prominent Golgi

apparatus around the nucleus and large quantities of rough endoplasmic reticulum. However definite proof of protein production, in comparison to that produced in vivo could be best illustrated by studying specific gene activity.

Membrane bounded structures containing many membrane profiles were observed frequently in both in vivo tissue and 'giant' yolk sac tissue, beyond the apical surface, attached to the microvillous border and within the intercellular spaces. They are organelles which may possibly be involved in membrane recycling and storage or in the destruction of damaged membrane through lysosomal digestion. Myelin figures, so called as their closely packed lamellar structure resembles myelin, are occasionally observed in both in vivo tissue and 'giant' yolk sac tissue, although their occurrence is by no means common. They are thought to arise following the partial breakdown of phospholipid containing cell components which cannot be fully digested in lysosomes.

One of the few significant differences between the cultured and in vivo yolk sac tissue is that the in vivo tissue contains large yet variable glycogen deposits in the majority of cells - such deposits are not seen in the 'giant' yolk sac. Glycogen is observed in both the clustered α configuration and the single particle β configuration. Each particle is approximately 30nm in diameter and it is their size (approx. twice that of free ribosomes) and the fact that they are not membrane associated, which allows identification. Glycogen can also be demonstrated histochemically using PAS stain. In the large glycogen deposits it was observed that the deposits were bordered by apparently clear cytoplasm. This is due to the partial or total dissolving of

glycogen during its processing.

Therefore the ultrastructure of the tissue of both 'giant' yolk sac systems appears normal and gives no indication of any unexpected abnormalities in cell function.

Comparing the external appearence of 'giant' yolk sacs cultured by the original and revised method, it is obvious that the purpose of modifying the technique was to remove any unknown effects caused by the presence of a dead, partially autolysed embryo. The revised culture method successfully removes such embryonic effects and the only apparent difference caused by the removal of the embryonic tissue is that the growth and development (in terms of yolk sac diameter) of the 'giant' yolk sac is retarded. In fact its final size was shown to be significantly lower than the 'giant' yolk sac containing a dead embryo. Direct measurement of the capacity of such yolk sacs to take up a range of ^{125}I-labelled substrates (chapters 5 - 7) will indicate if the removal of the embryonic tissue has any effect on the functioning of the visceral yolk sac. Hence the potential of this 'clean' system, free from embryonic effect will be evaluated.

An interesting observation in the culture of 'giant' yolk sacs after removal of the embryonic tissue is that blood islands still form in a corona around the ectoplacental cone. This correlates well with the results of Moore and Metcalf (1970) who cultured 7.5 day mouse embryos and yolk sacs together and separately. They found that haemopoesis could occur in the embryo but only after the migration of the stem cells from

the yolk sac, but that the yolk sac itself could initiate haemopoesis.

Blood cells arise in the peripheral mesoblast which probably derives its cells from the primitive streak. This ring of mesoblast is known as the area opaca of Kolliker and later becomes the wall of the yolk sac in most mammalian species. Hence the wall of the yolk sac is the site of blood cell formation (see review Bloom 1939). The primitive yolk sac mesodermal cells differentiate into the primordial blood cells and clump together to form a dense group - the blood islands. The more central cells forming the blood cells and the peripheral cells flattening to form the endothelium of the vessels. The primitive blood cells accumulate haemoglobin and become primitive erythrocytes. Blood cells are also formed in the liver.

In the rat blood formation starts on approximately day 9 with the migration of mesoderm in the yolk sac. Blood islands develop and by 11 days have become the distinct red colour after the onset of haemocytoblast formation. At 11.5 days the vitelline artery and vein are formed and they contain circulating blood cells.

The fact that blood islands develop in the 'giant' yolk sac after prior removal of the embryo at 9.5 days, indicates that the yolk sac alone can express the necessary genes for the development of the primitive erythrocytes in blood islands. However there is no major development of the vitelline circulation, as this is an epigenetic phenomenon related to haemodynamic factors. However recently some capillary development has been observed in 'giant' yolk sac tissue grown without the presence of an embryo (Rowlands - personal communication).

In conclusion therefore we can say that this morphological study has shown the tissue of the 'giant' yolk sac (cultured by both methods) to be apparently healthy and functionally active, with no significant amount of cell death or damage caused by the prolonged period in culture. Also although there are some differences between the cultured tissue and control tissue, removed immediately from the pregnant female, the potential advantages of the system with its unique spherical structure maintaining a separate exocoelom, make it ideal for the study of macromolecular uptake, haemopoesis and protein synthesis (to name just a few) and outweigh morphological differences.

SECTION 4

MORPHOMETRIC ANALYSIS OF THE VISCERAL YOLK SAC ENDODERM IN THE 'GIANT' YOLK SAC AND 18.5 DAY CONTROL YOLK SAC.

4.1 Introduction

The endoderm of the yolk sac placenta in the rat is a highly pinocytic tissue which has been shown to be important in histiotrophic nutrition, particularly during early organogenesis before the establishment of the chorioallantoic placenta. The importance of the visceral yolk sac in early embryonic nutrition was first suggested by Brunschwig (1927) and some of the important work carried out since then has been reviewed in section 1.8.

The pinocytic uptake of macromolecules by the visceral yolk sac has been investigated *in vitro* and to a lesser extent *in vivo* by many workers using a wide variety of techniques, which have already been discussed (section 1.8). The rate of pinocytic uptake and the digestion of radiolabelled macromolecules has also been measured directly and will be discussed in detail in chapters 5-7.

Many authors (Cohn 1966;Cohn and Parks 1967;Westwood and Langstaff 1976) have used morphometric analysis as a method of quantifying pinocytosis. They have related the number of pinocytic vacuoles or the vacuolar volume to the rate of pinocytosis. It has since been realised that the size of the vacuolar system cannot be directly related to the rate of pinocytic uptake by a cell as many other factors also contribute to the morphology of the vacuolar volume. However morphometric analysis of the vacuolar volume gives an overall picture of the functional state of the cell types being studied.

In this study the size of the vacuolar volume in the visceral endoderm of 'giant' yolk sacs was compared to that in control *in vivo* yolk sacs at the same gestational age. The study was carried out employing stereological principles from which it is possible to derive three-dimensional information from two-dimensional images (Loud et al 1965; Weibel 1969).

4.1.1 The principles of stereology.

Although stereology has been used for many years in geology it was only relatively recently that its application to biological studies has been fully realised (Weibel 1963). The mathematics on which it is based are much older. The parameters open to study include fractional component volumes and surface areas, mean particle volumes and surface area and particle numbers and size.

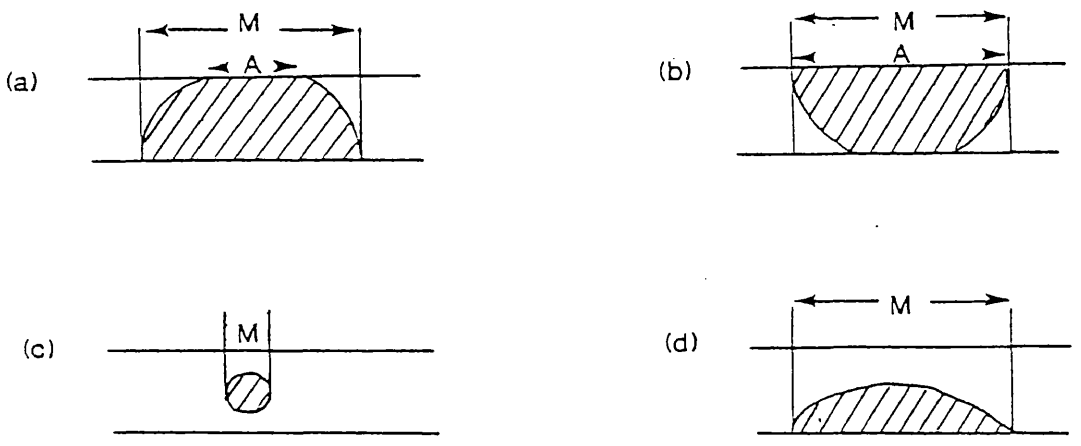
The nineteenth century French geologist Delesse and the English microscopist Henry Sorby independently established the principle that stated "on average the fractional area of a particular sort of component in a section taken of a solid body, is directly proportional to the fractional volume of that component in the original solid body"(Delesse 1848). The mathematical derivation of the Delesse principle will not be detailed here but is discussed at length by Williams (1977).

4.1.2 The importance of section thickness in stereology.

The ideal specimen for stereology would be a section of zero thickness the plane of which would pass through organelles of perfect

contrast. In practice sections for electron microscopy have a thickness of 40-100nm and therefore certain problems can arise.

Figure 4.1 The Holmes effect (Holmes 1927).



The calculations used in stereology are based on the notion that the objects measured are perfectly two-dimensional. The upper surface is taken to be the sectional plane from which measurements should be taken. Because the section has a finite thickness, the measured length M may exceed the true length A (Figure 4.1a). However in Figure 4.1b A=M. In Figure 4.1 c and d, although the particles do not intercept the measurement plane, they will still be recorded (A=0 but M is significant), because they can be seen in the micrograph. The magnitude of this effect depends on the size of the organelles relative to the section thickness.

Particles are rarely of perfect contrast and parts of the image are likely to be lost due to the limitations of staining techniques. Thus for most cellular objects both the Holmes effect and the "lack of contrast" effect will have an effect but since they oppose each other they will tend to cancel each other out to some extent.

4.1.3 Measurement of volume fractions.

Since the Delesse principle was derived and stereology has become an important method of morphometric analysis several methods of measuring volume fractions have been used.

(i) An early method was to cut up micrographs with scissors and weigh the different components. The weight of a particular component was expressed as a percentage of the total weight of the micrograph.

(ii) Using planimetry discrete particles can be measured rapidly and directly in mm^2.

(iii) Linear analysis makes use of an array of equally spaced parallel lines (Rosival 1898; Loud et al. 1965). The method involves measuring the total length of lines overlying the item in question and expressing this as a fraction of the total length of the lines overlying the whole micrograph.

(iv) Point counting makes use of a lattice of equally spaced points often in the form of a square lattice although triangular or hexagonal lattices can also be used. The total number of points which fall over the component are expressed as a fraction of the total points over the whole micrograph. The closer together the points are spaced the more accurate the analysis.

4.2 Materials and Methods

In this morphometric study the size of the vacuolar volume in the endoderm cells of the 18.5 day 'giant' yolk sac was compared to the vacuolar volume in the 18.5 day *in vivo* control visceral yolk sac, explanted directly from the pregnant female.

4.2.1 Explantation of 17.5 day in vivo yolk sacs and culture of 'giant' yolk sacs.

The explantation of 18.5 day *in vivo* control yolk sacs was carried out as described in section 2.9. The process was carried out as quickly as possible in sterile Hank's BSS with minimal tissue handling, to avoid tissue deterioration before fixation. The whole yolk sacs were washed thoroughly in fresh Hank's BSS and immediately placed in 3% glutaraldehyde in 0.1M phosphate buffer at 4°C for 2 hours as described in section 3.2.5.

'Giant' yolk sacs were cultured as described in section 2.5 and at 18.5 days were harvested as outlined in section 2.6. After measuring the yolk sac diameters and removing the extraembryonic coelomic fluid and the amniotic fluid, the remains of the partially autolysed embryo and the amnion were separated from the visceral yolk sac. The yolk sacs were washed well in fresh Hank's BSS and immediately placed in 3% glutaraldehyde in 0.1M phosphate buffer at 4°C for 2 hours.

4.2.2 Preparation of tissue for electron microscopy.

After initial fixation in 3% glutaraldehyde, specific areas of both tissue types were washed in sucrose buffer, post fixed in 2% osmium tetroxide for 1 hour at 4°C, dehydrated through graded ethanol solutions at room temperature and embedded in araldite. A detailed description of tissue processing for electron microscopy is given in section 3.2.5.

To carry out a meaningful comparison of the vacuolar volumes of the yolk sac endoderm in different experimental and control groups, it was essential to section identical areas of the visceral yolk sac. The highly folded region of both the 'giant' yolk sac and the *in vivo* yolk sac was the area selected for post-fixation in osmium tetroxide, dehydration and embedding.

4.2.3 Orientation of the tissue.

Semi-thin sections were cut on the Reichert-Jung OMU4 microtome and stained with toluidine blue. These sections were examined under the light microscope to assess the orientation of the endoderm cells of the yolk sac. For the purpose of this investigation it was essential that the plane of section was longitudinal, showing the endoderm cells as a single row of columnar epidermis with an apical microvillous border and a basally situated nucleus.

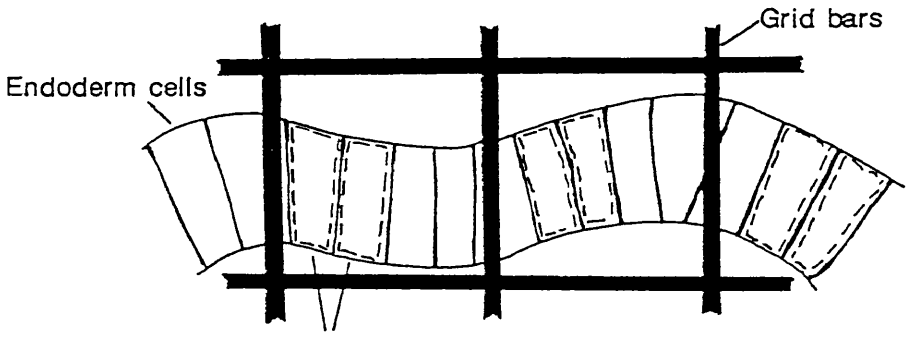
Having aligned the tissue block to obtain suitable semi-thin sections, ribbons of ultra-thin, pale gold/silver sections (70nm thick) were cut from each block and mounted on 200 micromesh copper grids.

These were stained with uranyl acetate and lead citrate using the method of Reynolds (1963).

4.2.4 Microscopy

Stained sections of both 'giant' yolk sac and control yolk sac tissue were observed under the Jeol 100S electron microscope at an accelerating voltage of 80KV. A series of micrographs were taken of whole endoderm cells at a magnification of 7000. These were a sample of cells randomly selected as shown in Figure 4.2. The micrographs were printed up to a final magnification of 14,000 and montages of whole endoderm cells were constructed.

Figure 4.2 Random sampling.



4.2.5 Morphometric analysis.

The MOP AM02 quantitative analyser and Apple computer (Plate 4.1) were used to simplify the mophometric analysis, which was based on a

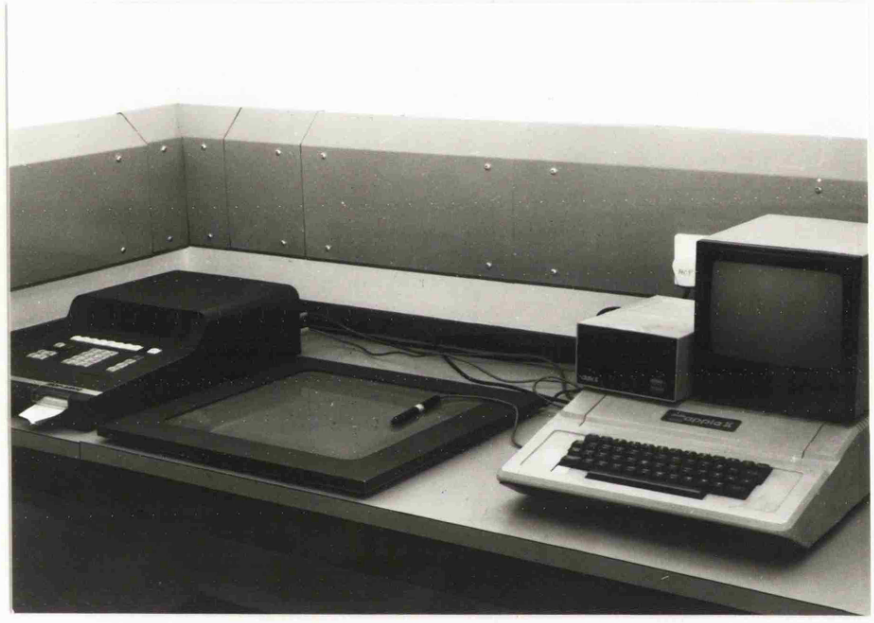


Plate 4.1 MOP AM02 quantitative analyser and Apple computer.

system of point counting. A programme adapted for the Apple computer, calculated the volume density of the vacuolar system as a percentage of the total cytoplasmic volume, from measurements of the relative areas of the cytoplasm and vacuolar system in each cell.

4.2.6 Sample size

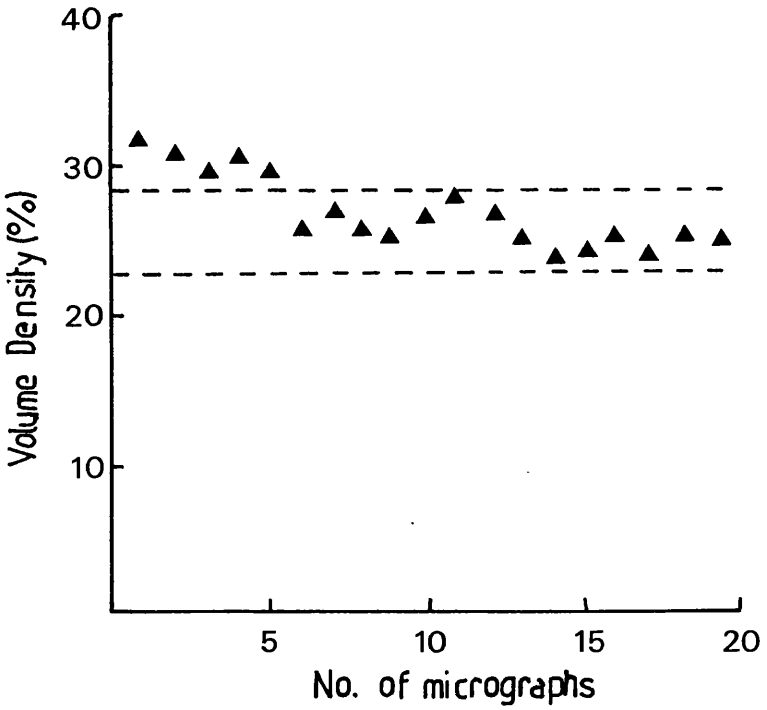
In this experiment two 'giant' yolk sacs cultured from 9.5 day embryos explanted from each of three different females were used (six in total) , and two yolk sacs which were obtained from three 18.5 day pregnant rats served as controls. Two ribbons of sections were cut from each tissue block.

The volume density of the vacuolar system in this randomly selected sample of cells from each yolk sac was measured. To ensure a large enough sample had been used, which would ensure good levels of accuracy, a cumulative mean plot was constructed for each yolk sac (Williams 1977). An example of a cumulative mean plot is shown in figure 4.3. The sample size required to achieve and remain within +/- 10% of the final cumulative mean was taken as the minimum sample size for the measurement of the volume density of the vacuolar system.

The cumulative mean plots obtained with the 18.5 day *in vivo* control yolk sacs showed that the cumulative mean reaches and remains within 10% of the final cumulative mean after analysis of 10-12 endoderm cells. The cumulative mean found using 'giant' yolk sac tissue remains within 10% of the final cumulative mean after analysis of approximately 15 endoderm cells. Therefore in order to obtain a representative sample

20-25 endoderm cells were analysed from each yolk sac. A mean value for the volume density of the vacuolar system in each yolk sac was found and the means from all the yolk sacs in each experimental group were combined to give the overall means and standard errors (SEM) quoted. Statistical comparisons of 'giant' yolk sac and control yolk sac tissue were then made using the Student's T-test.

Figure 4.3 Example of a cumulative mean plot.



4.3 Results

The morphological appearance of the visceral yolk sac endoderm cells of the 18.5 day *in vivo* control yolk sac and the 18.5 day 'giant' yolk sac is shown in Plates 4.2 and 4.3 and has been discussed in detail in chapter 3. In both tissues the cells are columnar, with a microvillous border at the apical surface and a basally situated nucleus. The apical cytoplasm contains the vacuolar system and from morphological observations this appears to be considerably larger in the cultured 'giant' yolk sac tissue than in the control yolk sacs. It should be noticed that in the 'giant' yolk sac tissue the number as well as the size of the vacuoles appears to have increased.

Gupta et al (1982) classified the endocytic vacuoles observed in the yolk sac endoderm cells of 9.5 and 11.5 day conceptuses according to their morphology and situation in the cell. These classes were (a) small vacuoles located in the apical region of the cell at the base of the microvilli; (b) larger vacuoles varying in form between completely empty types to those containing lightly flocculent material; (c) vacuoles containing flocculent material and a homogeneous electron-dense area; and (d) vacuoles consisting entirely of electron-dense material. All these classes of vacuoles were observed in both 'giant' yolk sac and control tissue and although the relative proportions of each varied between the tissues, in this study the vacuolar volume was measured as a whole and not subdivided. All the aforementioned classes of vacuoles have been shown to be involved in endocytosis by culturing the visceral yolk sac in the presence of iron dextran and ferritin (Gupta et al. 1979).

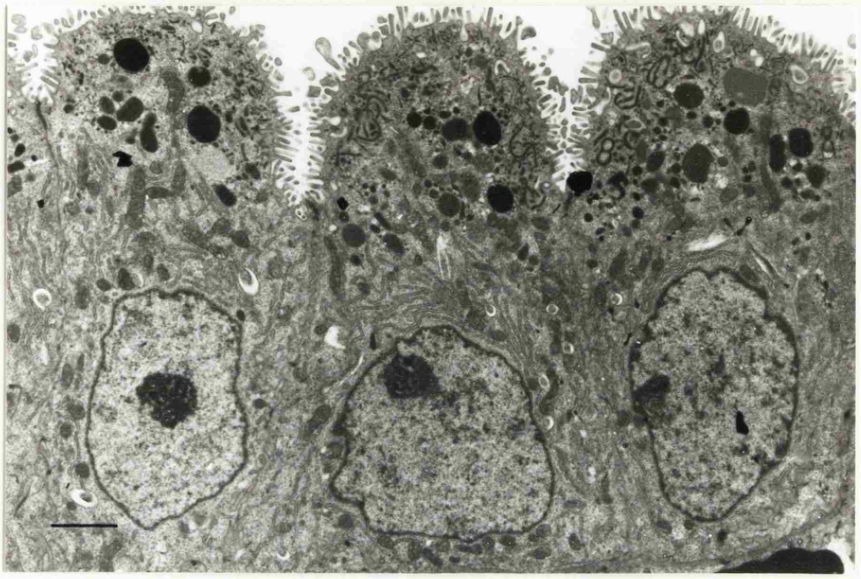


Plate 4.2 Electron micrograph of 17.5 day *in vivo* endoderm cells orientated for morphometric analysis. Bar = 2 μm.

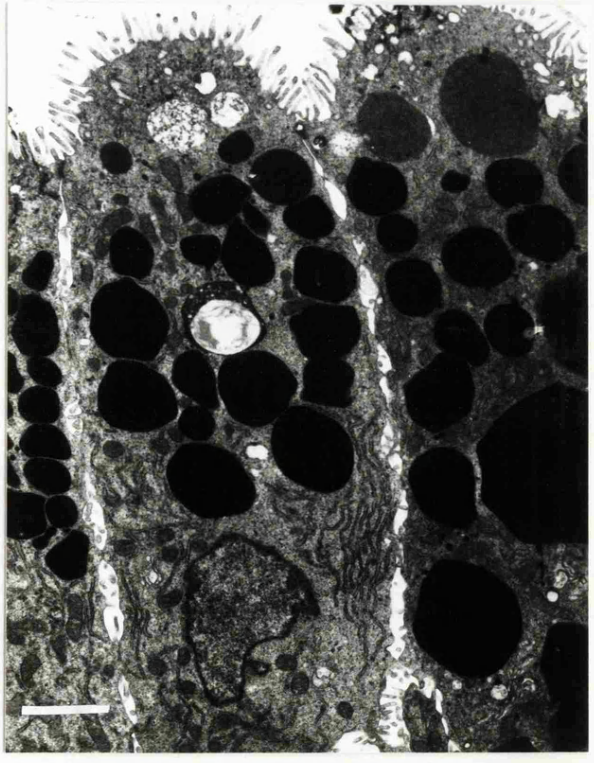


Plate 4.3 Electron micrograph of GYS endoderm cells orientated for morphometric analysis. Bar = 2 μm.

Table 4.1. Results of morphometric analysis on the rat visceral yolk sac endoderm.

Volume density of the vacuolar compartment expressed as a percentage of the total cytoplasm.

	In vivo	In vitro
Day 18.5	6.65 +/- 0.34	* 23.75 +/- 0.52

* Significantly different from in vivo, $P<0.01$.

Each value is a mean +/- SEM for 6 yolk sacs.

Table 4.2 Results of morphometric analysis on the rat visceral yolk sac endoderm (from Gupta, Gulamhusein and Beck 1982).

Volume density of the vacuolar compartment expressed as a percentage of the total cytoplasm.

	In vivo	In vitro
Day 9.5	15.82 +/- 0.51	16.61 +/- 0.59
Day 11.5	10.68 +/- 0.21	11.20 +/- 0.64

Each value is a mean +/- SEM for 6 yolk sacs.

The results obtained from the morphometric analysis of the vacuolar volume of endoderm cells of the 'giant' yolk sac and control 18.5 day *in vivo* yolk sacs are presented in Table 4.1. The vacuolar volume is expressed as a percentage of the total cytoplasmic volume in the two tissues. Table 4.2 shows results obtained in a similar study comparing the vacuolar volume in the visceral yolk sac endoderm cells of conceptuses removed directly from the mother with those cultured *in vitro* at 9.5 and 11.5 days (Gupta et al. 1982).

Gupta et al. (1982) found that there was no significant difference between the yolk sacs grown *in vivo* and those cultured *in vitro* both at 9.5 days and 11.5 days. However there was a significant difference ($P<0.01$) between the volume density *in vivo* at 9.5 and 11.5 days and there was a similar significant difference between the volume density *in vitro* at 9.5 and 11.5 days.

The results obtained in this study at 18.5 days showed a significant difference ($P<0.01$) in the vacuolar volume of *in vivo* yolk sacs and 'giant' yolk sacs. the latter having a significantly larger vacuolar volume.

The gradual build up of pinocytosed material in the endoderm cells of the 'giant' yolk sac during development *in vitro* has been observed, although morphometric analysis was not carried out at stages between 11.5 and 18.5 days. Plates 4.4 - 4.6 show endoderm cells from 'giant' yolk sacs at various stages of gestation. The vacuolar volume can be seen to increase gradually with gestational age.

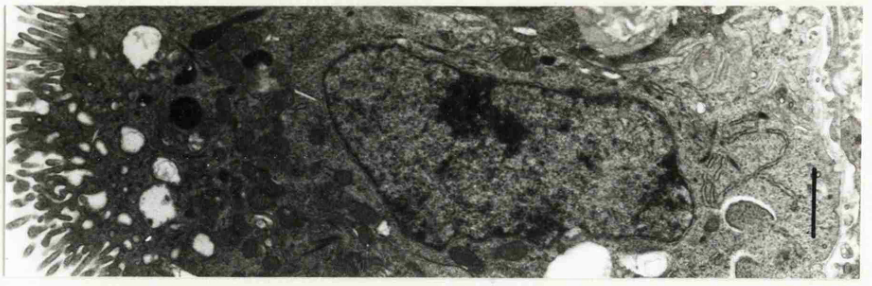


Plate 4.4 11.5 day 'giant' yolk sac endoderm cell. Note the long slender microvillous border and the greatly reduced vacuolar volume. Bar = 2 μm.

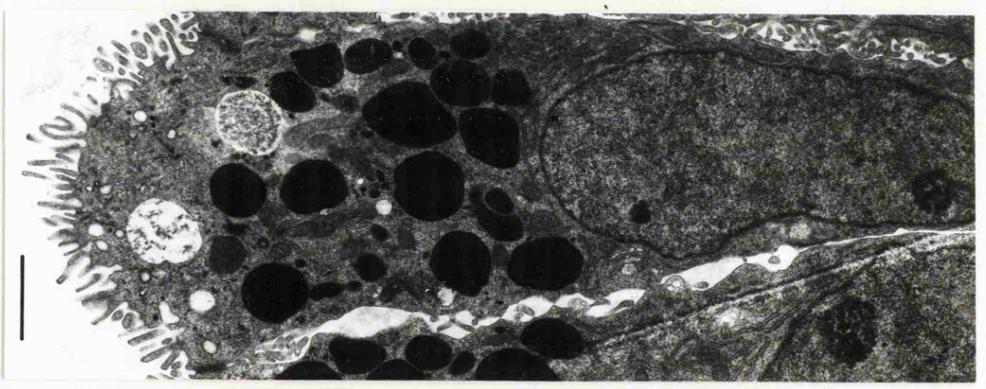


Plate 4.5 13.5 day 'giant' yolk sac endoderm cells. Already a 'typical' increase in vacuolar volume is apparent. Bar = 2 μm.

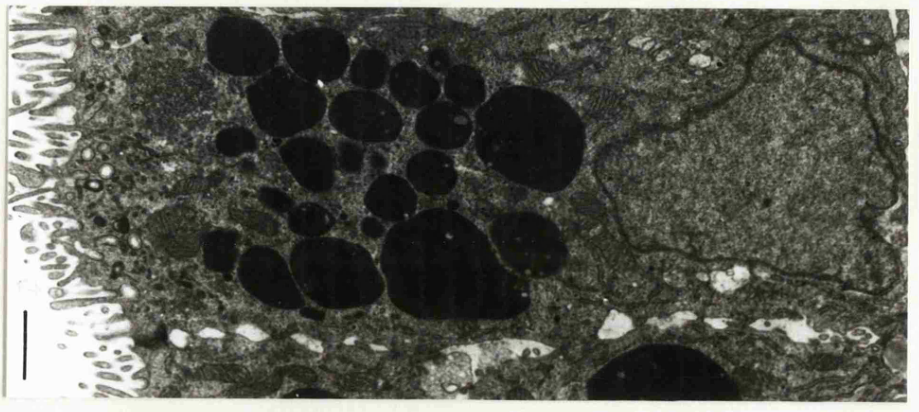


Plate 4.6 15.5 day 'giant' yolk sac endoderm cell, showing enlarged vacuolar system. Bar = 1 μm.

4.4 Discussion

When considering the morphological appearance of a cell it is essential to first assimilate as much information as possible about the condition of the cell and its environment, particularly if there are any differences from the situation *in vivo*. Conclusions can then be drawn from the results and any hypotheses made.

The lack of a developing embryo within the 'giant' yolk sac means that the nutritional requirements of the system are considerably lower than the system *in vivo*. Therefore the processed histiotroph is utilised only by the growth of the 'giant' yolk sac itself. This could result in an excess of internalised macromolecules which could accumulate within the tissue of the yolk sac, as the concentration of products in the exocoelom is increased. It might be postulated that a feedback mechanism is involved, based on a build up of the products of histiotrophic digestion, due to the intact nature of the 'giant' yolk sac.

We must consider the results obtained in this study in the light of results obtained previously as part of a similar study (Gupta et al. 1982) investigating the volume density of the vacuolar compartment of 9.5 and 11.5 day yolk sac endoderm cells cultured *in vitro* and *in vivo*. Looking first at the normal situation *in vivo* it is apparent that the vacuolar volume decreases in size with gestational age. This appears to fit in with the concept that histiotrophic nutrition supports the embryo totally until the establishment of the chorioallantoic placenta. As the chorioallantoic placenta begins to function at 11.5-12.5 days the nutritional requirements of the embryo are met by both the yolk sac

placenta and the chorioallantoic placenta; hence the rate of activity in the yolk sac endoderm may actually be reduced. In vivo the actively developing embryo utilises all the nutritional material provided by both placentas and therefore no excess material accumulates within the yolk sac endoderm cells.

In vitro the volume density of the vacuolar system decreases with gestational age between 9.5 and 11.5 days exactly as seen in vivo. As these two situations have been shown to be functionally very similar at these stages (Freeman et al. 1981) the biological condition of the in vivo yolk sacs should also apply in vitro. However, as has already been stressed in vitro the chorioallantoic placenta does not develop and although the yolk sac continues to grow and develop beyond 11.5 days, the embryo soon dies and is partially autolysed. This system clearly has two main differences from the situation in vivo and corresponding differences in the vacuolar system seem reasonable.

Various considerations need to be taken into account when assessing the value of such a study. It is essential to choose a magnification on the electron microscope which is great enough to measure the majority of the endocytic vacuoles and hence produce results which are accurate. However this must be counter balanced with the practical difficulties of handling and analysing montages of a very large size. Also it is essential to use the same magnification throughout so that comparisons between different specimens can be made. A final point to consider is that in a single section it is often impossible to distinguish between membrane bounded vesicles which are true intracellular vacuoles and those which are simply parts of the

cannalicular system in cross section and therefore external to the cell. It would be possible to distinguish between these two alternatives by taking serial sections of each cell or by staining the surface before sectioning. However it could be argued that the coated invaginations are themselves a functional part of the vacuolar system. For the purpose of this comparison the Golgi apparatus and canalicular system were not assessed as part of the vacuolar volume.

It is an oversimplification to say that the volume of the vacuolar compartment can be directly correlated to the rate of endocytosis in the visceral yolk sac. The functions of the whole vacuolar system are intimately linked to the age of the cell, the rate of lysosomal digestion and hence the activity of the lysosomal enzymes. The rate of exocytosis and membrane recycling are also important, as could be the subsequent utilisation of the processed histiotroph by a developing embryo if present and the yolk sac itself.

Although one cannot draw definite conclusions about the rate at which the *in vivo* yolk sac and 'giant' yolk sac are taking up and digesting macromolecules from their immediate environment, it is possible to gain some information.

In the *in vivo* 17.5 day yolk sac the presence of numerous coated pits and coated vesicles at the apical surface indicates pinocytosis is taking place. The comparatively small vacuolar volume emphasises the delicate equilibrium of efficient endocytic function. The membrane involved in endocytosis is being rapidly recycled.

However with the 'giant' yolk sac system several environmental factors differ from the situation *in vivo* (section 2.5) and corresponding differences in functional activity are inevitable. The presence of normal coated pits and vesicles indicates endocytosis is taking place. Also the presence of secondary lysosomes indicates digestion may be taking place. The preliminary information observed here together with a direct measure of the pinocytic uptake and digestion of certain radio-labelled macromolecules (chapters 5-7) should prove valuable in the elucidation of yolk sac function.

SECTION 5

THE UPTAKE OF ^{125}I-POLYVINYLPYRROLIDONE (PVP)

5.1 Introduction.

Pinocytosis is an important method by which cells take up substrates from their immediate external environment, within membrane limited vesicles (see section 1.3 for a full description). Jacques (1969) proposed two mechanisms by which a substrate can enter a cell pinocytically. Firstly uptake can be in the fluid phase, following the engulfment of microscopic droplets of extracellular fluid and secondly substrates can enter the cell adsorbed to the external surface of the plasma membrane, which subsequently invaginates to form the limiting membrane of a pinocytic vesicle.

For many years the mechanisms of pinocytosis remained unexplored until the process was analysed quantitatively. Many authors have studied pinocytic uptake using single cell systems such as macrophages (eg Kooistra et al. 1981). Details of these studies will not be discusssed here but are reviewed at length by Duncan and Pratten (1985).

The rat visceral yolk sac is an ideal organ on which to study the pinocytosis of a range of macromolecules as it is solely responsible for early embryonic nutrition in this species. The situation *in vivo* is very complex involving interaction between the embryo, the yolk sac placenta, the chorioallantoic placenta and the maternal system and to obtain meaningful results a method of organ culture is helpful.

Williams et al.(1975a,b) devised a method for studying quantitative aspects of pinocytosis, in which the visceral yolk sac at 17.5 days of gestation can be removed from the pregnant female and

maintained in organ culture. Over the incubation period the tissue was shown to remain morphologically and apparently functionally intact. Results using the system show that various ^{125}I-labelled substrates exhibited a reproducible, linear rate of uptake over an eight hour incubation period. Also a universal unit for describing the rate of pinocytosis was devised. The Endocytic Index (EI) was defined as the quantity of fluid (µl) whose contained substrate was captured per unit quantity of tissue (mg) per hour . The units are therefore µl/mg tissue protein/hour. Obviously when uptake is purely in the fluid phase the EI is an accurate representation of the volume of fluid captured. However when substrates are taken up bound to the plasma membrane the EI is a convenient unit for describing the uptake but does not indicate the true volume of fluid captured.

^{125}I-labelled polyvinylpyrrolidone (PVP) is a macromolecule with a mean molecular weight of 30,000 - 40,000 and has been used extensively as a marker of fluid phase pinocytosis. It is taken up by the endoderm cells and being resistant to lysosomal digestion accumulates within the tissue. As the release of ^{125}I-PVP from the tissue has been shown to be minimal, this accumulation within the tissue represents accurately the total uptake (Williams et al. 1975a).

Roberts et al. (1977) studied the uptake of three chemically diverse non-protein substrates by 17.5 day rat yolk sacs explanted directly from the mother. The substrates used were ^{125}I-PVP, U-^{14}C -sucrose and colloidal ^{198}Au gold and all were shown to have a similar EI in the identical incubation conditions used. In agreement with the earlier work of Williams et al. (1975a) the EI in each case was found to

be approximatelly 2µl/mg/hour. This shows that either all three enter the cell in the fluid phase or all enter the tissue bound to the plasma membrane to exactly the same extent. As the three have very different chemical properties and molecular weights, the latter seems unlikely and it seems that all are entering in the fluid phase. This was also indicated by the fact that the EI of these macromolecules was independent of substrate concentration in the incubation medium over a wide range (sucrose 0.1µg/ml - 10mg/ml and PVP 0.15µg/ml -1mg/ml). In contrast other macromolecules, for example formaldehyde denatured ^{125}I-labelled bovine serum albumin (discussed at length in section 6) was shown to have a much greater EI, which was seen to be dependent on the concentration of BSA in the incubation medium. There is competition for uptake between the labelled and unlabelled protein which is obviously taken up bound to the plasma membrane at saturable sites (Williams et al. 1975b)

Although this incubation technique has provided us with an insight into pinocytosis by the rat visceral yolk sac, the system does have its limitations. The 'giant' yolk sac however, maintained intact, in culture from 9.5 days - 17.5 days provides an ideal system for the study of pinocytic uptake as the visceral yolk sac is intact and maintains the exocoelomic fluid separately from the incubation medium. This means that the 'giant' yolk sac can be used to study the total uptake and release of macromolecules both back into the incubation medium and also into the exocoelomic fluid. Obviously this is of particular importance when studying functionally important macromolecules such as IgG some of which is taken up and broken down in the yolk sac tissue and some of which crosses the yolk sac intact. The directional nature of such transport

mechanisms, which until now has been impossible to study using the available methods of organ culture, can be investigated. The system does however have its limitations, as will become apparent in section 7.

However before investigating the complex uptake and processing of macromolecules digested within the visceral yolk sac it is important to establish the base line rates of fluid phase pinocytosis in a variety of incubation conditions. This serves to assess the functional activity of the 'giant' yolk sac systems in comparison to control yolk sacs at the same stage of gestation, removed immediately from the pregnant female. These experiments, as well as establishing the optimum conditions for pinocytic uptake, will also give us an insight into the potential of the 'giant' yolk sac as a viable system for studying other more efficient mechanisms of pinocytic uptake, namely adsorptive pinocytosis and specific receptor mediated pinocytosis.

5.2 Materials and Methods.

5.2.1. Rate of uptake of ^{125}I-PVP by 17.5 day in vivo yolk sacs.

Conceptuses were removed as quickly as possible from the pregnant females as outlined in section 2.9 and washed well in Medium 199 (See Appendix C for list of components). It was essential to remove all the amnion as any remaining would result in errors in the protein estimation of the yolk sac.

(i) Preparation of incubation bottles.

Nine mls of incubation medium (either Medium 199 or whole heat inactivated rat serum) was carefully introduced to 60 ml sterile culture bottles. These were gassed for approximately 30 secs. with a gas mixture usually of 95% O_2;5% CO_2. This has been shown to promote optimum rates of pinocytosis. The gas mixture has been varied in some experiments and details are given in the appropriate sections. The bottles were then sealed with a silicone bung and placed upright in a shaking water bath at 37^{o}C and allowed to equilibrate.

(ii) Incubation of the yolk sacs.

The washed yolk sacs were transferred to the pre-warmed and gassed incubation bottles, a maximum of 10 yolk sacs being placed in each bottle. The bungs were quickly replaced and the yolk sacs allowed to equilibrate in the shaking water bath for approximately 30 mins.

In some experiments the conditions of incubation have been varied, brief details are given in the appropriate experimental section.

(iii) Uptake of ^{125}I-PVP.

Throughout these studies the PVP used was ^{125}I-PVP(Obtained ready labelled from Amersham International) with a concentration of 2.65mg/ml. At time zero, 1ml of incubation medium containing 20µg/ml of ^{125}I-PVP was added to each incubation bottle, thus giving a concentration of 2µg/ml in the incubation medium. The bottles were then reincubated with an atmosphere of 95%O_2;5%CO_2, in the shaking water bath for varying periods up to 5 hours.

Two or three pregnant females were usually used in each experiment, providing between 23-35 yolk sacs. These were randomly allocated, a maximum of 10 yolk sacs per incubation bottle. Cultures were terminated at half hourly or hourly intervals between 0.5 and 5 hours of incubation. At the end of each time period the bottle or bottles were taken from the shaking water bath and the yolk sacs quickly removed and washed for 3x 2mins in beakers containing small quantities of fresh icecold 1M ice-cold 1M NaCl. The beakers were agitated gently to ensure an efficient washing. After washing, each yolk sac was removed from the NaCl, dried on clean tissue and placed separately in a stoppered plastic test tube containing 5ml 1M NaOH. Two 1ml samples of the incubation medium were also taken and placed in 3.5ml plastic tubes (Sarstedt). The samples of medium were frozen immediately after each had been taken and were stored at -20^{o}C until the radioactivity in each could be measured. The yolk sacs in 1M NaOH were vortexed

every half hour to assist in the breakdown of the yolk sac and at the end of the experiment were placed at 37°C for approximately 2 hours in an incubator to aid further protein dissociation. On occasions the yolk sacs were stored overnight at 4°C and were placed in the incubator for final dissociation the following day. This seemed to have no detrimental effect on the measured protein content of the yolk sacs or the detection of ^{125}I-PVP.

Two 1ml samples of each dissolved yolk sac were taken and placed in 3.5 ml plastic tubes for detection of radioactivity. The amount of ^{125}I-labelled PVP in both the yolk sac samples and the samples of medium were measured using an LKB RIA Automatic gamma counter. If measurements could not be carried out immediately all samples were stored at -20°C.

The protein content of each yolk sac was estimated using a modified version of the technique of Lowry et al.(1951) using bovine serum albumin (BSA) as the standard protein.(see Appendix B)

5.2.2 Rate of uptake of ^{125}I-PVP by the 17.5 day 'giant' yolk sac.

'Giant' yolk sacs were cultured in whole heat inactivated rat serum as outlined in section 2.5. After 8 days of culture the 17.5 day 'giant' yolk sacs were washed well with several changes of Medium 199 or fresh serum(depending on the incubation medium to be used in the particular experiment) as outlined in section 2.6. After a final wash as much as possible of the medium was removed and 6.3ml of fresh sterile Medium 199 or heat inactivated serum was added to each incubation bottle containing three intact 'giant' yolk sacs. The bottles were gassed with

a mixture containing 95%O_2;5%CO_2, sealed with a silicone bung and replaced horizontally in a roller incubator at 37°C to equilibrate for approximately 30mins. After the period of equilibration at time zero 0.7ml of incubation medium containing ^{125}I-PVP was added to give a final concentration of 2.0 µg/ml of incubation medium. The bottles were regassed and each incubated for a period between 0.5 and 5 hours.

At the end of each incubation period the bottle or bottles were carefully removed from the roller incubator taking care not to disrupt the delicate yolk sac membranes. The incubation medium was carefully removed using a bent needle and syringe and two 1ml samples collected and stored in 3.5ml plastic tubes at -20°C for the measurement of radioactivity. The yolk sacs were washed well in several changes of ice-cold 1% NaCl again using a bent needle and syringe to introduce and remove the NaCl. After washing the 'giant' yolk sacs were harvested as described in section 2.6 using a small tank full of 1% NaCl.

Each yolk sac in turn was observed under the dissecting microscope to ensure that it was still intact and the fluid from within the exocoelom was collected with a 1ml syringe and fine needle. A further indication that the yolk sac was intact was given by the colour of this fluid, which was usually a pale straw colour. When incubated in Medium 199 any minute damage to the yolk sac allowed the pink medium to leak inside and discolour the exocoelomic fluid. Any such fluid samples were discarded. The volume of fluid collected was noted and the fluid stored in 3.5ml plastic tubes at -20°C until the radioactivity was measured.

After removal of the fluid the 'giant' yolk sacs were opened up

and any remnants of the embryo together with the amnion and ectoplacental cone were removed. Both the inner and outer surfaces of the yolk sac were washed in ice-cold 1M NaCl. The yolk sacs were then transferred individually to plastic tubes each containing 2.5ml of 1M NaOH. The yolk sacs were vortexed every half hour to break up the tissue and then maintained at 37°C for 2 hours to assist in the total dissolving of the protein. Two 1ml samples were then taken and placed in 3.5 ml plastic tubes for the measurement of radioactivity. Samples were stored at -20°C until such measurements were made.

Protein estimations were also carried out on samples of the yolk sacs in 1M NaOH using a modified version of the technique of Lowry et al.(1951)

5.2.3 Rate of uptake of ^{125}I-PVP by the 'giant' yolk sac after removal of the embryonic tissue.

The technique of culturing 'giant' yolk sacs after prior removal of the embryonic tissue at 9.5 days is outlined in section 2.8. The method used for studying the uptake of ^{125}I-PVP by such yolk sacs was exactly the same as the method used for studying uptake in 'giant' yolk sacs which did contain an embryo(see section 5.2.2).

The methods outlined in this chapter are those routinely used, however adaptions to the basic technique have been used in some experiments. Details of any variation from the normal technique will be given in the individual experimental descriptions.

5.2.4 Calculating the rate of uptake of ^{125}I-labelled Polyvinylpyrrolidone (PVP) as an Endocytic Index (EI).

As briefly discussed in the introduction to this section the unit used to describe the rate of uptake of a particular substance by a certain tissue type is the Endocytic Index (EI) with the units expressed as microlitres of fluid whose contained substrate was captured per unit quantity of protein per hour (µl/mg protein/hour). The EI is a useful unit as it takes into account the difference in the amount of protein in each yolk sac, the slightly varying amounts of substrate in the incubation medium and also the effects of radio-isotope decay.

The uptake volume for a particular substrate is given by

$$\frac{Y}{M \times P}$$

Where Y is the total radioactivity(in cpm corrected for background) in the whole yolk sac. M is the radioactivity(in cpm corrected for background) per ul of incubation medium and P is the protein content(in mg) of the whole yolk sac. The rate of uptake of ^{125}I-PVP, the Endocytic Index, is obtained from the plot of uptake volume against time for each incubation.

For each individual experiment a plot was drawn of uptake volume against incubation time and the results subjected to linear regression analysis. The correlation coefficient, the slope and the intercept on the ordinate axis were found. The slope gives the EI for the uptake of ^{125}I-PVP. The results of all the experiments carried out under identical

conditions were compared and no significant difference was found. It was therefore possible to combine results from different experiments to find an overall mean +/- SE at the different incubation times and to find an overall mean value for the EI +/- SE.

When considering the rate of uptake of macromolecules by the intact 'giant' yolk sac it was essential to include any radioactivity detected in the exocoelomic fluid. However as ^{125}I-PVP is known to be taken up and accumulated in the tissue it was not surprising to find only a very small amount of ^{125}I-PVP had leaked into the exocoelom.

In experiments investigating the uptake of ^{125}I-PVP the concentration of PVP in the culture medium was found to fall by less than 1% during the 5 hour incubation period. This means that the concentration of ^{125}I-labelled PVP can be thought of as constant throughout the incubation period and therefore the value for M used to calculate the EI can be measured at the end of incubation.

5.2.5 Release of ^{125}I-PVP from pre-loaded 'giant' yolk sacs and 17.5 day *in vivo* yolk sacs.

(i) Release by 17.5 day *in vivo* yolk sacs.

17.5 day *in vivo* yolk sacs were explanted as previously described and placed in Medium 199 containing 2.65µg/ml ^{125}I-PVP. The incubation bottles were gassed with 95%O_2;5%CO_2 and incubated for 3 hours. At the end of this time the yolk sacs were removed from the incubation medium and washed for 3 x 2 minutes in Medium 199 before being re-incubated in

fresh Medium 199 for 3 hours. At intervals (15,30,60,120,180 minutes) after the start of incubation in fresh medium, 0.5ml samples of the medium were taken and the radioactivity associated with each was measured. At the end of the incubation the amount of radioactivity in each yolk sac was also measured as previously described. From this a graph was drawn of the ^{125}I-PVP released (as a percentage of the total tissue associated radioactivity at the start of the re-incubation) against time.

(ii) Release by 17.5 day 'giant' yolk sacs into the culture medium.

The release of ^{125}I-PVP from the 'giant' yolk sac was also studied after pre-incubation of 'giant' yolk sacs for 3 hours in Medium 199 containing 2.65µg/ml ^{125}I-PVP, with an atmosphere of 95%O_2;5%CO_2. At the end of this time the radioactive medium was removed as previously described (section 2.5). The yolk sacs were then washed in 3 changes of fresh Medium 199. Having removed as much as possible of the final washing, 7ml of fresh Medium 199 was placed in the bottles which were re-gassed and placed in a roller incubator. At zero time and at regular intervals (15,30,60,120,180 minutes) after the start of incubation in fresh medium, 0.5ml samples of the medium were taken and the radioactivity in each measured. At the end of the incubation period the 'giant' yolk sacs were removed from the bottles as previously described. The exocoelomic fluid from each yolk sac was collected, its volume noted and the radioactivity measured. The radioactivity associated with each yolk sac and its protein content were also measured. From the results a graph was drawn of the release of ^{125}I-PVP into the incubation medium(as a percentage of the total radioactivity associated with the 'giant' yolk

sacs at the start of re-incubation) against time.

5.3 Experimentation and Results.

5.3.1 Comparison of uptake of ^{125}I-PVP by in vivo yolk sacs incubated in the roller culture and shaking water bath.

This experiment aims to compare the rate of uptake of ^{125}I-PVP in control 17.5 day yolk sacs when incubated in Medium 199 in the roller incubator and the shaking water bath, to see if either condition adversely affects the rate of pinocytosis.

Three 17.5 day pregnant rats were killed and their yolk sacs explanted as previously described. Half the yolk sacs were placed in bottles containing 9ml of Medium 199 in a shaker water bath (Plate 5.1) and half in bottles containing 6.3ml of Medium 199 horizontally in a roller incubator (Plate 5.2). To each bottle ^{125}I-PVP was added to a final concentration of 2.65µg/ml and the incubations were gassed with a mixture of 95%O_2;5%CO_2. The rate of uptake of ^{125}I-PVP was measured over a 5 hour period in both incubation conditions. Graphs were plotted of the rate of uptake of the ^{125}I-PVP and the EI calculated.

Results.

Figure 5.1 shows the rate of uptake of PVP in the control yolk sac to be linear with time when incubated in both the roller incubator and the shaking water bath. From the slope of the graphs the EI was calculated in each case (Table 1), and subjected to statistical analysis (Student's t test).

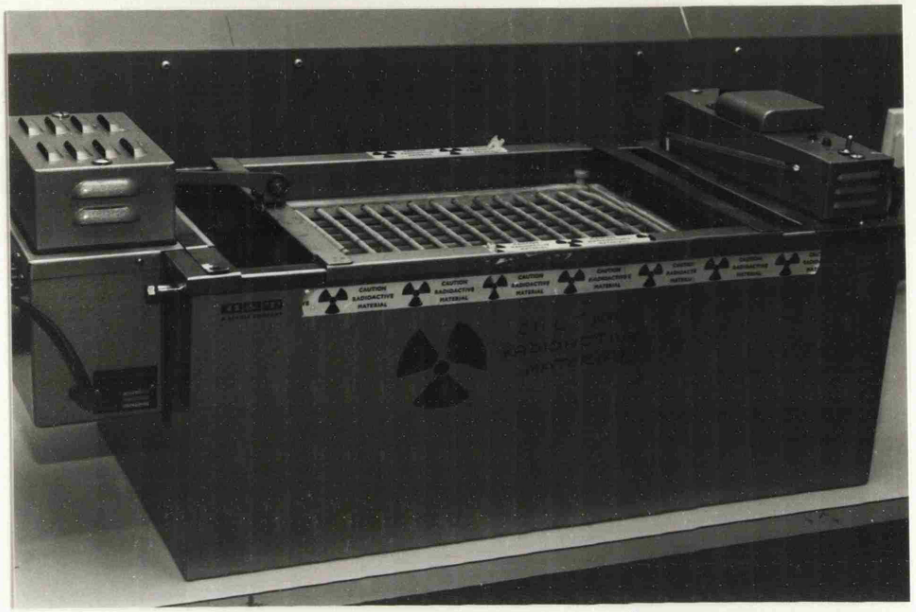


Plate 5.1 Shaking water bath maintained at 37°C.

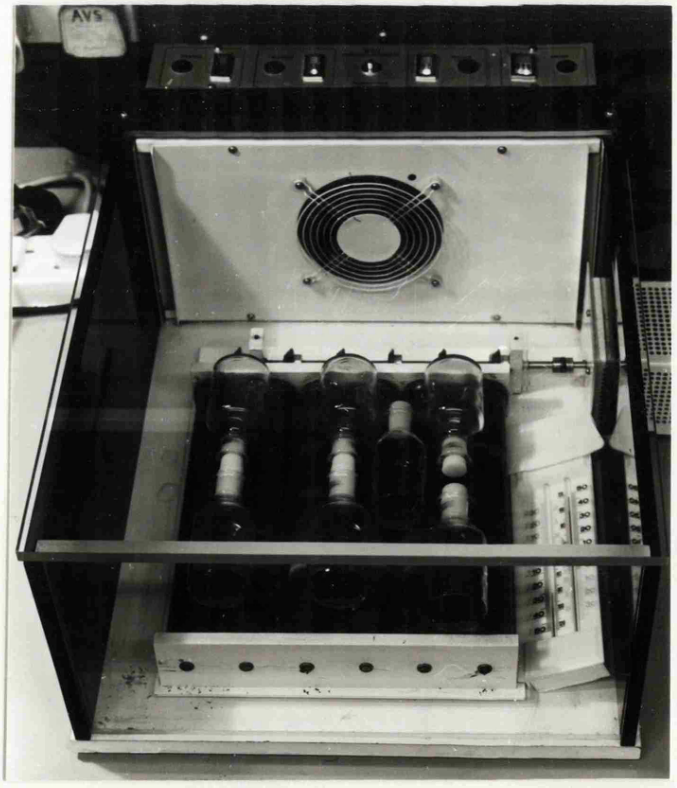


Plate 5.2 Roller incubator maintained at 37°C.

Figure 5.1 **Comparison of the uptake of ^{125}I-PVP by 17.5 day *in vivo* yolk sacs maintained in a roller incubator and shaking water bath.**

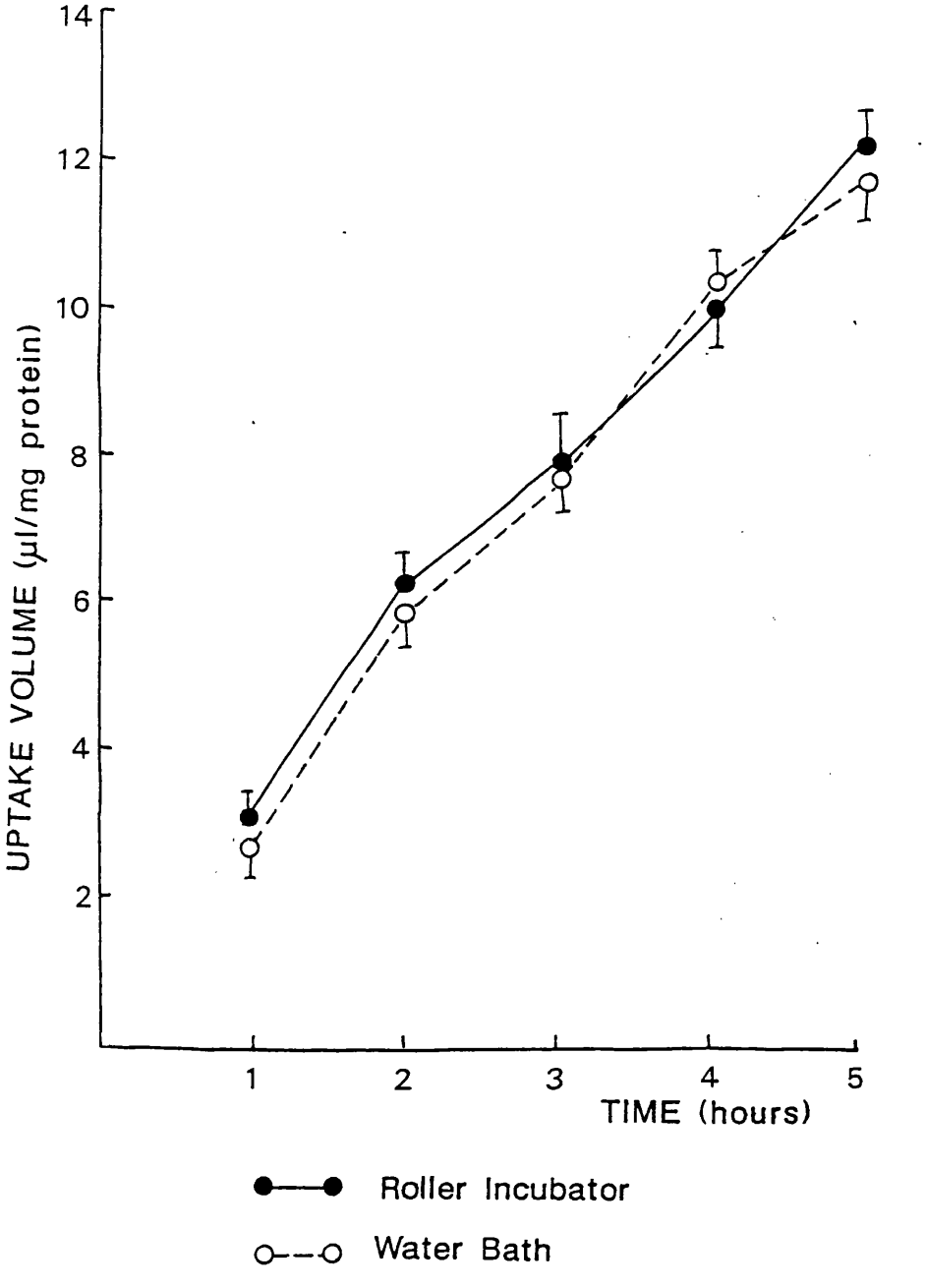


Table 5.1. Uptake of ^{125}I-PVP by 17.5 day *in vivo* yolk sacs in two types of incubator.

Incubation Conditions	Endocytic Index (µl/mg protein/hour)
Roller Incubator (Medium 199;95%O_2;5%CO_2)	**2.3 (n=14)**
Shaking Water Bath (Medium 199;95%O_2;5%CO_2)	**2.4 (n=14)**

It can be seen that there is no significant difference between the EI found in the two incubation conditions.

5.3.2 Comparison of ^{125}I-PVP uptake by *in vivo* 17.5 day yolk sacs and the 'giant' yolk sac when measured in four different sets of incubation conditions.

Previous workers (eg Williams et al. 1975) have studied the uptake of macromolecules using 17.5 day *in vivo* yolk sacs incubated in either serum free Medium 199 or Medium 199 containing 10% heat-inactivated calf serum, with an atmosphere of 95%O_2;5%CO_2. 'Giant' yolk sacs are routinely cultured in whole rat serum with an atmosphere of 20%O_2;5%CO_2;75%N_2. The uptake of ^{125}I-PVP by both *in vivo* yolk sacs and

'giant' yolk sacs in the different conditions was carried out to investigate how the yolk sacs reacted in the different conditions and hopefully indicate the optimum incubation conditions for a comparison of the two systems.

In this series of experiments 17.5 day 'giant' yolk sacs and 17.5 day *in vivo* yolk sacs explanted directly from the mother were each incubated in four different conditions. Incubations of 'giant' yolk sacs being carried out in the roller incubator and incubations of the control yolk sacs in a shaking water bath. The conditions used are outlined below:

(1) Serum free Medium 199 with an atmosphere of 95%O_2;5%CO_2.
(2) Serum free Medium 199 with an atmosphere of 20%O_2;5%CO_2;75%N_2.
(3) Whole rat serum with an atmosphere of 95%O_2;5%CO_2.
(4) Whole rat serum with an atmosphere of 20%O_2;5%CO_2;75%N_2.

The rate of uptake of ^{125}I-PVP by both the 'giant' yolk sacs and the 17.5 day *in vivo* yolk sacs was calculated in all four conditions as described in section 5.2.4. Graphs of the uptake volume against incubation time were plotted and from the slopes the Endocytic Index was found in each case. The series of experiments was repeated six times and mean values for all the results were obtained.

Results.

In all cases the plot of uptake volume against time was found to be linear and by obtaining the mean uptake volume at each time interval

the plots in Figure 5.2 (a and b) were obtained.

From the individual plots drawn after each experiment mean EI's were calculated. The mean EI +/-SE for each incubation condition are shown in table 5.2.

Table 5.2. Uptake of ^{125}I-PVP in different incubation conditions.

Incubation Conditions	Endocytic Index (µl/mg protein/hour)	
	in vivo yolk sac	'Giant' yolk sac
Medium 199;95%O_2	2.65+/-0.11(n=41)	0.96+/-0.04(n=30)
Medium 199;20%O_2	0.76+/-0.03(n=30)	0.51+/-0.02(n=27)
Rat serum ;95%O_2	1.05+/-0.09(n=27)	0.94+/-0.01(n=26)
Rat serum ;20%O_2	0.43+/-0.04(n=26)	0.46+/-0.06(n=25)

It can be seen that in Medium 199 with an atmosphere of 95%O_2;5%CO_2 the EI of *in vivo* yolk sacs is 2.65, however the EI of 'giant' yolk sacs in the same conditions is only 0.96. When incubated in Medium 199 and 20%O_2;5%CO_2;75%N_2 the EI in both the *in vivo* yolk sacs and the 'giant' yolk sacs fall to 0.76 and 0.51 respectively.

When incubations were carried out in whole rat serum and 95%O_2;5%CO_2 the EI of *in vivo* yolk sacs (1.05) and the 'giant' yolk sac (0.94) were very similar. The EI's of *in vivo* yolk sacs (0.47) and the 'giant' yolk sac (0.46) were again very similar although lower when incubated in whole rat serum with an atmosphere of 20%O_2;5%CO_2;75%N_2.

Figure 5.2(a) **The uptake of ^{125}I-PVP by 'giant' yolk sacs in different incubation conditions.**

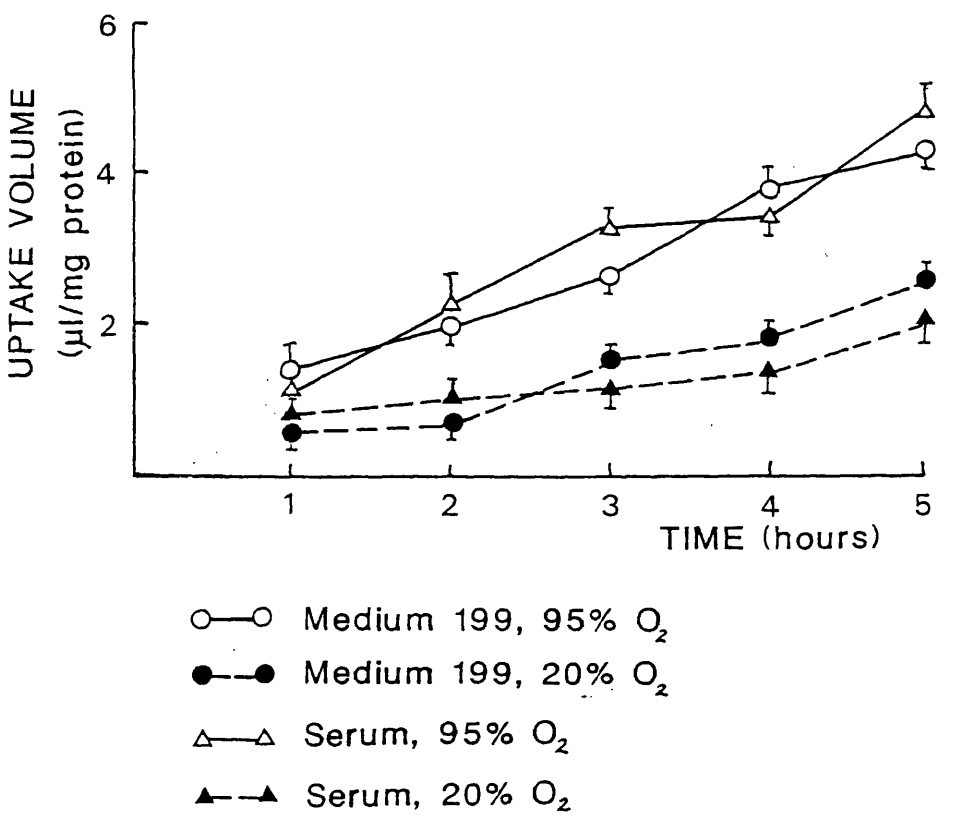
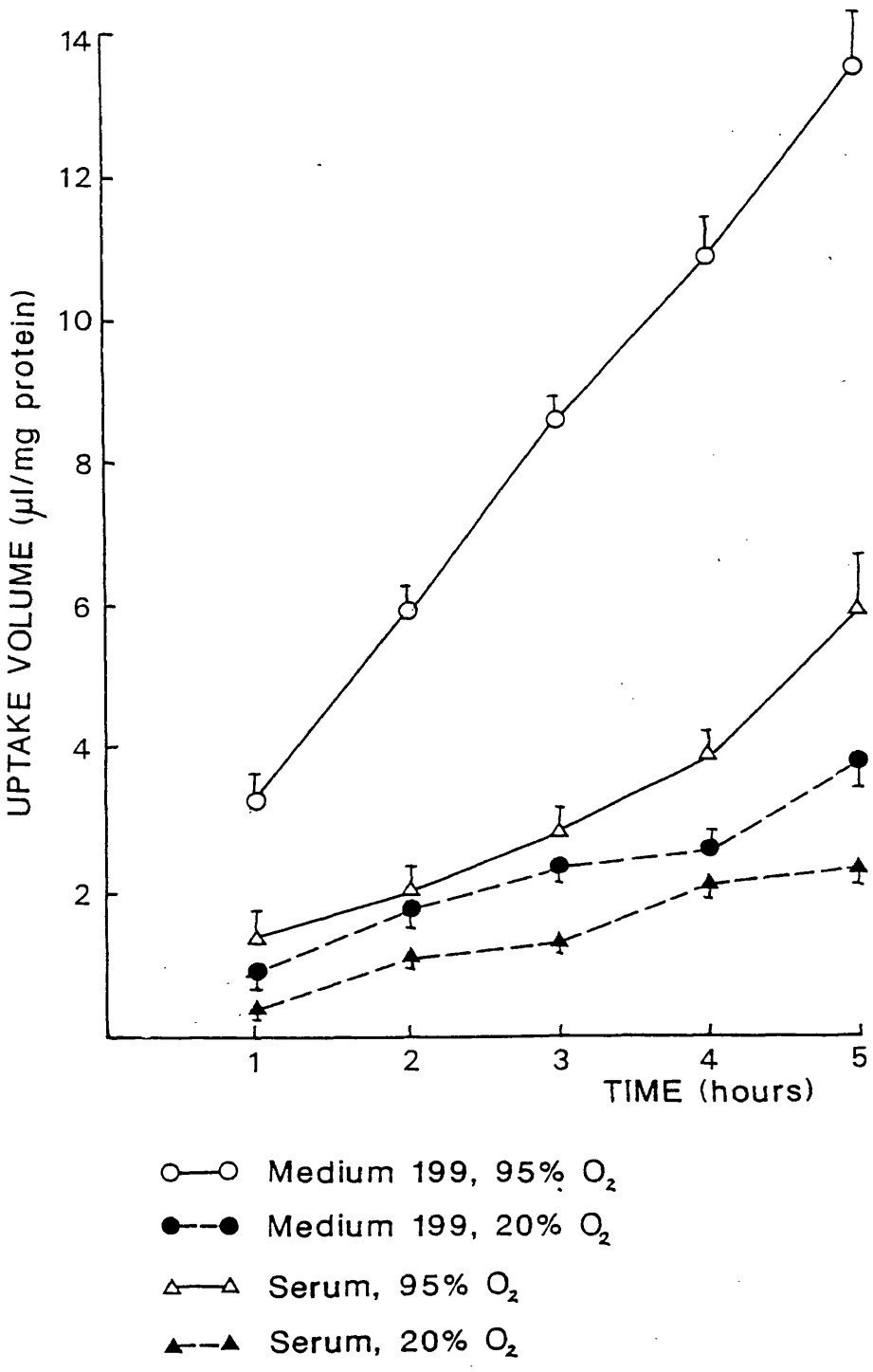


Figure 5.2(b) **The uptake of ^{125}I-PVP by 17.5 day *in vivo* yolk sacs in different incubation conditions.**



5.3.3 The physiological integrity of the 'giant' yolk sac.

The series of experiments to study the uptake of ^{125}I-PVP by the 'giant' yolk sac in the four incubation conditions outlined in the previous section, has also provided valuable information concerning the physiological integrity of the 'giant' yolk sac visceral endoderm.

As outlined in section 5.2.2 of the general materials and methods of this chapter, it was always necessary to collect the exocoelomic fluid from 'giant' yolk sacs during studies of ^{125}I-PVP uptake. The amount of radioactivity within the exocoelomic fluid was measured using an LKB Automatic gamma counter and was compared to the amount present in the incubation medium.

Results.

The results shown in Table 5.3 relate to samples collected from one experiment after 1 hour and 5 hours of incubation. These serve to illustrate the results found throughout all the experiments. The amounts of radioactivity found inside the exocoelomic fluid was negligible in comparison with the high amount in the incubation medium.

Table 5.3. Measurement of radioactivity in exocoelom of 'giant' yolk sac as an assessment of its physiological integrity.

Incubation time (Hours)	Radioactivity in Exocoelomic fluid (cpm/ml)	Radioactivity in Medium (cpm/ml)
1	81+/-16 (n=5)	200,370
5	170+/-21 (n=5)	211,240

5.3.4. The pre-incubation of in vivo yolk sacs in serum.

In experiment 5.3.2. the rate of fluid phase pinocytosis in control 17.5 day yolk sacs has been shown to be dependent on the composition of the medium in which they were incubated. The present experiment was designed to investigate how quickly control 17.5 day yolk sacs were able to adapt to changes in their environment and change the rate at which they pinocytosed ^{125}I-PVP.

17.5 day in vivo yolk sacs explanted as described in section 2.9, were randomly placed in bottles, half containing serum free Medium 199 and half whole rat serum. All were gassed with 95%O_2;5%CO_2 and allowed to equilibrate. The yolk sacs were pre-incubated for 4 hours. After this time the yolk sacs preincubated in Medium 199 were transferred to fresh Medium 199 gassed with 95%O_2;5%CO_2; these served as controls. Half the total number of yolk sacs pre-incubated in whole rat serum were placed into incubation bottles containing Medium 199 gassed with 95%O_2;5%CO_2

and the other half were placed in fresh heat inactivated serum. To each bottle ^{125}I-PVP was added to a final concentration of 2.65µg/ml and incubations were carried out for 5 hours as described in section 5.2.1. The mean EI in each incubation condition was calculated as outlined in section 5.2.4.

Results.

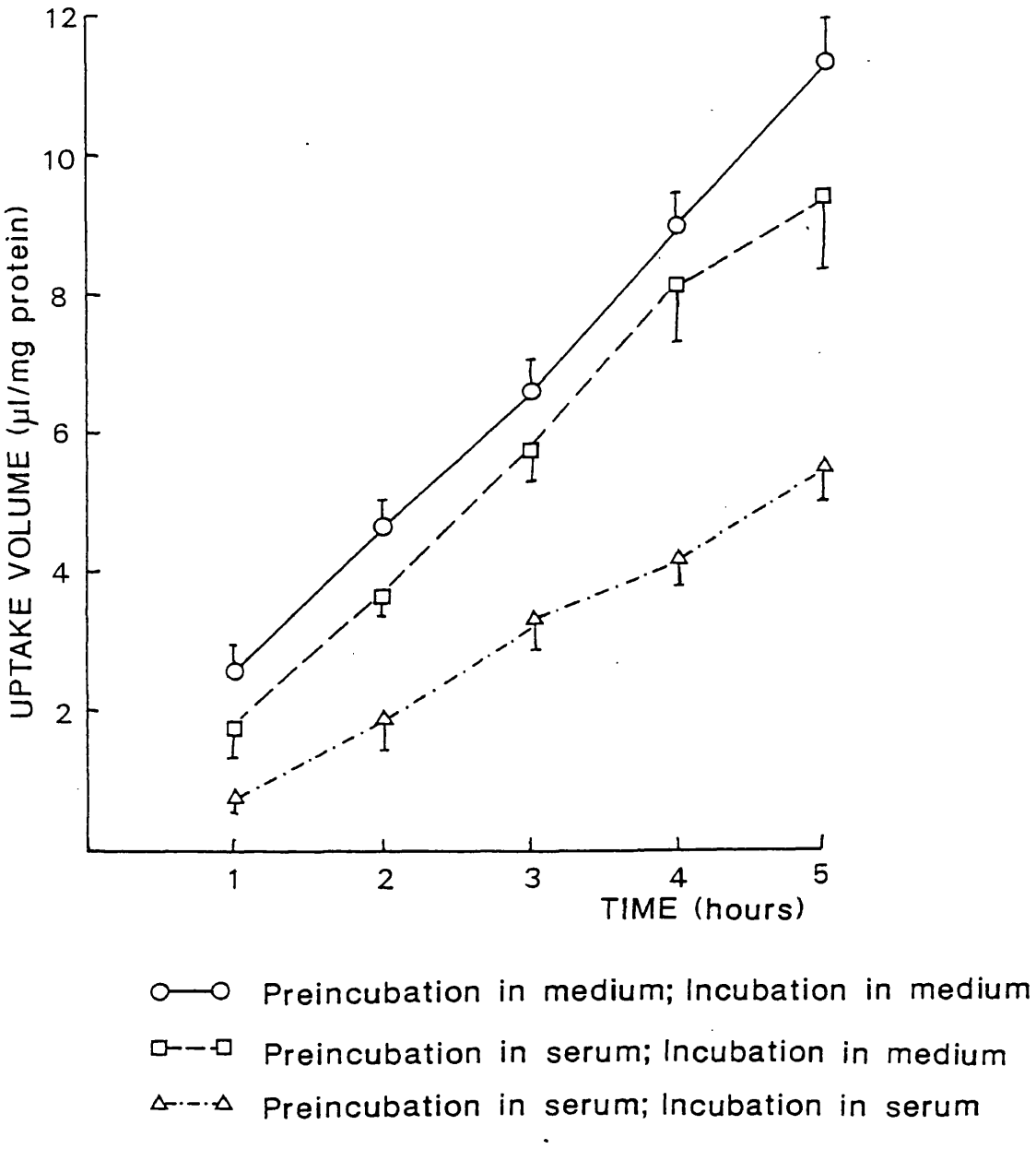
The plots of uptake volume against time were found to be linear in all three incubation conditions and are shown in figure 5.3. Each point being a mean +/- SE for approximately 35 yolk sacs from a number of experiments. Table 5.4 shows the EI calculated in each case as a mean of the slope from plots drawn after each experiment.

Table 5.4. Uptake of ^{125}I-PVP by *in vivo* yolk sacs after pre-incubation in Medium 199.

Pre-incubation Medium	Incubation Medium	Endocytic Index (µl/mg protein/hour)
Medium 199	Medium 199	2.26+/-0.18 (n=35)
Rat serum	Rat serum	1.05+/-0.02 (n=34)
Rat serum	Medium 199	2.02+/-0.10 (n=35)

The yolk sacs which were incubated throughout in Medium 199 had an EI of 2.26 µl/mg/hour and serve as a control. Yolk sacs incubated throughout in whole rat serum had an EI of 1.05 µl/mg/hour. Yolk sacs which were pre-incubated in whole rat serum for 4 hours and then transferred to Medium 199 had an EI of 2.02 µl/mg/hour. It can be seen

Figure 5.3 **The uptake of ^{125}I-PVP by 17.5 day *in vivo* yolk sacs after pre-incubation in whole rat serum and Medium 199.**



clearly from Figure 5.3 that the rate of uptake after pre-incubation in serum followed by incubation in Medium 199 is only slightly lower than that of control yolk sacs incubated throughout in Medium 199.

Variations in the conditions for the culture and incubation of the 'giant' yolk sac which may increase the rate of fluid phase pinocytosis.

In the following two experiments various small changes were made in the culture of the 'giant' yolk sac or in the method of incubation during ^{125}I-PVP uptake. The aim of the experiment was to see if any variation in the conditions could alter the rate of fluid phase pinocytosis by the 'giant' yolk sac, which we have found functions at a lower rate than *in vivo* when incubations are performed in serum free Medium 199.

5.3.5. Pre-incubation in Medium 199.

Experiments 5.3.2 and 5.3.4 have demonstrated that the control 17.5 day yolk sac is able to adapt very quickly to changes in the environment and that the rate of pinocytosis more than doubled when incubated in serum free Medium 199. However the rate of pinocytosis in the 'giant' yolk sac remained the same. It is possible that its ability to adapt has simply been slowed down. By pre-incubation in serum free Medium 199 for a period of time before incubation with ^{125}I-PVP the rate of pinocytosis may increase.

In this series of experiments a limited number of 'giant' yolk sacs were pre-incubated in serum free Medium 199 for either 4 hours or 24 hours. After this time the medium was replaced with fresh Medium 199 and ^{125}I-PVP added again to a final concentration of 2.65 µg/ml and the rate of uptake measured as described in section 5.2.2.

Results.

Table 5.5. Uptake of ^{125}I-PVP by GYS after pre-incubation in Medium.

Incubation Conditions of 'giant' yolk sacs	Endocytic Index (µl/mg protein/hour)
Pre-incubated 4 hours in Medium 199	0.94+/-0.03(n=9)
Pre-incubated 24 hours in Medium 199	0.60+/-0.10(n=9)
'Giant' yolk sacs No pre-incubation	0.97+/-0.04(n=9)

The results shown in Table 5.5 indicate that the 'giant' yolk sacs pre-incubated in Medium 199 for 4 hours before the normal 5 hour incubation period in ^{125}I-PVP, did not take up the macromolecule at a higher rate than the 'giant' yolk sacs cultured in serum and then incubated in medium 199 containing ^{125}I-PVP. The results of the two groups were found not to be significantly different using Student's T test. The 'giant' yolk sacs which were pre-incubated in Medium 199 for 24 hours prior to the normal 5 hour incubation in Medium 199 containing ^{125}I-PVP, were not found to have an increased rate of pinocytosis. The

Endocytic Index had in fact been reduced and was found to be significantly smaller than that of the control 'giant' yolk sacs using Student's T test.

Plate 5.3 shows typical morphology of 'giant' yolk sac endoderm cells after incubation in Medium 199 for 24 hours. Note that the number of large electron dense vacuoles is greatly reduced and infact most of the apical vacuoles appear electron lucent containing a small amount of flocculent material.

5.3.6 Opening the 'giant' yolk sac.

One major advantage of the 'giant' yolk sac system over other systems used for the study of uptake and transport by the visceral yolk sac, is that it is a closed system in which the fluid inside the exocoelom is maintained separately from the culture medium. However it may be that the closed nature of the vesicle and its prolonged culture without an actively developing embryo could account for a reduction in the rate of pinocytosis. there may be a build up of the products of histiotrophic nutrition. If this is the case, opening the 'giant' yolk sac vesicle would allow any accumulated products to disperse in the culture medium. This may reduce the large number of secondary lysosomes in the tissue and the rate of pinocytosis may increase.

In this experiment 'giant' yolk sacs were removed from their culture bottles as described in section 2.6. The exocoelomic fluid and amniotic fluid was collected, the visceral yolk sac torn apart and the

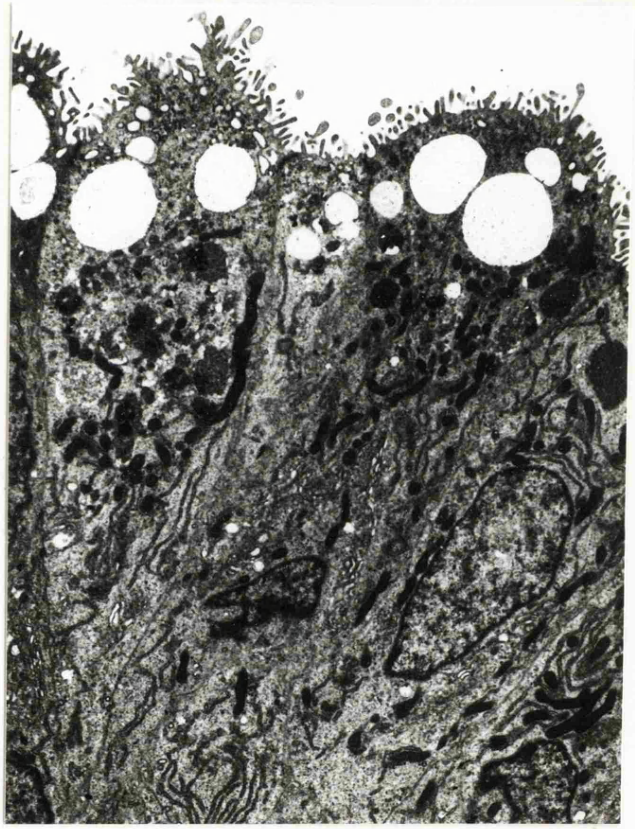


Plate 5.3 Typical morphology of GYS endoderm cells after incubation in Medium 199 for 24 hours. Note reduction in large electron dence vacuoles. Bar = 2μl

remains of the amnion, embryo and ectoplacental cone removed. The yolk sacs were rinsed in fresh Medium 199, replaced in incubation bottles containing Medium 199 and after equilibrating were incubated in ^{125}I-PVP as described in section 5.2.2. A second series of opened 'giant' yolk sacs were also pre-incubated in Medium 199 for 4 hours. Plots of uptake volume against time were constructed and the mean EI calculated in both sets of 'giant' yolk sacs. These were compared to the EI found with intact 'giant' yolk sacs incubated in Medium 199 and gassed with $95\%O_2;5\%CO_2$.

Results.

Table 5.6 shows that the EI of the opened 'giant' yolk sac was not significantly different from the closed 'giant' yolk sac even after a period of pre-incubation in Medium 199.

Table 5.6. Uptake of ^{125}I-PVP by opened 'giant' yolk sacs.

Incubation conditions	Endocytic Index (µl/mg protein/hour)
Control Intact 'giant' yolk sacs	0.96+/-0.04 (n=10)
Opened GYS (No pre-incubation)	0.91+/-0.06 (n=10)
Opened GYS (4 hour pre-incubation)	0.89+/-0.06 (n=10)

5.3.7 Uptake of ^{125}I-PVP by 'giant' yolk sacs at different gestational ages.

This series of experiments was devised to assess how the rate of pinocytosis of the 'giant' yolk sac changes over the period of culture. Previous workers have carried out a similar series of experiments using control yolk sacs explanted directly from the mother at different stages of gestation. They found a general increase in the rate of pinocytosis with gestational age.

'Giant' yolk sacs were cultured as described in section 2.5, to 13.5, 14.5 and 15.5 days and at each stage the rate of uptake of ^{125}I-PVP was measured over a 5 hour period and the EI calculated.

Results.

As can be seen from Table 5.7 the EI of 'giant' yolk sacs at various gestational ages was found to decrease with increasing age, when incubated in serum free Medium 199 and gassed with 95%O_2;5%CO_2.

Table 5.7. Uptake of ^{125}I-PVP by 'giant' yolk sacs at different stages of gestation.

Gestational Age	Endocytic Index (µl/mg protein/hour)
13.5 days	2.25+/-0.1 (n=7)
14.5 days	1.67+/-0.7 (n=8)
15.5 days	1.06+/-0.8 (n=8)
17.5 days	0.95+/-0.6 (n=10)

5.3.8. Uptake of ^{125}I-PVP by 'giant' yolk sacs after removal of the embryonic tissue.

The 'giant' yolk sac system described in section 2.5 has been used extensively and very successfully. It is a very good system for studying the uptake of macromolecules by the visceral yolk sac. However the one disadvantage with the system is that it contains the remains of a dead embryo within an amnion of varying size. A system not containing such embryonic remains would have obvious advantages.

Recently it has been found (Dunton et al 1986) possible to remove the embryonic tissue from an egg cylinder at 9.5 days and culture the remainder of the conceptus usually for 8 days as described in section 2.8 of the materials and methods. The yolk sac tissue grows as normal and although it is generally smaller than the 'giant' yolk sac containing an embryo, its morphology appears normal (Chapter 3).

In this experiment 'giant' yolk sacs were cultured to 17.5 days after removal of the embryonic tissue at 9.5 days as described in section 2.8.

At 17.5 days the rate of uptake of ^{125}I-PVP by these 'giant' yolk sacs was measured over a five hour period, as described in section 5.2.4. The uptake of PVP was measured in both serum and serum free Medium 199 with an atmosphere of 95%O_2;5%CO_2. These conditions gave an optimum rate of pinocytosis in 'giant' yolk sacs containing remains of a dead embryo (Experiment 5.4). Graphs were drawn of uptake volume against incubation time and from each plot an EI was calculated. The mean EI +/- SE was found from a series of experiments.

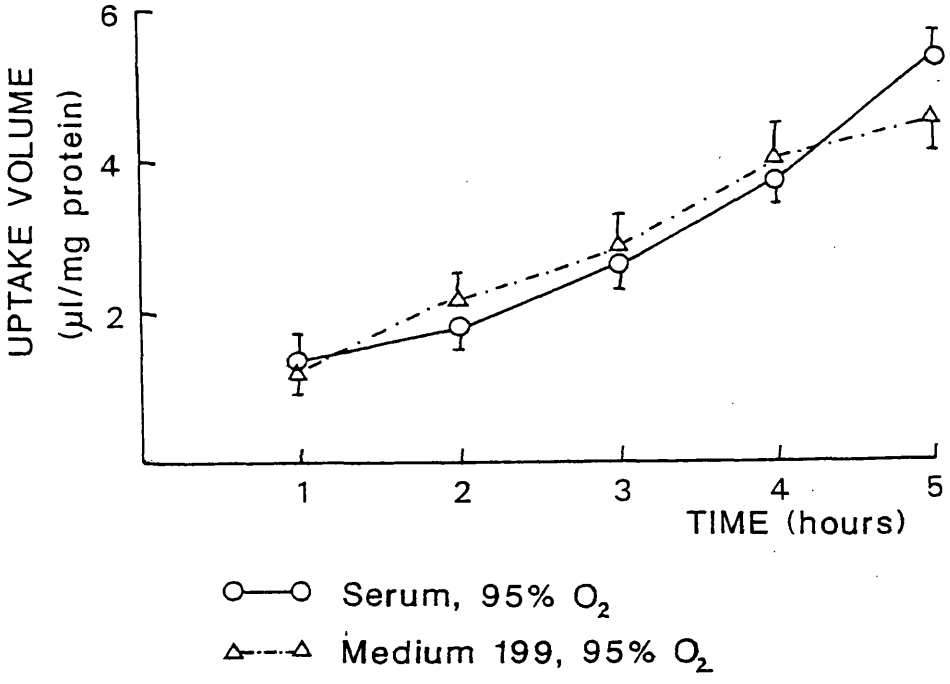
Results.

Table 5.8. Uptake of ^{125}I-PVP by 'giant' yolk sacs without embryos.

Incubation conditions	Endocytic Index (µl/mg protein/hour)
Medium 199;95%O_2;5%CO_2	1.03+/-0.07 (n=20)
Rat Serum ;95%O_2;5%CO_2	0.96+/-0.06 (n=19)

The uptake of ^{125}I-PVP by these 'giant' yolk sacs was found to be linear with time in both incubation conditions used (see Figure 5.4) and the mean EI calculated from such plots was found not to be significantly different from the results obtained using 'giant' yolk sacs which had contained a developing embryo.

Figure 5.4 **The uptake of ^{125}I-PVP in different incubation conditions by 'giant' yolk sacs cultured after prior removal of the embryo.**



5.3.9. Release of ^{125}I-PVP by the 'giant' yolk sac and 17.5 day in vivo yolk sac.

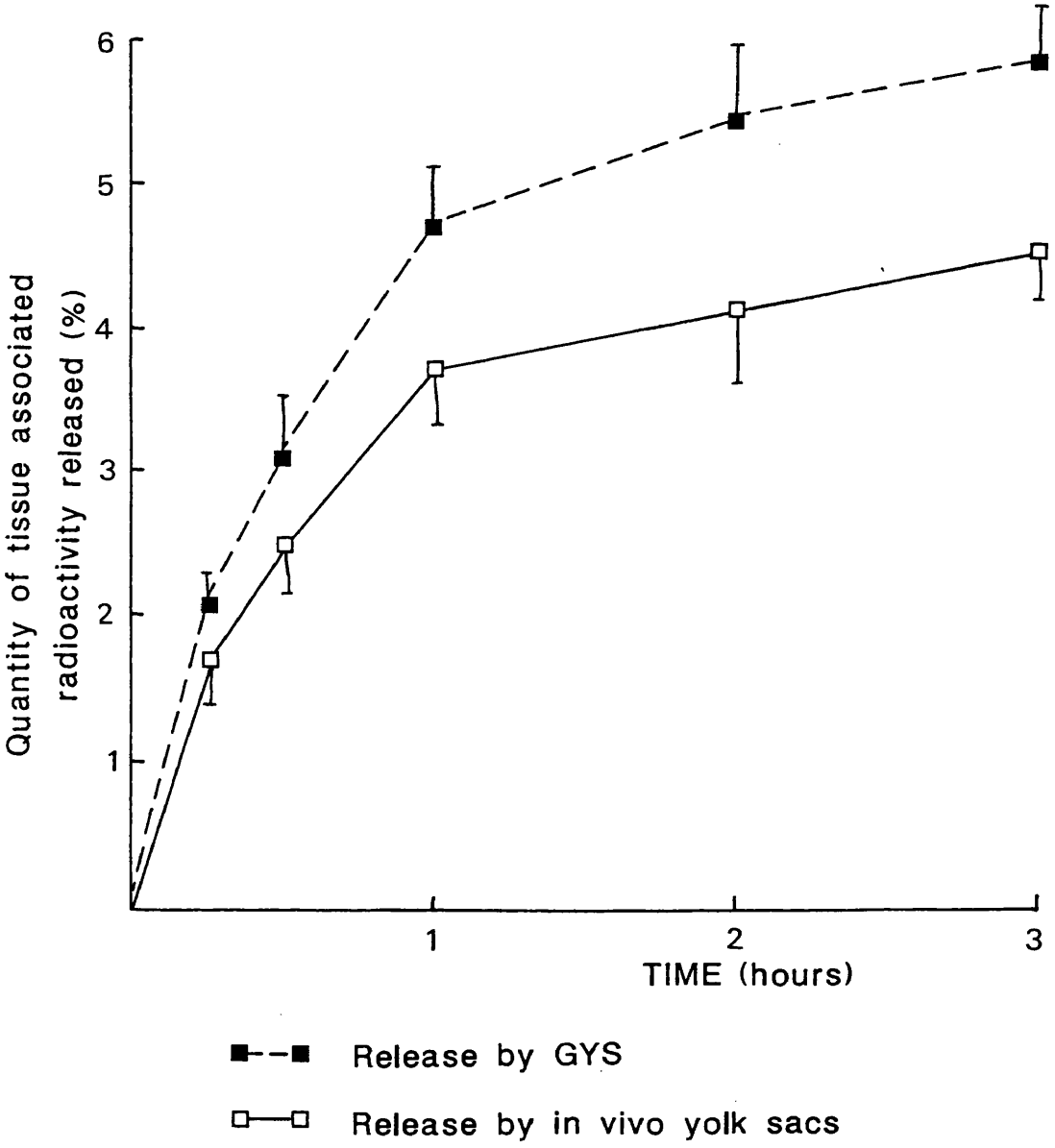
In all experiments such as those to measure the uptake of ^{125}I-PVP it is essential to carry out a study of release. This is because the very nature of the calculation assumes that the amount of ^{125}I-PVP taken up is the same as the amount accumulated in the tissue. If a substantial amount was being released this would not be so and the calculation of ^{125}I-PVP uptake would have to be amended to take into account any released ^{125}I-PVP.

Yolk sacs were pre-loaded with ^{125}I-PVP, placed in fresh incubation medium and the release of radioactivity into the fresh incubation medium was studied, as outlined in detail in section 5.2.5.

Results.

The graph (Figure 5.5) shows that over the 3 hour period of re-incubation in fresh Medium 199 only a very small fraction of the total radioactivity associated with the control yolk sacs and 'giant' yolk sacs was released. With the 'giant' yolk sacs the release after 3 hours can be seen to be just less than 6% and with control yolk sacs release after 3 hours was approximately 4.5%.

Figure 5.5 **The release of ^{125}I-PVP by 'giant'yolk sacs and 17.5 day *in vivo* yolk sacs into the incubation medium.**



5.4. Discussion.

Many workers (eg Williams et al 1975a,b) have used the 17.5 day yolk sac for studying the pinocytic uptake of a variety of substances. They have carried out incubations of the control yolk sac in culture bottles upright in a shaking water bath. However because of the relatively delicate nature of the 'giant' yolk sac and because it is essential to maintain its intact structure, incubations must be carried out with the culture bottles in a horizontal position and a roller incubator is used for this purpose.

The results of this comparative experiment show that there is no significant difference in the rate of uptake of ^{125}I-PVP by *in vivo* yolk sacs incubated in a roller incubator and a shaking water bath. Therefore it seems reasonable to compare the uptake of ^{125}I-PVP in the 'giant' yolk sac routinely cultured in a roller incubator to maintain their intact spherical structure, with 17.5 day *in vivo* yolk sacs incubated in a shaking water bath. This is helpful as space in the roller incubators is restricted.

The results of experiment 5.3.1 established that the two methods routinely used for the incubation of yolk sacs during uptake, have no adverse effect on the efficiency of uptake of ^{125}I-PVP by *in vivo* yolk sacs. It was important therefore to consider the incubation conditions in more detail and assess uptake in all combinations of the conditions routinely used for 'giant' yolk sac culture and the conditions previously used to study the uptake of macromolecules by *in vivo* yolk sacs (Williams et al. 1975a).

As expected the rate of uptake of PVP by the *in vivo* yolk sac is lower when incubations were carried out in whole serum than in serum free Medium 199. This correlates well with previous results (Ibbotson and Williams, 1979; Forster and Williams 1984). These workers showed that the concentration of serum in the incubation medium affects the rate of uptake in the 17.5 day yolk sac. Using different concentrations of heat-inactivated calf serum in Medium 199 they found an increase in the concentration of serum caused a decrease in the rate of fluid phase pinocytosis and adsorptive pinocytosis.

However what is surprising in my experiments, is that the composition of the incubation medium seems to have little effect on the rate of uptake of ^{125}I-PVP by the 'giant' yolk sac. Perhaps the reason is that the system overall has a lower nutritional requirement than yolk sacs *in vivo* due to the lack of a developing embryo which *in vivo* would utilise all the products of histotrophic nutrition. It should be noted that carrying out incubations in serum is more physiological although not as "clean" as incubating in Medium 199. Analysis of results obtained after incubation in serum is more complicated as so many unknown factors are or could be involved.

We could postulate that the large vacuolar volume observed morphometrically (chapter 3) is in fact indicative of a system congested with the products of histotrophic nutrition. With no developing embryo to utilise this nutritional pool, it accumulates within the tissue and may lead to some sort of feedback mechanism operating to regulate the rate of pinocytic uptake. Another possibility is that the decrease in

the rate of pinocytosis could be due to the lack of available membrane.

Valuable information concerning the physiological integrity of the 'giant' yolk sac can also be obtained from studying the uptake of ^{125}I-PVP. As the macromolecule does not cross the visceral yolk sac and enter the exocoelom, any large quantities of radioactivity detected within the exocoelomic fluid are indicative of leakage.

The results show that the 'giant' yolk sac is indeed an intact structure and no significant leakage of ^{125}I-PVP into the exocoelom has occurred. This follows on from the work of Al-Alousi(1983) in which the yolk sac epithelium was shown, by the use of lanthanum nitrate, to provide a barrier to passive diffusion. A thick electron dense layer of lanthanum staining material was seen to cover the surface of the cells up to, but not beyond the level of the tight junctions. Being naturally intact the 'giant' yolk sac has obvious advantages over other systems used to study the passage of proteins across the yolk sac and other extra-embryonic membranes. One such system involved clamping membranes between two perspex chambers and observing the transport of maternal serum proteins from one chamber to the other. Evans blue was added to the maternal side to indicate any membrane defects (Wild 1965).

Experiment 5.3.4 has shown again the difference in fluid phase uptake when incubations are carried out in whole serum and serum free Medium 199. It also shows that the *in vivo* yolk sacs have the ability to adapt very quickly as the incubation conditions are changed from those in which pinocytosis occurs relatively slowly. It would perhaps have been interesting to study in more detail the first hour after changing

from the pre-incubation in serum to incubation in Medium 199, to assess how quickly the change in the rate of pinocytosis occurred. However from this experiment it is clear that pinocytosis is a finely controlled mechanism which responds very quickly to any changes in the external conditions which may adversely affect the homeostasis of the yolk sac endoderm cells and their functions in embryonic nutrition.

The results of experiment 5.3.5 emphasise that the 'giant' yolk sac does not respond in the same way as 17.5 day *in vivo* yolk sacs, to a serum free incubation medium and extending the exposure time to Medium 199 has no effect on the rate of ^{125}I-PVP uptake. Indeed a deterioration in the functional activity of the cells occurred after a pre-incubation for 24 hours in Medium 199,which contains only low concentrations of nutrients. A significant effect was observed on the morphology of the endoderm cells. As the period of pre-incubation was increased so the number of darkly stained secondary lysosomes decreased and the apical cytoplasm was seen to contain mainly small electron lucent vesicles. Even after 24 hours in Medium 199 the cells appeared healthy and illustrate this gradual morphological change most effectively.

The results of experiment 5.3.6 show that opening the 'giant' yolk sac vesicle has no effect on its rate of pinocytosis. It appears that the 'giant' yolk sac is pinocytosing at a fixed rate regardless of its external and internal environment. It could be the nature of the extended culture period that alters its facility to increase and decrease its rate of pinocytosis as changes occur in the environment.

An alternative method of trying to increase the rate of

pinocytosis may be to culture the 'giant' yolk sac throughout the 8 days in a medium consisting of a lower serum content - perhaps 50% serum and 50% Medium 199 or Hank's BSS. It has been shown previously (Stubbs Unpublished) that 50% serum in Hank's BSS is more than adequate to support the normal growth and development of an embryo from 9.5-11.5 days and should therefore be adequate to support the 'giant' yolk sac. A reduction in the available protein in the culture medium may yield a more functionally active 'giant' yolk sac system, with a less enlarged vacuolar volume. A lack of time prevented me putting this theory into practice.

At 13.5 days, one day after the death of the embryo the 'giant' yolk sacs appear to be pinocytosing at a rate very similar to that of 17.5 day control yolk sacs explanted directly from the mother. However as the culture period is extended to 14.5 and 15.5 days the rate of pinocytosis decreases further towards the rate normally associated with the 17.5 day 'giant' yolk sac. This decrease in the rate of pinocytosis could therefore be related to the length of time the yolk sacs are in culture and could also be due to a reduction in the nutritional requirements of the system after the death of the embryo.

I would have expected the rate of pinocytosis in the control yolk sac explanted directly from the mother to either function at a constant EI throughout gestation or for the EI to decrease slightly as the relative importance of the chorioallantoic placenta in embryonic nutrition, increases.

However the results obtained previously (Williams et al. 1976),

using yolk sacs explanted directly from the mother indicate that the EI increases with gestational age between 13.5 and 17.5 days. However the figures they obtained cannot be directly compared to these results as the incubation medium used was Medium 199 supplemented with 10% heat-inactivated calf serum.

It is also important to note the results of morphometric analysis described in section 4.3 which showed a general increase in the vacuolar volume with gestational age, indicating perhaps the overloading of a system with low nutritional requirements.

'Giant' yolk sacs cultured using the modified method (section 2.8) involving the removal of all the embryonic tissue at 9.5 days, are shown in experiment 5.3.7 to be functionally active. The rate of uptake of ^{125}I-PVP was not significantly different from that found in 'giant' yolk sacs in which the embryo is allowed to develop to 11.5 days and then die. However the system is useful as the additional unknown factors connected with the dead and partially autolysed embryo, have been removed.

Results from experiment 5.3.8 show that in the case of both the *in vivo* yolk sac and 'giant' yolk sac it is clear that the majority of the 4% ^{125}I-PVP released is lost during the first 0.5-1.0 hours of re-incubation, and after this initial period release is relatively low. This may well be due to the release of ^{125}I-PVP trapped in the highly convoluted surface structures of the yolk sac tissue, which remains after normal washing procedures. The reason that the amount of ^{125}I-PVP released by the 'giant' yolk sac is higher than that released by the

control yolk sacs could well be that because of efforts made to maintain the intact nature of the 'giant' yolk sac, it is impossible to wash the structure as efficiently as control yolk sacs. Also for this reason a sample of incubation medium was taken immediately after the addition of the fresh medium ; this gave an indication of the efficiency of washing. However this initial sample was usually found not to contain an exceptionally high level of radioactivity, usually just above backgroung levels.

These results also fit in well with the observation made in many experiments involving the uptake of ^{125}I-PVP, that when a regression line was drawn through the data there was a small but positive intercept on the ordinate axis.

This experiment has emphasised that ^{125}I-PVP is indeed taken up by the yolk sac and accumulates within the tissue. As release is very low it is legitimate to calculate the rate of uptake of ^{125}I-PVP by measuring the rate of its accumulation within the tissue.

The importance of the 'giant' yolk sac system is that it has been shown to be functionally active in the uptake of ^{125}I-PVP. The rate of pinocytosis is not too important, in fact it would have been naive to expect a yolk sac maintained in culture for 8 days to function at an identical rate to the yolk sac *in vivo*, especially in view of the fact that the cultured 'giant' yolk sac does not contain an actively developing embryo. The basal rate of fluid phase pinocytosis in the 'giant' yolk sac system in optimum incubation conditions and its physiological integrity has been established. However one great

potential of this closed system is in the ellucidation of more complex uptake mechanisms, involving macromolecular transport, digestion and release and perhaps giving an insight into the vectorial nature of these processes.

SECTION 6

THE UPTAKE OF ^{125}I-BOVINE SERUM ALBUMIN (BSA)

6.1 Introduction

Many animal cell systems have been used for the study of protein uptake. Early workers used cell fractions of tissues from rats injected with a variety of markers including ^{125}I-labelled albumin and transferrin (Gordon and Jacques 1966); yeast invertase (Jacques 1968); Horseradish peroxidase (Jacques 1968). These studies showed that proteins congregate in particulate cell fractions with sedimentation coefficients similar to lysosomes. Histological methods have confirmed these findings (Beck et al. 1971; Wild 1973). Ingested exogenous protein was seen first in the peripheral vacuoles not containing lysosomal enzymes and later in secondary lysosomes (Straus 1964).

After uptake, it was found in quite early studies, that ingested protein gradually dissapears from the tissue at a rate characteristic for each protein (Schumaker 1958; Jacques 1968). It was long believed that this was a result of protein degradation rather than the exocytosis of intact protein (Beck et al. 1967), as it was known that lysosomes contain proteinases, peptidases and many other acid hydrolases. Indeed in 1967 Ehrenreich and Cohn showed that macrophages incubated in the presence of ^{125}I-labelled human serum albumin, ingested protein and released iodotyrosine. This was later confirmed with sarcoma S-180 cells (Gabathuler and Ryser 1969). Mego et al. (1967) showed that exogenous protein is digested in osmotically active subcellular fractions prepared by differential centrifugation.

It has also been shown by several authors that amino acids and some dipeptides can pass through the lysosomal membranes but intact

proteins cannot (Lloyd 1971)

Recently much work has been carried out using the 17.5 day visceral yolk sac organ culture system of Williams et al (1975a,b). The authors showed that using the 17.5 day rat visceral yolk sac maintained in medium 199 containing 10% heat inactivated calf serum, ^{125}I-labelled BSA added to the culture medium was taken up, digested and the digestion products (mainly iodotyrosine) released back into the incubation medium. Only a very small amount of intact ^{125}I-BSA was released back into the medium (approximately 3%). These results agreed with those found in earlier *in vivo* studies (Williams et al. 1971). It was also found that the amount of radioactivity detected in the tissue remained constant after rising in the initial 1-2 hour period, which indicates that the uptake of ^{125}I-BSA was the rate determining step and not its subsequent digestion. As iodotyrosine cannot be incorporated into newly synthesised protein, ^{125}I-BSA is an ideal protein for studies into uptake, digestion and release.

In the majority of studies it has been assumed that all protein digestion is occurring intralysosomally, and although the lysosomes have been shown to contain the necessary proteinases, extracellular proteolysis is a possibility. Some authors (eg Tokes and Sorgente 1976) claim to have obtained such extracellular degradation of ^{125}I-labelled casein bound to latex beads by several cell types including T-lymphocytes and macrophages. Also degradation of ^{125}I-labelled peptide hormones by extracellular membrane bound peptidase has been observed (eg Freychet et al. 1972). Livesey and Williams (1979) have shown that the

products of BSA digestion only appear in the incubation medium after being detected in the yolk sac tissue and only after a progressive accumulation of TCA insoluble protein has been detected within the tissue, therefore implying that in this case proteolysis cannot be attributed to proteinases in the incubation medium or indeed to membrane bound extracellular proteinases.

As described in the previous chapter ^{125}I-labelled PVP has long been used as a marker of fluid phase pinocytosis and as it is non-degradable it accumulates within the tissue. The uptake of PVP has been proved to occur via fluid phase pinocytosis in the 17.5 day rat visceral yolk sac endoderm (Roberts et al. 1977; Ibbotson and Williams 1979) because when non-labelled PVP is added to the culture medium the rate of uptake of ^{125}I-PVP is not significantly reduced. Also when the 10% calf serum normally added to incubation medium is removed, the rate of uptake of PVP only increases by 45%. In contrast to these results Ibbotson and Williams (1979) found that the rate of uptake of ^{125}I-BSA and (U^{14}-C)sucrose by 17.5 day yolk sacs was greatly increased by up to 5 fold when incubations were carried out in serum-free Medium 199 rather than medium containing 10%calf serum. Again in contrast to PVP uptake, the rate of BSA uptake was progressively decreased by up to 75% as the concentration of non-labelled BSA in the incubation medium was increased. These results were not due to any pharmacological effect of BSA concentration on pinosome formation as PVP capture was not altered by a similar addition of BSA to the medium. These results must therefore indicate a binding of BSA to the plasma membrane.

Livesey (PhD 1979) found the surface of rat yolk sac possessed

binding sites for a number of basic proteins. Kooistra et al.(1981) investigated the rates of uptake of different ^{125}I-labelled forms of lactate dehydrogenase (LDH) and BSA by rat yolk sac and rat peritoneal macrophages *in vitro*. They found that in the rat yolk sac the rate of uptake of LDH H_4 (more positively charged) was only slightly higher than the negatively charged LDH M_4, but in macrophages the uptake of LDH H_4 was approximately three times that of LDH M_4. Both cell types showed little affinity for BSA but after formaldehyde denaturation (resulting in an increased hydrophobicity) there was a several fold increase in uptake, indicating yolk sacs and macrophages have binding sites for both hydrophobic and positively charged proteins but that in the yolk sac hydrophobicity is relatively more important. It is therefore appropriate when studying the uptake of BSA by the rat visceral yolk sac to use a more hydrophobic formaldehyde denatured form which is taken up with a greater affinity.

In this study the uptake of BSA has been investigated in the 'giant' yolk sac system and compared to uptake in control 17.5 day yolk sacs using the technique of Williams et al.(1975). The organotypic growth of the 'giant' yolk sac system has proved ideal for investigating further the uptake and subsequent digestion of this protein and also studying the vectorial nature of the mechanism involved (Dunton et al. 1988).

6.2 Materials and Methods.

6.2.1 Labelling of Bovine Serum Albumin with radioactive iodine.

BSA obtained from Sigma Chemical Company was labelled with ^{125}I using a modification of the Chloramine-T method (Greenwood and Hunter 1963). This method was chosen because of the sucessful results obtained by previous workers (eg Williams et al. 1975a,b), the ease of the technique and the low cost involved in comparison to other labelling methods.

20mg of BSA was dissolved in 9ml of phosphate buffer and labelled with 1mCi of ^{125}I as outlined in Appendix D. The albumin was denatured for 72 hours at $4^{o}C$ with 10% formaldehyde, and then dialysed extensively against 1% NaCl, until the amount of radioactivity detected in the dialysate was negligable.

The BSA containing solution was then removed from the Visking tubing using a needle and syringe, placed in 5ml glass bijou bottles and stored at $-20^{o}C$ until required.

6.2.2 Efficiency of Labelling.

A sample of labelled BSA solution before and after dialysis was analysed to assess the efficiency of the labelling process (see Appendix D).

6.2.3 Assessment of % Trichloroacetic Acid soluble radioactivity present in solution after extensive dialysis.

It is essential to check a sample of the BSA solution used in each experiment to ensure that the amount of TCA soluble radioactivity is not above the acceptable limit before the start of the experiment. See Appendix D for experimental details.

6.2.4 Uptake of ^{125}I-BSA by 17.5 day yolk sacs explanted directly from the mother.

(i)Explantation of 17.5 day control yolk sacs.

Conceptuses were removed as quickly as possible from the pregnant female as outlined in section 2.9. The yolk sacs were separated from the placental disc, amnion and embryo and washed in incubation medium.

(ii)Preparation of incubation bottles.

10ml of incubation medium (either Medium 199 or whole, heat inactivated rat serum) was introduced to 60ml sterile culture bottles. These were gassed for approximately 30 seconds with a gas mixture of $95\%O_2;5\%CO_2$. In the previous chapter (experiment 5.4) $95\%O_2;5\%CO_2$ was shown to produce an optimum rate of pinocytosis. The bottles were sealed with a silicone bung, placed upright in a shaking water bath at 37° C and allowed to equilibrate.

(iii)Uptake of ^{125}I-labelled BSA

The washed, whole yolk sacs were transferred to the pre-warmed incubation bottles, a maximum of 4 yolk sacs being placed in each bottle. the bungs were quickly replaced and the incubations allowed to equilibrate for approximately 30 min.

When studying the uptake of BSA by control yolk sacs it has been found (Williams et al. 1975b) essential to limit the number of yolk sacs per bottle. This is because the yolk sacs take up BSA at a much greater rate than PVP and if large numbers are incubated in each bottle the concentration of BSA in the incubation medium is severely depleted.

Throughout the experiments described in this chapter the BSA used was that labelled as described in section 6.2.1 and Appendix D. The concentration of ^{125}I-BSA in the stock was 625µg/ml. ^{125}I-BSA was added to a final concentration of 10µg/ml in each incubation bottle at time zero. The bottles were re-gassed with a mixture of 95%O_2;5%CO_2 and the silicone bungs quickly replaced. The bottles were then incubated in the shaking water bath for periods up to 5 hours, incubations being terminated at half hourly or hourly intervals.

At the end of each incubation period the bottles were taken from the water bath and the yolk sacs quickly removed and washed for 3 x 2mins in fresh ice-cold 1% NaCl. After washing each yolk sac was dried on clean tissue and placed separately in a stoppered tube containing 5ml 1M NaOH. 2 x 0.25ml samples of incubation medium were also taken and placed in 3.5ml plastic tubes and made up to 1ml with distilled water.

These samples of medium were immediately frozen to prevent any deterioration of the sample. The yolk sacs in 1M NaOH were vortexed every half hour to assist in tissue breakdown and at the end of the experiment were placed at 37°C for 2 hours to aid further the protein degradation. 2 x 1ml samples of each dissolved yolk sac were taken and placed in 3.5ml plastic tubes for the detection of total radioactivity. The amount of radioactivity in both the yolk sac samples and samples of medium were measured using an LKB Ria Automatic gamma counter. If measurements could not be carried out immediately the samples were stored at -20°C.

After the total radioactivity of each sample had been measured it was also necessary to determine the amount of iodotyrosine in both the tissue and culture medium (the product of the lysosomal digestion of ^{125}I-BSA). This is possible because ^{125}I-tyrosine is soluble in TCA but whole BSA is not.

To assess the TCA soluble and insoluble fractions of radioactivity in the yolk sac the tissue was homogenised in 5ml distilled water for approximately 10 seconds, using an MSE Ultra-sonicator, rather than dissolving the tissue protein in NaOH.

Therefore to each 1ml sample of tissue or medium, 0.5ml rat serum was added to increase the protein concentration of the sample and hence aid in the precipitation of the whole protein. If the incubation medium was rat serum its protein content was already high and this step unnecessary. 0.5ml of 10% TCA was also added to each tube and after replacing the lids the tubes were inverted to thoroughly mix the

contents. A white precipitate formed. Each tube was spun at 3000rpm for 30mins.. The clear supernatant (approx. 1.2ml) could then be decanted off into fresh 3.5ml plastic tubes, leaving the white precipitate behind. The TCA soluble radioactivity in the supernatant from each sample was then measured again using the LKB Ria Automatic gamma counter.

The protein content of each yolk sac was estimated using a modified version of the technique of Lowry et al.(1951) using bovine serum albumin (BSA) as the standard protein (see Appendix B).

6.2.5 Rate of uptake of ^{125}I-BSA by the 17.5 day 'giant' yolk sac.

(i)Culture and preparation of 'giant' yolk sacs.

'Giant' yolk sacs were cultured in whole heat inactivated rat serum as outlined in section 2.5 and washed as described in section 5.2.2. 7ml of fresh incubation medium (either Medium 199 or serum) was added, each bottle containing 3 yolk sacs was gassed with a mixture of 95%O_2;5%CO_2 and allowed to equilibrate at 37^{o}C.

(ii) Uptake of ^{125}I-BSA by 17.5 day 'giant' yolk sacs.

At time zero ^{125}I-labelled BSA was added to each incubation bottle to a final concentration of 10µg/ml. At this stage a sample of medium was taken to assess the amount of radioactivity at the start of incubation; the bottles were regassed and incubated in a roller

incubator for periods up to 5 hours, incubations being terminated at half hourly or hourly intervals.

At the end of each incubation period the bottle or bottles were removed from the roller incubator, care being taken not to disrupt the delicate yolk sac membranes. 2 x 0.25ml samples of the medium were taken and each made up to 1ml with distilled water. The radioactivity in each was measured. The 'giant' yolk sacs were washed, the exocoelomic fluid removed and both prepared for the assessment of radioactivity as outlined in section 5.2.2.

The samples of culture medium, extraembryonic coelomic fluid and yolk sac tissue were assessed for total radioactivity and TCA soluble radioactivity (as outlined in section 6.2.5), thus giving an indication of any breakdown products of BSA digestion present in the yolk sac tissue, the extraembryonic coelomic fluid and culture medium.

6.2.6 Rate of uptake of ^{125}I-BSA by 'giant' yolk sacs grown after removal of the embryonic tissue.

The method of culture of 'giant' yolk sacs after prior removal of the embryonic tissue has been outlined in section 2.8. The method of studying the uptake of ^{125}I-BSA by such 'giant' yolk sacs is exactly as for 'giant' yolk sacs containing remnants of a dead embryo (section 6.2.5)

6.2.7 Calculating the rate of uptake of ^{125}I-BSA as an Endocytic Index(EI).

The Endocytic Index (EI), first described by Williams et al.(1975a) is the unit used to express the rate of uptake of ^{125}I-BSA so comparisons can be made with other substrates.

However as BSA is a digestible substrate we would expect the majority of the ingested radioactivity to be released from the tissue after digestion and not retained by the tissue as was the case with the non-degradable PVP. To calculate the total uptake it is therefore necessary to sum the amount of radioactivity within the yolk sac tissue and also the amount of radioactive digestion products released from the tissue. Experiments have shown that the amount of intact ^{125}I-BSA released from the tissue is minimal (Experiment 3 section 6.3.3; Williams et al. 1975).

(i) Calculating the rate of uptake by *in vivo* yolk sacs.

In vivo the uptake of ^{125}I-BSA occurs at a much greater rate than was found with the uptake of ^{125}I-PVP. Despite the amount of tissue per incubation being limited some depletion of substrate in the medium may occur. Therefore to take into account the release of products from the tissue and the possible depletion of the medium radioactivity, a modified equation is used to express the uptake volume of BSA at a particular incubation time:-

$$\frac{(Y + 10S)}{M'P}$$

Where Y is the total amount of radioactivity retained in the tissue (cpm corrected for background), and S is the total quantity of TCA soluble radioactivity per ml in the culture medium at the end of incubation (cpm/ml, corrected for background and the amount of TCA soluble radioactivity present naturally in the BSA preparation). There was 10ml of incubation medium in each bottle. M' is the mean TCA insoluble radioactivity in the culture medium over a particular incubation period (cpm/µl medium corrected for background) and P is the protein content of the yolk sac (mg).

In most experiments 4 yolk sacs were incubated in each bottle and therefore it was necessary to consider all four yolk sacs and their digestion products as one unit. A plot of uptake volume/time was then drawn for each experiment and from the slope a value for the EI was found. After a series of experiments a mean EI +/- SE was calculated. Also mean values for the uptake volume at each time point were calculated and an overall plot of uptake volume/time was constructed for each series of experiments. In each case results were subjected to linear regression analysis and the correlation coefficient, slope and intercept on the ordinate axis found. Statistical analysis of the results from different experiments showed no significant difference using Students-t test and therefore it was legitimate to plot an overall mean.

(ii) Calculating the uptake of ^{125}I-BSA by control yolk sacs with the aid of a microcomputer programme.

To aid the process of calculating the uptake of ^{125}I-BSA by control 17.5 day yolk sacs it was possible to use a computer programme. A programme kindly donated by the Biochemical Research Unit, University of Keele, was adapted for use on an 'apple' computer. This programme corrects for a depletion in ^{125}I-BSA concentration in the incubation medium and includes the correlation coefficient, slope and intercept on the ordinate axis, of the data. A copy of the programme is given in Appendix E.

(iii) Calculating the rate of uptake of ^{125}I-labelled BSA by 17.5 day 'giant' yolk sacs.

As the 'giant' yolk sac is an intact structure, radioactivity can be released either into the exocoelom or back into the incubation medium. This necessitates an additional step in the calculation of uptake volume, to assess the amount of radioactive products inside the 'giant' yolk sac. The calculation is further altered as the volume of incubation medium used was 7ml rather than 10ml (the size of the incubation bottles being the limiting factor). Initial experiments indicated that the rate of uptake of ^{125}I-BSA was not so rapid as to deplete the concentration of substrate in the incubation medium and therefore the amount of substrate (^{125}I-BSA) in the medium can accurately be measured at the end of each incubation period.

The modified equation for calculating uptake volume in the 'giant'

yolk sac is:

$$\frac{Y + 7S^m + S^e}{MP}$$

In this case the numerator is the sum of the radioactivity associated with the yolk sac tissue, the TCA soluble radioactivity released into the 7ml of incubation medium ($7s^m$) and the solubles released into the exocoelomic fluid (S^e). In more detail, Y is the total amount of radioactivity retained by the yolk sac tissue (cpm corrected for background), S^m is the total quantity of TCA soluble radioactivity in the culture medium at the end of incubation (cpm/ml corrected for background and the amount of TCA soluble radioactivity naturally present in the BSA preparation) and S^e is the total quantity of radioactivity released into the exocoelom of the 'giant' yolk sac (cpm corrected for background). M is the TCA insoluble radioactivity in the incubation medium at the end of a particular incubation period (cpm/µl corrected for background) and P is the protein content of the yolk sac tissue (mg). Uptake volume has the units µl/mg protein.

In all experiments 3 'giant' yolk sacs were incubated together in one bottle and therefore it was necessary to consider all three yolk sacs and the digestion products they produce as one unit. The results were processed exactly as described in section 6.2.7(i), graphs being plotted to find the EI after each experiment and means calculated. The results were also subjected to linear regression and statistical analysis.

6.3 Uptake of ^{125}I-labelled BSA by 17.5 day GYS and in vivo yolk sacs incubated in Medium 199 and whole rat serum.

6.3.1 Introduction.

In this series of experiments, as with the uptake of ^{125}I-PVP (section 5.4) the rate of uptake of formaldehyde-denatured ^{125}I-BSA was compared in the 'giant' yolk sac and 17.5 day control yolk sac. Incubations were carried out using an atmosphere of 95%O_2;5%CO_2, found in section 5.4 to give an optimum rate of PVP uptake. The incubation media used were serum free Medium 199 and the more physiological whole rat serum. As previously described (Introduction, section 6.1) Ibbotson and Williams (1979) investigated the effect of removing the 10% calf serum, which they routinely added to their incubations. They found that the rate of uptake of formaldehyde-denatured ^{125}I-BSA was greatly increased in serum free Medium 199. Forster and Williams (1984) investigated the effect of increasing the amount of calf serum in the incubation medium to a maximum of 50% (v/v). They found the rate of uptake in 50% calf serum was only 5% that found in Medium 199.

6.3.2 Materials and Methods.

In this experiment, 17.5 day 'giant' yolk sacs (cultured as outlined in section 2.5 and 2.8) and 17.5 day control yolk sacs (explanted directly from the female as outlined in section 2.9) were each incubated in two different sets of incubation conditions.

The incubation conditions used were :-

(i) Serum free Medium 199 with an atmosphere of 95%O_2;5%CO_2,

(ii) Whole rat serum with an atmosphere of 95%O_2;5%CO_2.

In vivo 17.5 day yolk sacs explanted from 2 or 3 pregnant females were randomly allocated to 60ml culture bottles containing 10ml of medium. A maximum of 4 yolk sacs being placed in each bottle (see section 6.2.4 for full details).

'Giant' yolk sacs at 17.5 days gestation were placed at random (3 yolk sacs per 60ml bottle) in the two incubation conditions (see section 6.2.5).

The rate of uptake of formaldehyde-denatured BSA and its subsequent digestion by the 'giant' yolk sacs and control yolk sac systems, was calculated in both cases as described in section 6.2.7. Graphs of the uptake volume against incubation time were plotted for each experiment and from each slope an EI found. The series of experiments was repeated several times and mean values for all results obtained +/- SE. In the 17.5 day control yolk sac the relative amounts of tissue associated radioactivity and radioactivity released back into the culture medium was calculated. In the 'giant' yolk sac system as the exocoelomic fluid is maintained separately from the incubation medium, the amount of radioactive digestion products released into each could be measured and expressed separately.

6.3.3 Results.

In all cases the plots of uptake volume against time were found to be linear and by obtaining the mean uptake volume at each time interval, overall plots were constructed. Figure 6.1 shows that the uptake of ^{125}I-BSA by both the 'giant' yolk sac and the *in vivo* yolk sac in whole rat serum are very similar. However what is interesting is that in the *in vivo* yolk sac the amount of tissue associated radioactivity rises only very slightly over the 5 hour incubation period where as the tissue associated radioactivity in the 'giant' yolk sac tissue rises more rapidly and is a greater proportion of the total.

From the individual plots drawn after each experiment mean EI's were calculated. The mean EI+/- SE in both incubation conditions are presented in Table 6.1.

Table 6.1. Uptake of ^{125}I-BSA by *in vivo* and 'giant' yolk sacs.

	Endocytic Index (µl/mg protein/hour)		
Incubations	*In vivo* 17.5 day YS	'Giant' yolk sac	GYS - embryo
Medium 199; 95%O_2;5%CO_2	28.40 +/- 2.50 (n = 55)	1.48 +/- 0.05 (n = 48)	1.55 +/- 0.07 (n = 40)
Rat serum; 95%O_2;5%CO_2	1.34 +/- 0.04 (n = 46)	1.41.+/- 0.07 (n = 34)	1.44 +/- 0.08 (n = 40)

We can see from Table 6.1 that the rate of uptake of ^{125}I-BSA by

Figure 6.1 **The uptake of ^{125}I-BSA by 'giant' yolk sacs and 17.5 day *in vivo* yolk sacs incubated in whole rat serum.**

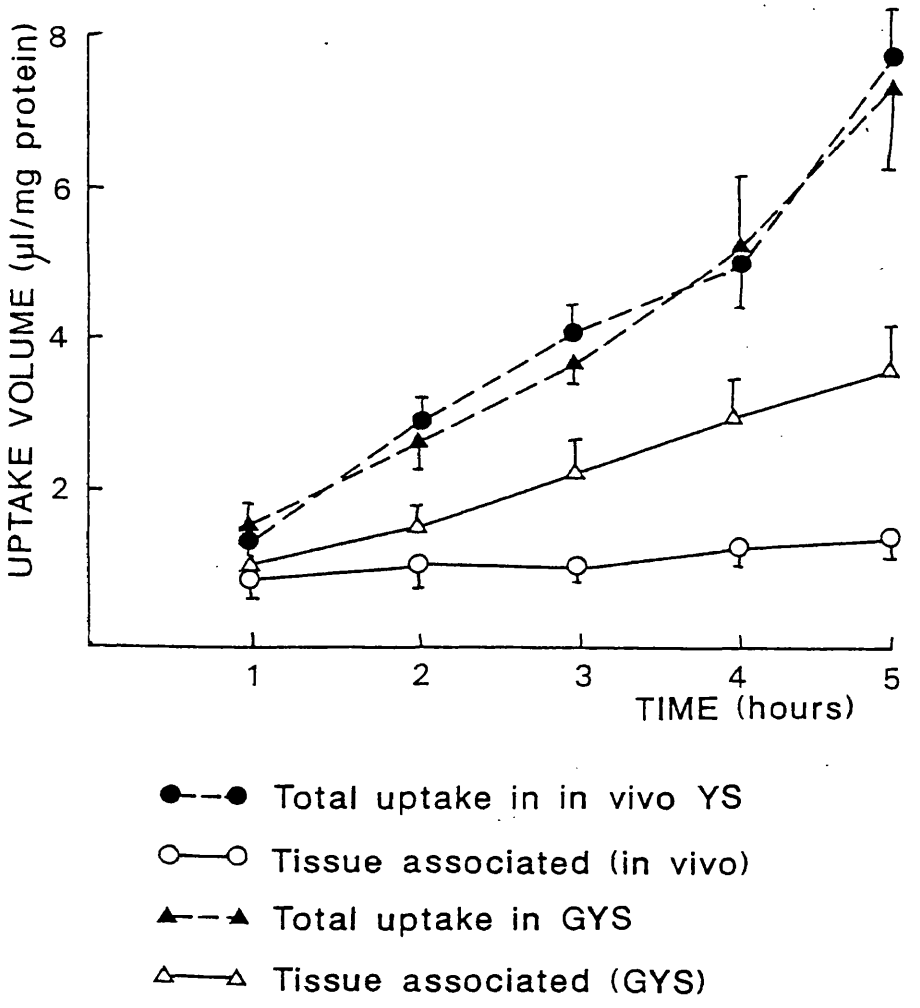
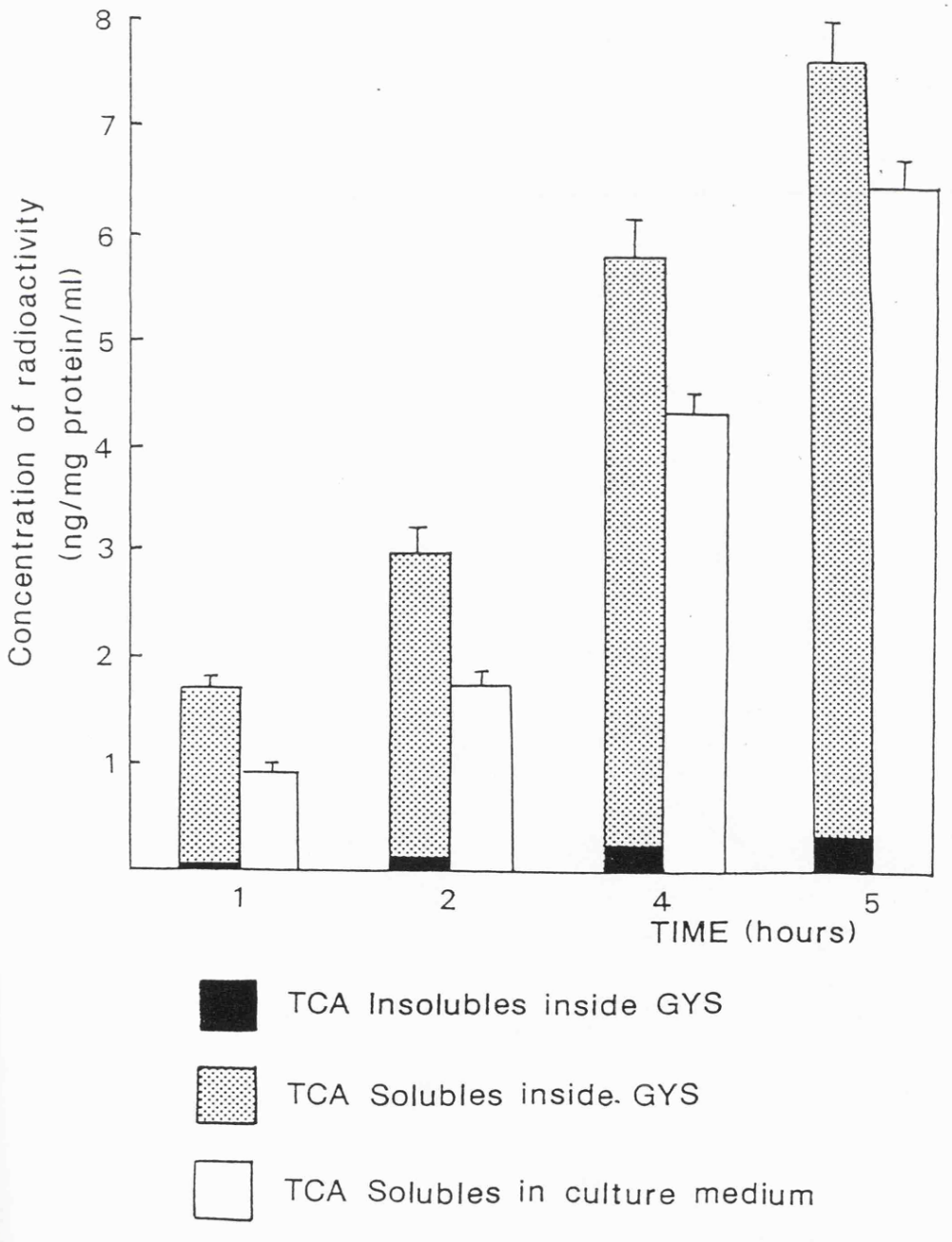


Figure 6.2 **The distribution of radioactivity to the inside and outside of the 'giant' yolk sac during incubation in medium containing ^{125}I-BSA.**



Soluble material expressed as a protein equivalent i.e. the amount of protein from which the measured solubles arose.

in vivo yolk sacs is approximately 20 times as great in serum free Medium 199 as in whole rat serum. Yet with the 'giant' yolk sacs, the rate of uptake of ^{125}I-BSA is only slightly higher in the Medium 199 than in whole rat serum. Of greater significance and as already mentioned, is that in the more physiological conditions of whole rat serum uptake by the 'giant' yolk sac and in vivo yolk sac are very similar.

Figure 6.2 shows the relative concentrations (ng/ml/mg tissue protein) of radioactive digestion products released to the inside and outside of the 'giant' yolk sac throughout the 5 hour incubation period. It can be seen that the concentration of iodo-tyrosine is always higher inside the exocoelomic fluid than in the incubation medium, and after 1 hour incubation the concentration inside is almost twice that outside. Gradually over the remainder of the incubation this concentration difference decreases although the concentration always remains greater inside than outside.

6.3.4 Discussion.

This experiment shows that the rate of uptake of ^{125}I-BSA in both the 'giant' yolk sac and control yolk sac is similar when incubations are carried out in whole rat serum with an atmosphere of 95%O_2;5%CO_2. Also it is apparant that in all tissues the rate of uptake of ^{125}I-BSA is greater than the rate of uptake of ^{125}I-PVP in identical incubation conditions (EI for uptake of ^{125}I-PVP by GYS - 0.94 +/- 0.01; EI for uptake of ^{125}I-PVP by control YS - 1.05 +/- 0.09).

In the control yolk sac incubation in serum free Medium 199 results in the rate of uptake of ^{125}I-BSA to increase almost 20 fold to 28.4 µl/mg/hour. This indicates that in the control situation there is competition for plasma membrane binding sites between the unlabelled serum proteins and the labelled BSA when incubated in serum but when incubated in Medium 199 there is no competition. It is unclear from these experiments how much, if any, ^{125}I-BSA is being taken up bound to the plasma membrane when incubations are carried out in serum. A series of competition experiments varying the concentration of unlabelled protein relative to the labelled protein may help to elucidate this.

The results using the 'giant' yolk sac are rather surprising - no competition similar to that described above was observed using the system. When incubated in serum free medium 199 the rate of uptake of ^{125}I-BSA does not increase by a similar sizable factor to that observed in the controls. If the uptake of ^{125}I-BSA is occurring by adsorptive pinocytosis this is difficult to explain. The results obtained using 'giant' yolk sacs cultured by the methods outlined in section 2.5 (containing remains of an embryo) and section 2.8 (after prior removal of the embryo) were not significantly different.

It could be postulated that in the 'giant' yolk sac systems, which lack an actively developing embryo with its high nutritional requirements, the demand for a rapid uptake and digestion of protein has been reduced. The endoderm cells of 'giant' yolk sac tissue have been shown to have a greatly enlarged vacuolar system (see section 4.3) comprised mainly of a large number of electron dense secondary

lysosomes; the system apparently has an excess of protein available for lysosomal digestion. Therefore the endocytic rate of the 'giant' yolk sac may have been down regulated either by a reduction in the rate of pinosome formation or by an actual reduction in the number of sites on the plasma membrane available for the binding of protein molecules, perhaps by a change in surface structure.

Another important observation (see figure 6.1) is that in the control yolk sac the tissue associated radioactivity rises at the start of incubation and then gradually plateaus as an equilibrium between the rate of uptake and the rate of digestion is reached. However in the 'giant' yolk sac system the tissue associated radioactivity rises throughout incubation and is a greater proportion of the final total. This indicates that no equilibrium state is reached and that it is the lysosomal digestion of the protein which is the rate limiting stage of the process and not its rate of uptake. This correlates well with comments made previously and also the morphological appearance of the 'giant' yolk sac tissue (section 3.3.3).

The distribution of digestion products throughout the incubation period shown in Figure 6.2 indicates that the concentration of iodotyrosine (ng/mg tissue protein/ml) in the exocoelomic fluid is maintained at a higher level than the concentration in the incubation medium. The results indicate transport against a concentration gradient and in this sense iodotyrosine seems to be directed preferentially into the exocoelom.

Therefore what has become clear from these experiments is that the

'giant' yolk sac system does show that the intact visceral yolk sac can take up protein, rapidly digest it and transfer the products both into the exocoelom and back out into the culture medium. It appears that although iodotyrosine could not be utilised by the yolk sac itself or by a developing embryo for the rebuilding of new protein, it is still preferentially transferred into the exocoelom, thus proving the long suspected theory that the yolk sac endodermal cells exhibit polarity in the uptake of protein and the release of the products of lysosomal digestion.

6.4 Release of radioactivity from the GYS and 17.5 day *in vivo* yolk sac after pre-loading with ^{125}I-BSA.

6.4.1 Introduction.

Any investigation into the uptake of a degradable protein such as ^{125}I-BSA should include a study of the patterns of ^{125}I-BSA release, to assess what percentage of the protein taken up by the tissue is released back into the culture medium in an intact form. In normal uptake studies this would be indistinguishable from the pool of ^{125}I-BSA already present in the incubation medium.

Therefore it is necessary to pre-load both 'giant' yolk sacs and control 17.5 day yolk sacs with ^{125}I-BSA, place them in fresh medium and study any release of radioactivity (both TCA soluble and TCA insoluble) into the fresh culture medium.

6.4.2 Materials and Methods.

(i) Release by control yolk sacs.

17.5 day *in vivo* yolk sacs were explanted as previously described in section 2.9 and were incubated for 3 hours in Medium 199 containing 10µg/ml formaldehyde denatured ^{125}I-BSA as described in section 6.2.4. At the end of this time the yolk sacs were removed from the incubation medium, washed extensively for 3 x 2 minutes in fresh warmed Medium 199, before being reincubated in 10ml of fresh Medium 199 for a further 3 hours. At regular intervals after the start of re-incubation (0, 15, 30, 60, 120, 180 minutes) 0.5ml samples of the medium were taken and the

total radioactivty associated with each was measured using an LKB Ria Automatic gamma counter. The TCA soluble radioactivity was also measured as previously described (section 6.2.4(iii)). At the end of the re-incubation the amount of radioactivity in each yolk sac was also measured. From these results a graph was drawn of the radioactivity released (both TCA insoluble whole BSA and the TCA soluble digestion products) as a percentage of the total radioactivity previously associated with the tissue.

(ii) Release by 'giant' yolk sacs into re-incubation medium.

The release of radioactivity from the 'giant' yolk sacs into the re-incubation medium was also studied after pre-incubation of yolk sacs for 3 hours in 7ml of Medium 199 containing 10ug/ml ^{125}I-BSA. At the end of this period a 0.5 ml sample of the medium was collected and the remainder removed. The 'giant' yolk sacs were washed in fresh Medium 199 and finally re-incubated in 7ml of fresh Medium 199 for a further 3 hours. At regular intervals after the start of re-incubation (0, 15, 30, 60, 120, 180 minutes) 0.5ml samples of the medium were taken and the total radioactivity and TCA soluble radioactivity present in each was measured. At the end of each interval some 'giant' yolk sacs were also harvested, the exocoelomic fluid was collected and the amount of radioactivity associated with it and the yolk sac tissue was measured.

From the results a graph was drawn of the release of radioactivity (TCA soluble and TCA insoluble) as a percentage of the total radioactivity associated with the tissue at the start of re-incubation.

6.4.3 Results.

The two graphs (Figures 6.4 (a) and (b)) show that over the 3 hour re-incubation period in fresh Medium 199 the amount of TCA insoluble radioactivity (intact ^{125}I-BSA) released back into the medium was very low in both 'giant' yolk sacs and control yolk sacs. After the 3 hour re-incubation period the release of ^{125}I-BSA was less than 7% of the total radioactivity previously associated with the tissue.

6.4.4 Discussion.

In both cases the low level release of whole ^{125}I-BSA was probably due to small amounts of ^{125}I-BSA remaining trapped in the convoluted regions of the yolk sac surface, and not removed during the washing. This is particularly likely in the case of the 'giant' yolk sac where effective washing is relatively difficult to achieve whilst maintaining the structure of the system. Any remaining release of ^{125}I-BSA is probably due to exocytosis, the leakage from dead or damaged cells and even the detachment of fragments of the yolk sac tissue.

Figure 6.3(a) **Release of radioactivity by 'giant' yolk sacs after pre-incubation in medium containing ^{125}I-BSA.**

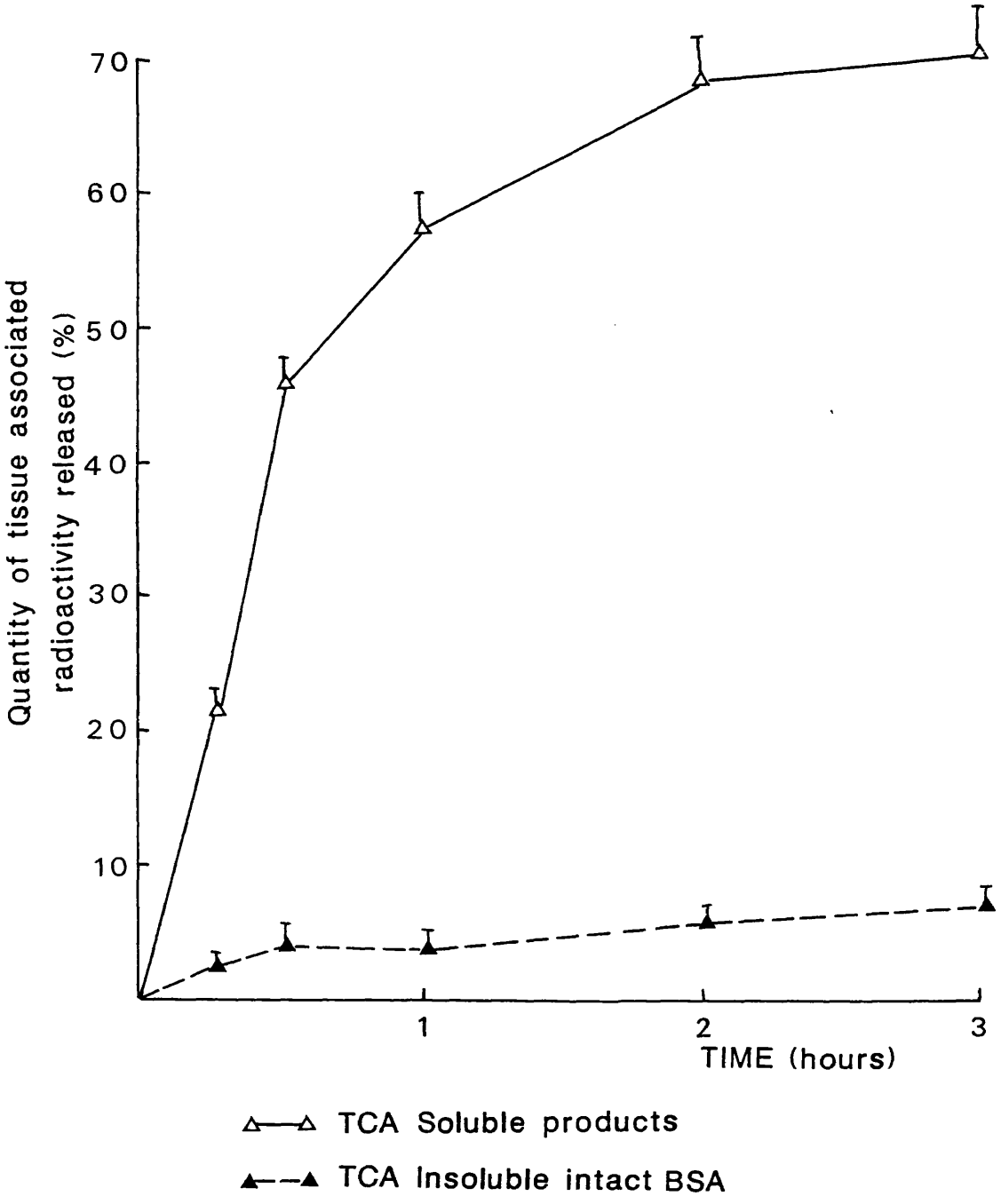
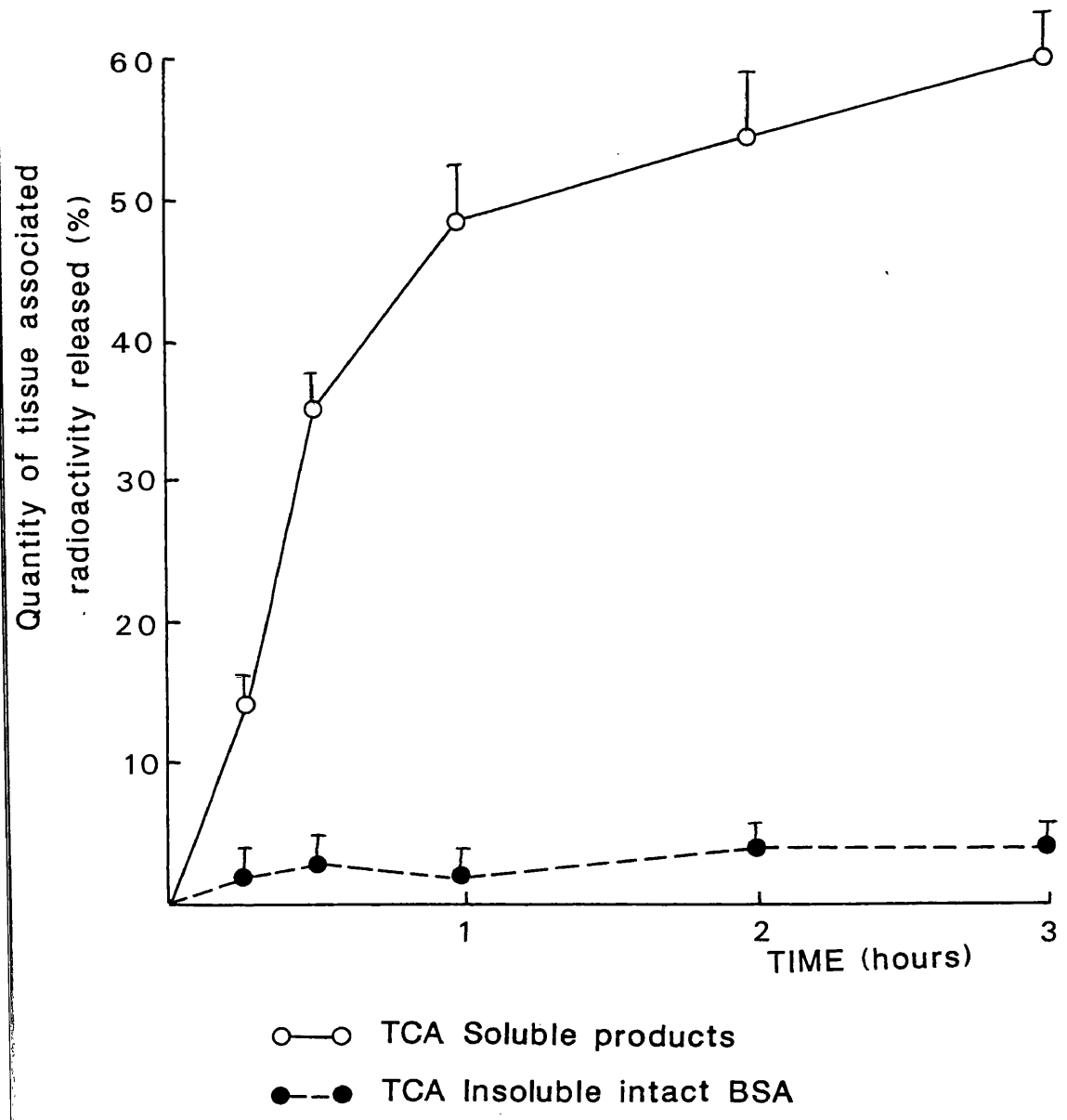


Figure 6.3(b) **Release of radioactivity by 17.5 day *in vivo* yolk sacs after pre-incubation in medium containing ^{125}I-BSA.**



6.5 Inhibition of ^{125}I-labelled BSA uptake by EGTA.

6.5.1 Introduction.

The chelating agent ethyleneglycol-bis(aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) is a known inhibitor of endocytosis. It has been shown previously to inhibit both fluid phase and adsorptive pinocytosis in the yolk sac (Duncan and Lloyd 1978). EGTA collects Ca^{2+} and causes a depletion of these essential ions in the membrane regions, thus inhibiting endocytosis. An advantage of this reagent is that the addition of equimolar calcium reverses the effect and restores the endocytic capacity of the tissue. EGTA has been used here to illustrate that the results previously presented do represent true uptake and intra lysosomal processing of the substrate.

6.5.2 Materials and Methods.

In this experiment 17.5 day 'giant' yolk sacs (cultured as outlined in section 2.5) and 17.5 day *in vivo* yolk sacs (explanted directly from the female as outlined in section 2.9) were each incubated in three different sets of conditions. The incubation conditions used were:

(i) Medium 199,

(ii) Medium 199 + 5mM EGTA,

(iii) Medium 199 + 5mM EGTA + 5mM $CaCl_2$,

In all cases yolk sacs were incubated in an atmosphere of 95% O_2;5%CO_2.

'Giant' yolk sacs, cultured from egg cylinders explanted from 3 females at 9.5 days, were placed at random (3 yolk sacs per 60ml bottle) in the three incubation conditions.

17.5 day *in vivo* yolk sacs explanted directly from 2 or 3 pregnant females were randomly allocated to 60ml culture bottles containing 10ml of medium.

All yolk sacs were incubated in the three different media as described in sections 6.2.4 and 6.2.5 over a 5 hour period, and the uptake of ^{125}I-BSA was measured. From these results an EI was calculated in each case as previously described. The series of experiments was repeated twice and mean values for all results obtained +/- SE.

6.5.3 Results.

From the individual plots of uptake volume against time constructed after each experiment, mean EI's were calculated. These results +/- SE in all three incubation conditions and with both tissue types are presented in Table 6.2.

Table 6.1 The inhibition ^{125}I-BSA uptake by EGTA.

Incubations	Endocytic Index (µl/mg protein/hour)	
	In vivo yolk sas	'Giant' yolk sacs
Medium 199	25.20 +/- 1.40 (n=20)	1.51 +/- 0.08 (n=20)
Medium 199 + 5mM EGTA	1.61 +/- 0.06 (n-24)	0.21 +/- 0.01 (n=21)
Medium 199 + 5mM EGTA + 5mM $CaCl_2$	23.73 +/- 1.24 (n=21)	1.34 +/- 0.05 (n=19)

We can see from Table 6.2 that the uptake of ^{125}I-BSA in Medium 199 is greatly reduced (to less than 15% of levels observed in the absence of the inhibitor) by 5mM EGTA added to the incubation medium. This effect is reversed by 5mM $CaCl_2$. A similar effect is observed in both 'giant' yolk sac and in vivo yolk sacs despite endocytosis being at a higher basal level in the in vivo yolk sac incubated in Medium 199.

6.5.4 Discussion.

The presence of EGTA in the incubation medium caused a significant decrease in the pinocytic uptake of ^{125}I-BSA in both the 'giant' yolk sac and 17.5 day in vivo yolk sac. As the inhibitor has also been shown previously to inhibit both fluid phase pinocytosis and adsorptive pinocytosis (Duncan and Lloyd (1978), this is good evidence that the substrate is internalised by the cells via a pinocytic route and is

processed intracellularly.

The simple addition of equimolar $CaCl_2$ to the incubation medium containing EGTA represses the action of the chelating agent and allows both *in vivo* yolk sacs and 'giant' yolk sacs to take up ^{125}I-BSA at a rate very similar to that observed in the control situation. The inhibitor was therefore in no way toxic to the tissue.

SECTION 7

THE UPTAKE OF ^{125}I-IMMUNOGLOBULIN G (IgG)

7.1 Introduction.

Receptor mediated endocytosis is a specialised form of endocytosis found in many animal cells by means of which nutritional, regulatory and other macromolecules can be selectively assimilated from the extracellular milieu with high efficiency. Invariably the process involves binding of the protein to specific receptors on the cell surface followed by rapid internalisation. A detailed account of the process is given in the General Introduction (sections 1.5 and 1.6). Macromolecules taken up by this process include plasma transport proteins (eg. low density lipoprotein), certain polypeptide hormones (eg. insulin, epidermal growth factor) and immunoglobulins.

The visceral yolk sac is the major site of transport for maternal immunoglobulins in several mammalian species particularly the rabbit and the guinea pig (Brambell 1970). However it is also important in the rat and mouse (Brambell and Halliday 1956) in which the transfer of IgG also takes place via the neonatal gut.

The visceral yolk sac has been shown to transfer large quantities of both homologous and heterologous IgG, the amount of the latter dependent on the species of origin (Brambell,Hemmings and Rowland 1948; Koch,Boesman and Gitlin 1967). It is the Fc fraction of IgG which is recognised by the cell surface receptors and the Fab fraction is not transported efficiently (Brambell,Hemmings,Oakley and Porter 1960). Further evidence of an IgG receptor comes from competition work in which unlabelled IgG competes for binding sites with labelled IgG (Gitlin and Morphis 1969). Also it has been observed that transport can reach

saturation.

Much work has been carried out on the uptake of IgG by the neonatal and young rat gut epithelium (Rodewald 1980; Abrahamson and Rodewald 1981) and, as well as providing details of uptake by this particular tissue type, their work also gives more general information regarding the receptor mediated endocytosis of IgG.

Using IgG conjugated to tracers, electron microscopic studies showed that IgG binds to receptors at the brush border (Rodewald 1970,1973,1976) and that binding is pH dependent. Binding occurs at pH 6-6.5 (normal pH of luminal fluid) but not at pH 7.4.

It is important to note that some IgG also enters the cell unbound, in the fluid phase. Morris and Morris (1974,1976) found that in jejunal segments of newborn rats only 40% of the administered ^{125}I-IgG was transmitted intact to the circulation. This was also shown by Rodewald (1973) who observed IgG-Ferritin in apical multivesicular bodies. After receptor bound transfer the normal abluminal pH of 7.4 causes IgG to dissociate from its receptor.

Huxham and Beck(1981) studied the transport of IgG across 11.5 day rat visceral yolk sacs. Using colloidal gold as an electron dense marker they found transport to be receptor mediated and to involve coated vesicles. Also they found that the number of particles seen in apical coated vesicles was much greater than the number observed to have been transported to the basolateral surface.

More recent work has been carried out by Weisbecker et al. (1983) using the 17.5 day visceral yolk sac maintained in short term organ culture, to measure the uptake of ^{125}I-IgG. These experiments have shown IgG to be taken up by the visceral yolk sac at a significantly greater rate than the fluid phase marker PVP. Also results indicate that approximately half of the IgG taken up is digested before being released back into the culture medium - half appears to be transported intact. By incubating in ^{125}I-IgG in the presence of the cathepsin inhibitor leupeptin, the rate of release of intact ^{125}I-IgG on reincubation in fresh medium remained unchanged but the rate of proteolysis fell by approximately 50%. This indicates that the 'sorting' of IgG for transport and IgG for digestion in the lysosomes occurs before fusion with the lysosomal system.

In the present study the uptake of ^{125}I-labelled IgG by the 'giant' yolk sac is compared to the uptake in the control 17.5 day yolk sac, using a modification of the method of Williams et al. (1975a,b). Also a preliminary electron microscopic study of IgG uptake by the two systems has been made using colloidal gold as an electron dense marker.

7.2 Materials and Methods.

7.2.1 Labelling of IgG.

A modified version of the Chloramine-T method (Greenwood and Hunter 1963) was the method chosen for the radio-iodination of rat IgG, based on the successful results obtained by other authors (eg. Weisbecker et al. 1983), its low cost, the high efficiency of labelling and the small amount of damage to the molecule.

10mg rat IgG (Sigma chemical Company) was dissolved in 5ml of 0.05M phosphate buffer, pH 8 in a sterile glass bijou bottle, and was labelled with a solution of ^{125}Iodine following the method outlined in Appendix D.

7.2.2 Efficiency of labelling.

A small sample of the IgG solution before and after dialysis were analysed to assess the efficiency of the labelling technique, as outlined in Appendix D.

7.2.3 Assessment of % TCA soluble radioactivity after dialysis.

The amount of TCA soluble radioactivity (ie. free iodide) present in the IgG solution after dialysis must be low and remains low throughout all experiments. Therefore an assessment of the % TCA soluble radioactivity in the IgG preparation was carried out after dialysis and during each experiment to ensure levels do not become unaceptably high

(see Appendix D).

7.2.4 Uptake of ^{125}I-IgG by 17.5 day *in vivo* yolk sacs explanted directly from the mother.

(i) Explantation of yolk sacs.

Conceptuses were removed as quickly as possible from the pregnant female as outlined in the General Materials and Methods (section 2.9).

(ii) Preparation of incubation medium.

10ml of Medium 199 was carefully introduced to 60 ml sterile culture bottles which were gassed with a mixture of 95%O_2;5%CO_2 for approximately 30 seconds and sealed with silicone bungs. The bottles were then placed upright in a shaking water bath at 37^{o}C and allowed to equilibrate.

(iii) Uptake of ^{125}I-IgG.

The washed yolk sacs were transferred to pre-warmed incubation bottles, a maximum of 4 yolk sacs being placed in each bottle, and were allowed to equilibrate for 30 minutes. Limiting the number of yolk sacs per bottle to four was essential as the rate of uptake of ^{125}I-IgG by yolk sacs has previously been found to be at a high level (Weisbecker et al. 1983); A larger number of yolk sacs per bottle may significantly deplete the concentration of ^{125}I-IgG in the incubation medium, and hence effect the overall rate of IgG uptake.

Throughout experiments described in this chapter ^{125}I-IgG was added to incubations to a final concentration of 1µg/ml at time zero. The bottles were re-gassed with a mixture of 95%O_2;5%CO_2 (found to promote an optimum rate of pinocytosis) and the silicone bungs replaced. The bottles were incubated in the shaking water bath for periods up to 5 hours and were terminated at half hourly or hourly intervals.

At the end of each incubation period the amount of radioactivity in all samples was measured as outlined in section 6.2.4(iii).

7.2.5 Uptake of ^{125}I-IgG by 17.5 day 'Giant' yolk sacs.

'Giant' yolk sacs were cultured as outlined in section 2.5, washed and 7ml of fresh Medium 199 was introduced to each incubation bottle containing three yolk sacs.

After the period of equilibration ^{125}I-IgG was added to each incubation botle to a final concentration of 1µg/ml, the bottles were regassed with 95%O_2;5%CO_2 and incubated at 37°C for periods up to 5 hours, incubations being terminated at half hourly and hourly intervals.

At the end of each incubation period the bottles were removed from the incubator, taking care not to disrupt the delicate yolk sac membranes. The amount of radioactivity (total and TCA soluble) in the incubation medium and the exoceolomic fluid and the total radioactivity of the 'giant' yolk sac tissue was measured as described in detail for the uptake of ^{125}I-BSA (section 6.2.5(ii)).

7.2.6 Uptake of ^{125}I-IgG by 17.5 day 'giant' yolk sacs after removal of the embryonic tissue.

'Giant' yolk sacs were cultured after removal of the embryonic tissue by the method outlined in section 2.8. The uptake of ^{125}I-IgG by these yolk sacs and the subsequent measurement of radioactivity was carried out exactly as described above for the 'giant' yolk sacs containing a dead embryo.

7.2.7 Calculating the uptake of ^{125}I-IgG as an Endocytic Index(EI).

The Endocytic Index(EI) is the unit we have used throughout to express the rate of uptake of a particular substrate by a certain tissue type (Williams et al. 1975a,b). It has the units µl of fluid whose contained substrate was captured/ unit quantity of tissue/ hour (µl/mg protein/hour).

(i) Calculating the uptake of ^{125}I-IgG by *in vivo* 17.5 day yolk sacs.

The calculation involved here is essentially identical to that used for the uptake of ^{125}I-BSA. The only difference is that IgG as well as being digested can also be released in an intact form; however the nature of the uptake experiment makes it impossible to differentiate between ^{125}I-IgG present in the incubation medium and that released from the tissue intact. It can therefore not be quantified here.

The equation used for calculating the uptake of ^{125}I-IgG is therefore:-

$$\frac{(Y + 10S^{m})}{M' \times P}$$

Where Y is the total amount of radioactivity retained by the tissue (cpm corrected for background), S^{m} is the total quantity of TCA soluble radioactivity in the culture medium at the end of incubation (cpm/ml corrected for background and the amount of TCA soluble radioactivity present naturally in the IgG preparation) M' is the mean TCA insoluble radioactivity in the culture medium over a particular incubation period (cpm/µl medium corrected for background) and P is the protein content of the yolk sac tissue (mg).

Uptake therefore has the units µl/mg protein. Plots of uptake volume/time were then constructed as described in section 6.2.7(i) and an EI obtained from the slope of each plot.

(ii) Calculating the rate of uptake of ^{125}I-IgG by the 17.5 day 'giant' yolk sac and 'giant' yolk sac grown after prior removal of the embryonic tissue.

The intact nature of the 'giant' yolk sac allows radioactivity to be present in the yolk sac membranes and the products of IgG digestion and intact IgG to be transported either into the exocoelom or back into the incubation medium. Therefore the method of calculating the rate of uptake of ^{125}I-IgG by the 'giant' yolk sac is very similar to that for

^{125}I-BSA and involves the calculation of an uptake volume after different incubation times and the plotting uptake volume against time to obtain an EI (section 6.2.7(iii)). Although it is possible to measure the TCA insoluble ^{125}I-IgG released into the exocoelom this was not included in the equation to allow a direct comparison of EI for GYS and in vivo yolk sacs. In section 7.5 a theoretical calculation representing a possibly more accurate measure of EI is discussed. Again as the rate of uptake by the 'giant' yolk sac is relatively low, there is no depletion of substrate in the medium and therefore the amount of radioactivity in the medium can be measured at the end of incubation.

The modified equation used is:-

$$\frac{(Y + 7S^{m} + S^{e})}{M \times P}$$

Where Y is the total amount of radioactivity retained by the yolk sac tissue (cpm corrected for background); S^{m} is the total quantity of TCA soluble radioactivity in the incubation medium at the end of each incubation (cpm/ml corrected for background and the amount of TCA soluble radioactivity naturally in the ^{125}I-IgG preparation) and S^{e} is the total quantity of TCA soluble radioactivity released into the exocoelomic fluid (cpm corrected for background). M is the total TCA insoluble radioactivity present in the incubation medium at the end of each incubation (cpm/ul corrected for background) and P is the protein content of the yolk sac tissue (mg).

Experimentation.

7.3 The uptake and processing of ^{125}I-IgG by 17.5 day 'giant' yolk sacs and in vivo yolk sacs when incubated in Medium 199.

7.3.1 Introduction

The uptake and processing of IgG by the cells of the visceral yolk sac is known to involve the binding of IgG to specific receptors. This process known as specific receptor mediated pinocytosis (see sections 1.5 and 1.6) is a highly efficient method of uptake and allows a proportion of the total IgG taken up to cross the yolk sac intact. Previous workers (eg Weisbecker et al. 1983) have demonstrated quantitatively using ^{125}I-IgG that some intact ^{125}I-IgG as well as some radiolabelled digestion products are released from rat visceral yolk sacs, back into the incubation medium, using the technique of Williams et al. (1975a,b). However, as was the case with the release of the products of ^{125}I-BSA digestion (section 6.3), the unique nature of the giant yolk sac enables the directional nature of ^{125}I-IgG to be monitored.

In this experiment both giant yolk sacs (with and without embryos) and 17.5 day in vivo yolk sacs are incubated in Medium 199 containing ^{125}I-IgG. Medium 199 was chosen as an incubation medium despite being less physiological than whole serum, because it removed the possibility of competition for binding sites between the labelled and unlabelled proteins and hence made analysis of the results easier.

7.3.2 Materials and Methods.

In this experiment 17.5 day 'giant' yolk sacs cultured both with and without embryonic tissue and 17.5 *in vivo* yolk sacs explanted directly from the mother, were incubated in Medium 199. The incubation of the 'giant' yolk sacs being carried out in a roller incubator and the incubation of *in vivo* yolk sacs in a shaking water bath ; in section 5.3.1 the differences in the two methods of incubation were shown to have no effect on pinocytic uptake.

17.5 day *in vivo* yolk sacs from 2 or 3 pregnant females were removed and randomly allocated to incubation bottles during each experimental run. The number of yolk sacs per bottle was limited to four as uptake has previously been shown to occur at a high rate which could cause the depletion of substrate concentration.

9.5 day conceptuses explanted from 2 pregnant females were grown to 17.5 day 'giant' yolk sacs (as detailed in section 2.5) and randomly used in each experimental run.

Also 9.5 day conceptuses were explanted from 2 pregnant females and the embryonic tissue removed as outlined in section 2.8. The remainder of the conceptus in each case was cultured to 17.5 days as previously outlined. They were randomly used in each experimental run.

The rate of uptake of ^{125}I-IgG and its subsequent digestion and transport by both the 'giant' yolk sac and 17.5 day *in vivo* yolk sac was

measured and the modified EI calculated in each case as described in section 7.2.

In the control yolk sac the uptake was calculated from the total amount of radioactivity present in the yolk sac tissue and the TCA soluble radioactivity released back into the incubation medium.

In the 'giant' yolk sac uptake was calculated from the total amount of radioactivity in the yolk sac tissue, the total amount of TCA soluble radioactivity in the exocoelomic fluid and the TCA soluble radioactivity in the incubation medium. Graphs of the uptake volume/incubation time were plotted for each experiment and from the slope, the EI was found. The experiments were repeated a number of times and the mean values +/- SE for all results found.

In order to elucidate the complex processes involved in IgG transport and digestion, the relative amount of TCA soluble and insoluble radioactivity in the different compartments of the 'giant' yolk sac system (exocoelomic fluid and yolk sac tissue) were analysed separately and histograms plotted of the distribution of products in the different compartments.

7.3.3 Results.

In all cases the plots of uptake volume against time were found to be linear and by obtaining the mean volume uptake at each time interval, plots were constructed.

From each individual plot drawn after each experiment mean EI values were calculated. The mean EI +/- SE for both 'giant' yolk sac systems and *in vivo* 17.5 day yolk sac are shown in table 7.1.

Table 7.1. Uptake of ^{125}I-IgG by *in vivo* and 'giant' yolk sacs.

	Endocytic Index (µl/mg tissue protein/hour)		
	'Giant' yolk sac	'Giant' yolk sac without embryo	*in vivo* 17.5 day Y.S
Medium 199; 95%O_2;5%CO_2	4.6 +/- 0.4 (n = 92)	4.38 +/- 0.22 (n=52)	56.65 +/- 3.12 (n = 85)

Table 7.1 shows that the rate of uptake of ^{125}I-IgG by both 'giant' yolk sacs cultured following the original method and those cultured after prior removal of the embryonic tissue are not significantly different. The rate of uptake by *in vivo* yolk sacs in medium 199 and an atmosphere of 95%O_2;5%CO_2 is much greater than the rate of uptake by either 'giant' yolk sac system in the same conditions, as was the case with the uptake of ^{125}I-BSA (section 6.3.3). Also it is important to note that the rate of uptake of ^{125}I-IgG in all three systems is significantly higher than the uptake of ^{125}I-BSA in the same conditions.

The main advantages of the 'giant' yolk sac for studying the transport/digestion of macromolecules is that it is a closed system in which the exocoelomic fluid is maintained within the continuous epithelium of the yolk sac separate from the incubation medium, thus allowing the directional nature of transport to be studied.

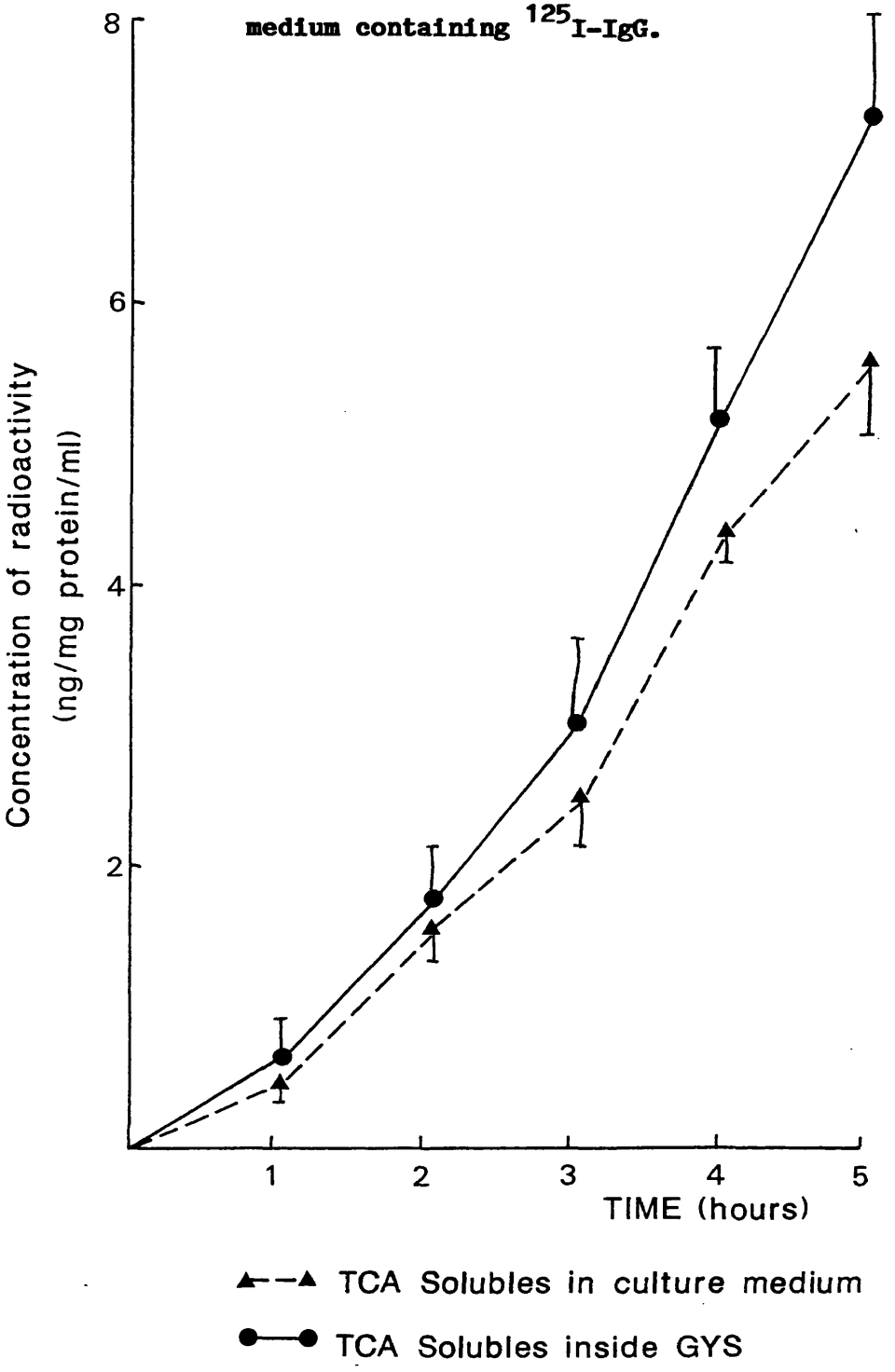
From these experiments it is therefore possible to obtain information about the vectorial nature of ^{125}I-IgG transport. Figure 7.1 shows the relative amounts of radioactive digestion products released to the inside and outside of the giant yolk sac throughout the 5 hour incubation period. The results are expressed in the form of a concentration (ng/ml/mg tissue protein). Soluble radioactive material is expressed as a protein equivalent (i.e the amount of protein from which the measured solubles arose). As with the distribution of ^{125}I-BSA digestion products (Figure 6.2,section 6.3.3) the concentration of iodotyrosine was found to be higher in the exocoelomic fluid than in the incubation medium throughout the 5 hour incubation period.

Results indicate that in the exocoelomic fluid the amount of TCA insoluble intact ^{125}I-IgG is always equal to or greater than the amount of TCA soluble digestion products.

Discussion.

This experiment is discussed in conjunction with experiment 7.4 (The release of IgG and iodotyrosine from the tissue) in section 7.4.4.

Figure 7.1 **Distribution of radioactivity to inside and outside of 'giant' yolk sac throughout incubation in medium containing ^{125}I-IgG.**



7.4 Release of ^{125}I-IgG and iodotyrosine after pre-loading the tissue.

7.4.1 Introduction.

To complete the picture of the directional nature of ^{125}I-IgG transport, the release of intact IgG and its digestion products into the incubation medium was studied. In the case of BSA, release was shown to be almost totally in the form of the products of lysosomal digestion, however in this case some whole IgG is likely to be released back into the incubation medium by both 'giant' yolk sacs and *in vivo* yolk sacs. As any whole IgG released back into the incubation medium cannot be distinguished from the IgG already in the medium (as described in section 7.2.7), it is impossible to include this in the EI calculation. Therefore the value for the rate of uptake of ^{125}I-IgG calculated in the previous experiment is an underestimate. However the results of this experiment have been combined theoretically with those obtained in section 7.3 to give an approximation of a more accurate measure of EI.

7.4.2 Materials and Methods.

(a) Release by 17.5 day *in vivo* yolk sacs.

17.5 day *in vivo* yolk sacs explanted directly from the mother were pre-incubated in medium containing 1μg/ml ^{125}I-IgG and gassed with 95%O_2;5%CO_2 in a shaking water bath at 37°C. Pre-incubation was carried out for 3 hours. At the end of this time the yolk sacs were removed from the incubation medium and washed extensively for 3 x 2mins. in fresh Medium 199 before being re-incubated in bottles containing 10ml fresh

Medium 199 for a further 3 hours. At intervals of 0, 15, 30, 60, 120 and 180mins. after the start of re-incubation in fresh medium a 0.5ml sample of the medium was taken and the total radioactivity and the TCA soluble radioactivity in each was measured. At the end of the re-incubation the amount of radioactivity in each yolk sac was also measured.(See section 6.5.2 for a more detailed description of this method.) From these results a plot was drawn of the ^{125}I-radioactivity released into the medium as a % of the total radioactivity associated with the yolk sac tissue at the start of re-incubation. The TCA soluble and insoluble fractions were calculated separately.

(b) Release by 17.5 day 'giant' yolk sacs into the culture medium.

The release of ^{125}I-IgG from both 'giant' yolk sac systems into the culture medium was also studied independently following the same proceedure, after a pre-incubation period of 3 hours in Medium 199 containing 1µl/ml ^{125}I-IgG, with an atmosphere of 95%O_2;5%CO_2. After pre-incubation, the ^{125}I-IgG containing medium was carefully removed and the 'giant' yolk sacs washed for 3 x 2mins. in fresh Medium 199 using a syringe and bent needle. Having removed as much as possible of the last washing, 7ml of fresh Medium 199 was introduced to each bottle containing three yolk sacs. The bottles were re-gassed with 95%O_2;5%CO_2 and replaced at 37°C in the roller incubator. 0.5ml samples of re-incubation medium were taken from each bottle at regular intervals(0, 15, 30, 60, 120 and 180mins.) after the start of re-incubation. The total amount of radioactivity and the TCA soluble radioactivity in each sample was measured. Also at the end of each time period some 'giant' yolk sacs were harvested and the radioactivity (TCA soluble and insoluble) present

in the exocoelomic fluid and the yolk sac tissue was measured. From the results a plot was drawn of the release of radioactivity into the re-incubation medium as a percentage of the total radioactivity associated with the 'giant' yolk sac at the start of re-incubation.

7.4.3 Results.

The two plots (Figure 7.2a and b) show that over the 3 hour re-incubation period in fresh medium, the in vivo yolk sac released approximately 80% of the tissue associated radioactivity present at the start of re-incubation and both 'giant' yolk sac models released approximately 70% of the tissue associated radioactivity. In the control yolk sac approximately 2/3 of the total released was in the form of TCA insoluble intact IgG and 1/3 TCA soluble iodotyrosine. In the 'giant' yolk sac approximately equal amounts of iodotyrosine and intact ^{125}I-IgG made up the total release of radioactivity.

7.4.4 Discussion of the uptake and release of ^{125}I-IgG by the 'giant' yolk sac and the 17.5 day in vivo yolk sac.

The results of experiment 7.3 indicate that in both in vivo yolk sacs and 'giant' yolk sacs ^{125}I-IgG is taken up and processed with greater efficiency than either ^{125}I-PVP known to be taken up only in the fluid phase and ^{125}I-BSA taken up mainly adsorbed to the plasma membrane. The EI's for ^{125}I-IgG uptake (calculated as described in section 7.2.7) were found to be at least twice the values found for the uptake of ^{125}I-BSA (calculated as described in section 6.2.7). This agrees with the results

Figure 7.2(a) **Release of radioactivity from 'giant' yolk sacs after pre-incubation in medium containing ^{125}I-IgG.**

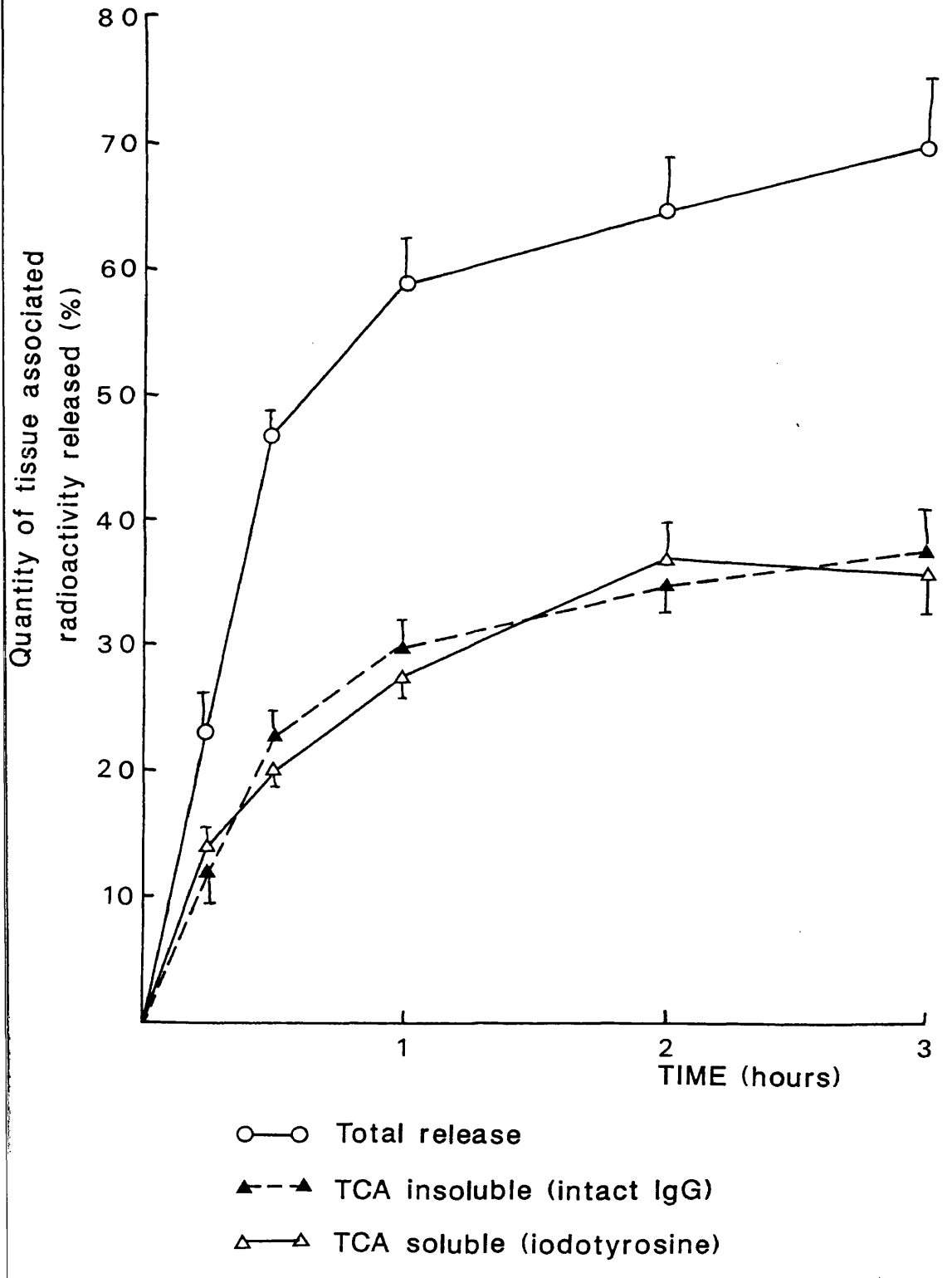
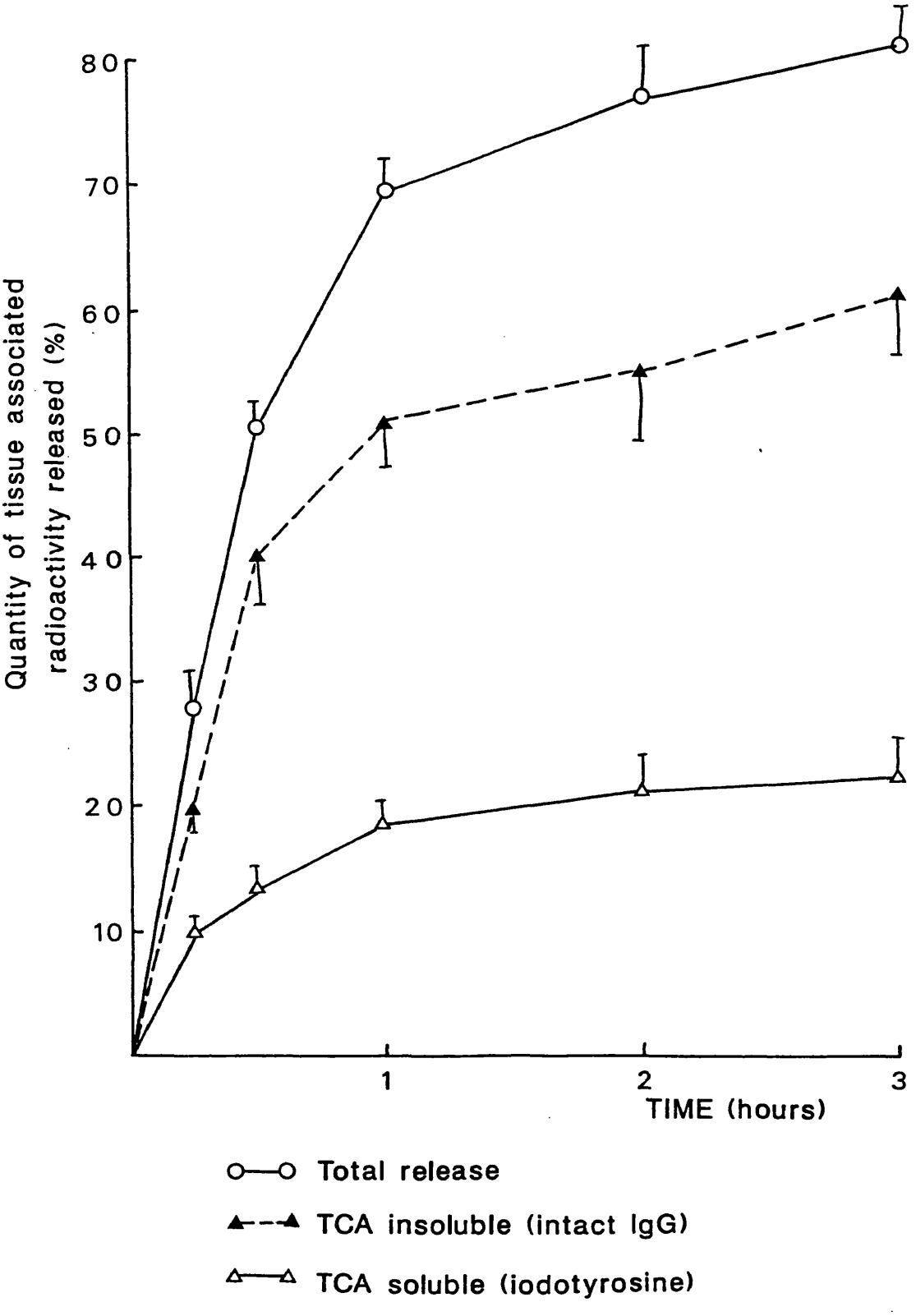


Figure 7.2(b) **Release of radioactivity from 17.5 day *in vivo* yolk sacs after pre-incubation in medium containing ^{125}I-IgG.**



of previous workers (eg Weisbecker et al. 1983) who demonstrated a highly efficient method of uptake of biologically important macromolecules - specific receptor mediated endocytosis.

The results also show clearly that 'giant' yolk sacs cultured after removal of the embryonic tissue at 9.5 days, seem to function identically to those cultured by the original method. The microdissection has no significant effect on the rate of uptake of ^{125}I-IgG by the yolk sacs.

It is very important to stress that ^{125}I-IgG differs from other macromolecules studied in that some of the substrate is taken up by the yolk sac tissue and released intact. In the rat the visceral yolk sac is an important route for the transfer of prenatal passive immunity.

During the incubation of 17.5 day <u>in vivo</u> yolk sac tissue, any intact ^{125}I-IgG released from either the apical or basal surfaces of the visceral yolk sac will be returned to the incubation medium. Such released ^{125}I-IgG is indistinguishable from the high concentration of labelled substrate already present. In the 'giant' yolk sac system intact ^{125}I-IgG released into the exocoelomic fluid can be successfully measured, but again any intact ^{125}I-IgG released via the apical surface of the yolk sac into the medium cannot be measured. This means that the Endocytic Index calculated for both tissues is greatly underestimated, and impossible to measure accurately using the techniques outlined in this thesis. However by preloading the tissue with ^{125}I-IgG, reincubating in fresh medium and studying its release (experiment 7.4), further information can be obtained and will subsequently be discussed.

Also by combining the results of uptake (experiment 7.3) and release (experiment 7.4) it may be possible to calculate in theory a closer approximation of the true rate of uptake of ^{125}I-IgG in both tissue types (see section 7.5).

As already stated the major advantage of the 'giant' yolk sac system is that the continuous epithelium of the 'giant' yolk sac maintains the exocoelomic fluid in an enclosed compartment. This allows us to study the direction of any transport mechanisms. Figure 7.1 shows that the concentration of TCA soluble radioactivity released to the inside of the 'giant' yolk sac was maintained throughout the incubation at a higher level than the concentration in the incubation medium. Also results indicate that throughout the incubation period the amount of TCA insoluble whole IgG transported into the exocoelomic fluid was equal to or greater than the amount of TCA soluble digestion products. Indicating some method of preferentially transporting intact IgG which avoids lysosomal digestion.

The studies into the release if ^{125}I-IgG (experiment 7.3) have shown that over a 3 hour period TCA insoluble whole IgG is also released back into the incubation medium. In the *in vivo* 17.5 day yolk sac the amount of whole IgG released was approximately twice the quantity of iodotyrosine. However the route through the cell by which the ^{125}I-IgG was transported back into the medium remains uncertain. In the 'giant' yolk sac the amount of iodotyrosine and intact ^{125}I-IgG released were approximately the same. The difference observed in the release of intact ^{125}I-IgG might be due to the fact that release back into the medium, during incubation of *in vivo* yolk sacs can be from both apical and basal

surfaces. The intact giant yolk sac allows release into the incubation medium only via the apical cell surface. Any release of intact ^{125}I-IgG from the basal surface accumulates within the exocoelom.

By extrapolation of the results of uptake and release (discussed at greater length in section 7.5) it can be concluded that whole ^{125}I-IgG which has been taken up and transported intact by the giant yolk sac appears to be maintained at a higher concentration in the exocoelomic fluid than in the culture medium. Thus showing that IgG is transported against a concentration gradient and in this sense seems to be directed preferentially into the exocoelom to maintain the concentration gradient.This is likely in view of the importance of IgG for the immunity of the developing embryo.

7.5 Theoretical calculation of total ^{125}I-IgG uptake.

In this section I have estimated what should be a closer approximation of the true rate of uptake of ^{125}I-IgG expressed as an EI. For the purposes of this theoretical calculation approximate mean figures have been used throughout.

(i) 17.5 day in vitro yolk sac.

In vivo yolk sacs are incubated as opened sacs (necessitated by the removal of the embryo immediately before incubation) and therefore everything is released back into the incubation medium. This simplifies the calculation of the EI but but does not provide as much information as the closed 'giant' yolk sac system.

In experiment 7.3 the following equation was used to calculate an uptake volume, from the measurement of total radioactivity in the tissue and TCA soluble digestion products accumulating in the medium:

$$\frac{Y + 10S^{m}}{M \times P}$$

(Key for abbreviations used in equations - see p176.) From the slope of a plot of uptake volume against time a value for the EI was obtained.

The release experiments (section 7.4) demonstrated that the ratio of TCA soluble : TCA insoluble radioactivity released was approximately 3 : 1.

A modified equation could therefore read:

$$\frac{Y + 40S^m}{M \times P}$$

(ii) 'Giant' yolk sac.

As 'giant' yolk sacs are closed systems, the exocoelom forms an additional compartment into which both intact ^{125}I-IgG and digestion products may be released after intracellular processing.

In experiment 7.3 the following equation was used to calculate an uptake volume from measurements of the TCA soluble radioactivity in the medium and exocoelomic fluid and total radioactivity in the yolk sac tissue:

$$\frac{Y + 7S^m + S^e}{M \times P}$$

From the slope of a plot of uptake volume against time a value for the EI was obtained.

Although the amount of TCA insoluble whole ^{125}I-IgG transported into the exocoelom during uptake was not used in the above equation (to allow a meaningful comparison with the *in vivo* yolk sac) it was measured and the concentration of TCA insoluble intact ^{125}I-IgG was always slightly higher than the TCA soluble digestion products in the exocoelom. In the the release experiments (7.4) the amount of TCA

insoluble ^{125}I-IgG and TCA soluble digestion products in the exocoelom were found to be approximately equal.

A modified equation for the uptake volume could therefore read:

$$\frac{Y + 2(7S^m) + S^e + I^e}{M \times P}$$

Key to all equations.

Y - Total radioactivity in yolk sac tissue.

M - TCA insoluble radioactivity (per ml) in medium.

P - Protein content of yolk sac tissue (mg).

S^m - TCA soluble radioactivity (per ml) in medium.

S^e - TCA soluble radioactivity (per ml) in exocoelom.

I^m - Total TCA insoluble radioactivity in medium.

I^e - Total TCA insoluble radioactivity in exocoelom.

Theoretical calculation using newly modified equations.

By substituting mean % values of the radioactivity in each compartment it is possible to calculate (by combining the results of experiments 7.3 and 7.4) a more accurate estimate of the true EI for ^{125}I-IgG.

Table 7.2 Distribution of TCA soluble and insoluble radioactivity in yolk sac systems.

Radioactivity	Mean % of total radioactivity	
	GYS	*in vivo* YS
Yolk sac tissue (Y)	28	19
Medium solubles (S^m)	53	81
Exocoelom solubles (S^e)	9	-
Exocoelom insols. (S^i)	10	-

Using the newly modified equations estimates of the true EI were calculated and Table 7.3 compares these results with those obtained in experiment 7.3. As has been found previously the capacity of the *in vivo* yolk sac for endocytic uptake is far greater than that of the 'giant' yolk sac.

Table 7.3 EI for uptake of ^{125}I-IgG.

	EI (µl/mg tissue protein/hour)	
	GYS	*in vivo* YS
EI calculated in experiment 7.3	4.6	56.6
Theoretical EI	7.8	220.9

Although these results are only estimates they do indicate the likely high efficiency of the specific receptor mediated uptake of ^{125}I-IgG in both 'giant' yolk sacs and 17.5 day *in vivo* yolk sacs.

7.6 Electron microscopic study of the uptake of colloidal gold and IgG-colloidal gold in the 'giant' yolk sac and the 17.5 day in vivo yolk sac.

7.6.1 Introduction.

A short study has been carried out to compare the uptake of colloidal gold and IgG-colloidal gold in the giant yolk sac and 17.5 day *in vivo* yolk sac systems. In this experiment human IgG was used as it was routinely being labelled in the laboratory and was readily available. However as has been shown (Brambell et al. 1948) both heterogenous and homogenous IgG is taken up rapidly by yolk sac tissue and this experiment should illustrate well the difference between the fluid phase uptake of colloidal gold and the receptor mediated uptake of IgG-colloidal gold.

7.6.2 Materials and Methods.

(i)Preparation of colloidal gold solutions

Colloidal gold particles with a diameter of approximately 18nm were produced in solution by the method of Horrisberger and Rossett(1977) modified by Huxham and Beck(1981). This was achieved by the sodium citrate reduction of chloroauric acid under reflux. Two colloidal gold solutions were made:-

a. In the first the pH was adjusted to pH 7.0 with 0.2M K_2CO_3 and stabilised with 20M Polyethylene glycol (PEG) at a

concentration of 0.6mg PEG/ ml colloid.

b. In the second solution the pH was adjusted to 7.0 with 0.2M K_2CO_3 and mixed for 5mins. with 1mg human IgG, previously dialysed against 0.005M NaCl. The solution was stabilised with 20M PEG (0.6mg/ml colloid).

Both solutions were extensively washed with Hank's balanced salt solution (BSS), to give a solution of approximately 20 x 10^{12} particles/ml.

(ii)Incubation in colloidal gold solutions.

17.5 day in vivo yolk sacs were explanted directly from the female as described in section 2.9 and were washed for 3 x 2mins. in fresh Hank's BSS.

'Giant' yolk sacs were cultured as described in section 2.5 and at 17.5 days were washed for 3 x 2mins. in Hank's BSS.

Both in vivo yolk sacs and 'giant' yolk sacs were incubated in 5ml. of colloidal gold solution and human IgG-colloidal gold solution at 37°C, with an atmosphere of 95%O_2;5%CO_2, in a roller incubator. Incubations were terminated after 5mins. and 60mins.

After the incubation periods all yolk sacs were washed extensively in Hank's BSS and rapidly fixed with 3% gluteraldehyde in 0.1M sodium/phosphate buffer and processed for electron microscopic examination as described in section 3.2.5. Pale gold/silver sections

were cut from each block and stained with uranyl acetate and lead citrate. The sections were observed using a Jeol 100s electron microscope at 80KV and photographs taken.

7.6.3 Results.

The general morphology of the 'giant' yolk sac and 17.5 day in vivo yolk sac tissue has been described in detail in chapter 3. After 1 hour incubation in the colloidal gold solutions no morphological changes indicative of any damage or cell death were observed in the tissue.

(i) Uptake of colloidal gold.

Plate 7.1a and b show the presence of 18nm colloidal gold particles in coated vesicles and a coated pit at the surface of both 'giant' yolk sac and in vivo yolk sac tissue after incubation in colloidal gold solution for 5mins.

After 1 hour incubation, colloidal gold particles were observed in the larger vacuoles containing flocculent material and also in the large electron dense secondary lysosomes, as well as the apical coated and non-coated vesicles, in both the 'giant' yolk sac and in vivo yolk sac tissue. Plate 7.2 shows colloidal gold particles in vesicles deeper in the cytoplasm of 'giant' yolk sac tissue.

No colloidal gold particles were observed free within the cytoplasm and none were seen at the basolateral surface. Although the amount of colloidal gold uptake was not accurately quantified, it was

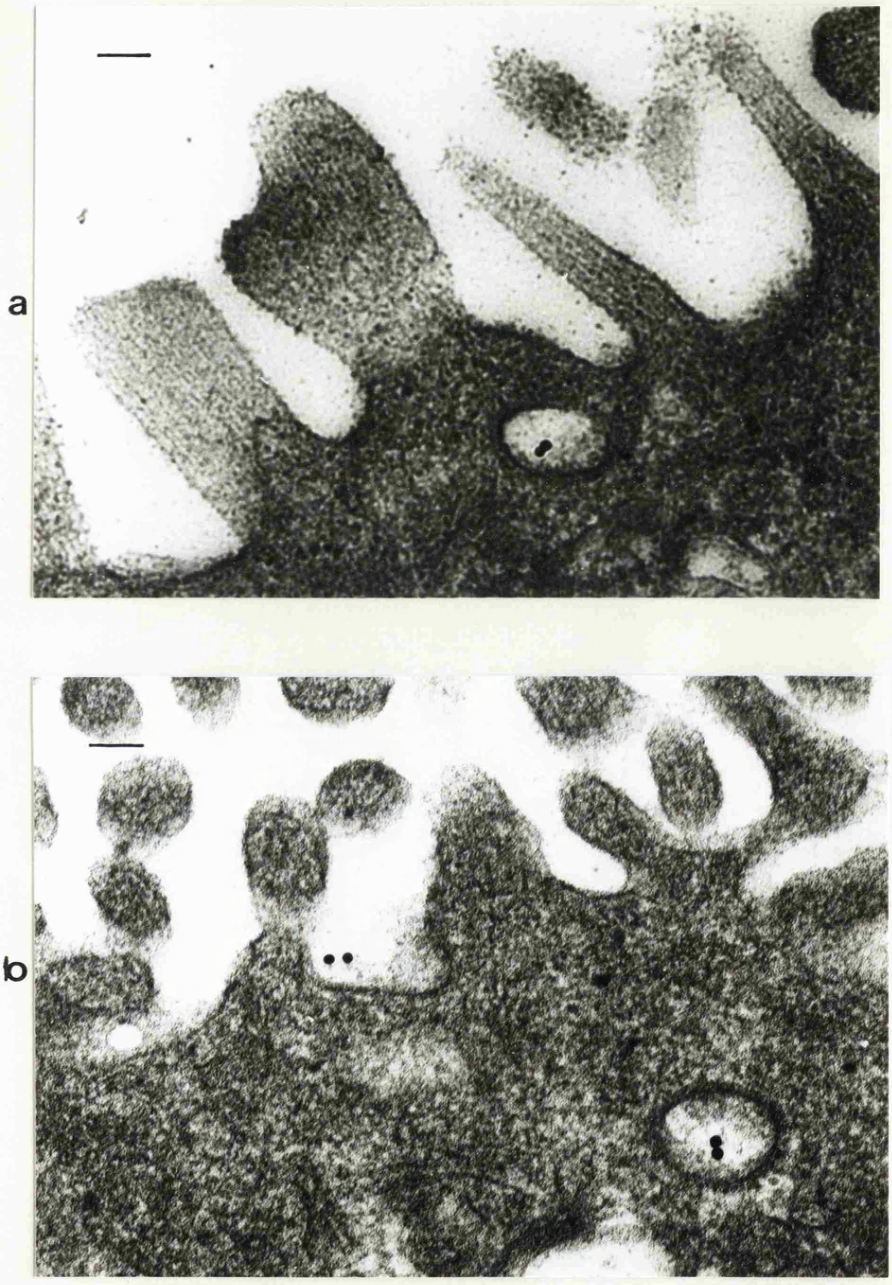


Plate 7.1 Uptake of 18 nm colloidal gold particles in
(a) 'giant' yolk sac tissue, bar = 0.1 μm,
(b) 17.5 day *in vivo* tissue. Bar = 0.1 μm.
Gold particles are present singly or in pairs in coated pits and apical coated vesicles after 5 minutes incubation in colloidal gold solution.

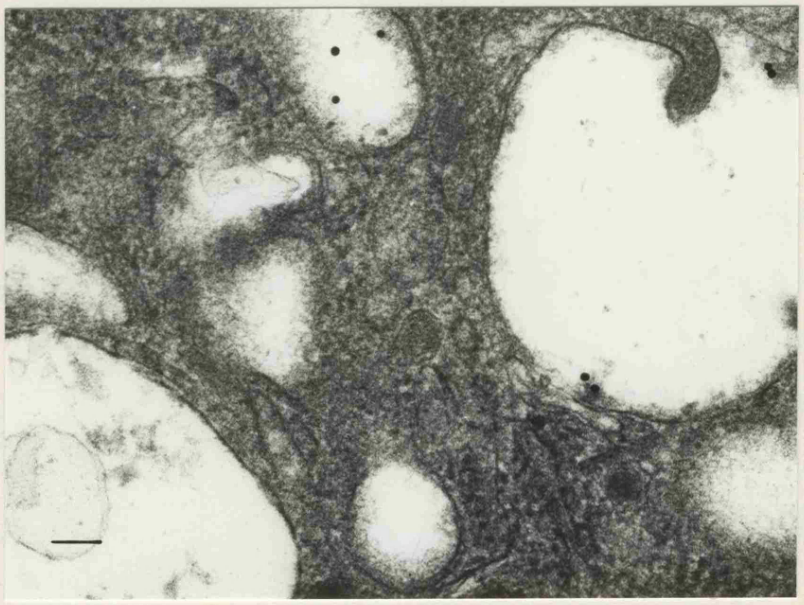


Plate 7.2 Uptake of 18 nm colloidal gold. After 1 hour incubation gold particles are present in the larger vacuoles and lysosomes deeper in the cytoplasm. Bar = 0.1 μm.

noted that colloidal gold uptake in both tissue types was relatively infrequently observed - after an incubation of 5mins. particles were observed in only perhaps 1 in 20 cells. Also colloidal gold particles were observed singly or occasionally in pairs in coated vesicles.

(ii) Uptake of human IgG-colloidal gold.

Plates 7.3a and b show the presence of human IgG-colloidal gold particles clustered in coated pits and apical coated vesicles of both 'giant' yolk sac tissue and control 17.5 day yolk sac tissue after incubation for 5mins. Note that some of the IgG-colloidal gold particles seem more closely associated with the membrane of the coated vesicle than do colloidal gold particles (Plate 7.3) perhaps indicating the former is still bound to specific membrane receptors. Human IgG-colloidal gold particles were observed clustered in numerous coated pits and coated vesicles in the majority of cells in both tissue types.

After 1 hour of incubation IgG-colloidal gold was also observed in the vacuoles containing flocculent material situated deeper in the cytoplasm and also in secondary lysosomes and residual bodies (Plate 7.4). In the larger secondary lysosomes the particles were observed to be not only in association with the membrane but also distributed within the organelle.

In both 'giant' yolk sac tissue and 17.5 day <u>in vivo</u> tissue IgG-colloidal gold was observed at the basolateral surface both in coated vesicles and in the inter cellular space after exocytosis from the cell. However the amount seem at the basolateral surface was

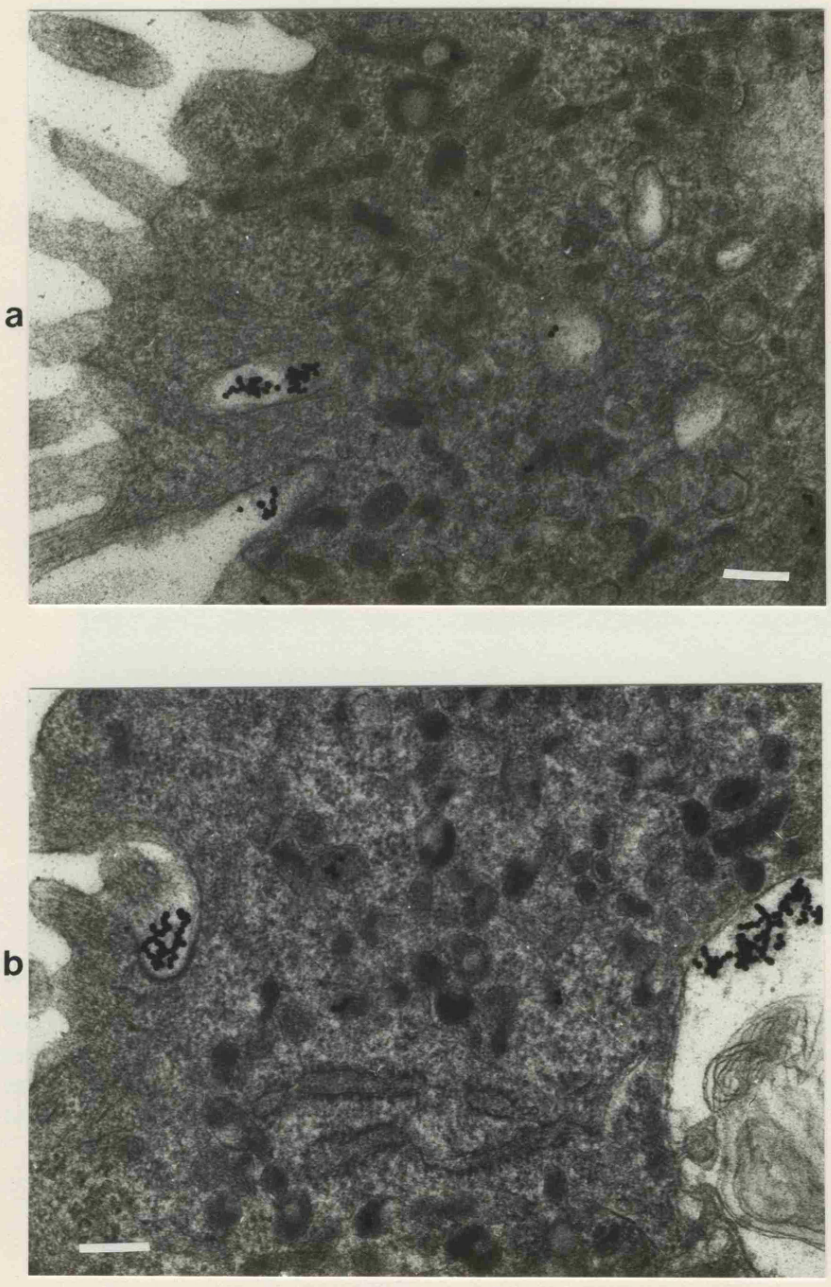


Plate 7.3 Uptake of human IgG-colloidal gold in
(a) 'giant' yolk sac tissue, bar = 0.2 μm,
(b) 17.5 day *in vivo* tissue. Bar = 0.2 μm.
After 5 minutes incubation particles are seen clustered in surface pits and apical coated vesicles.

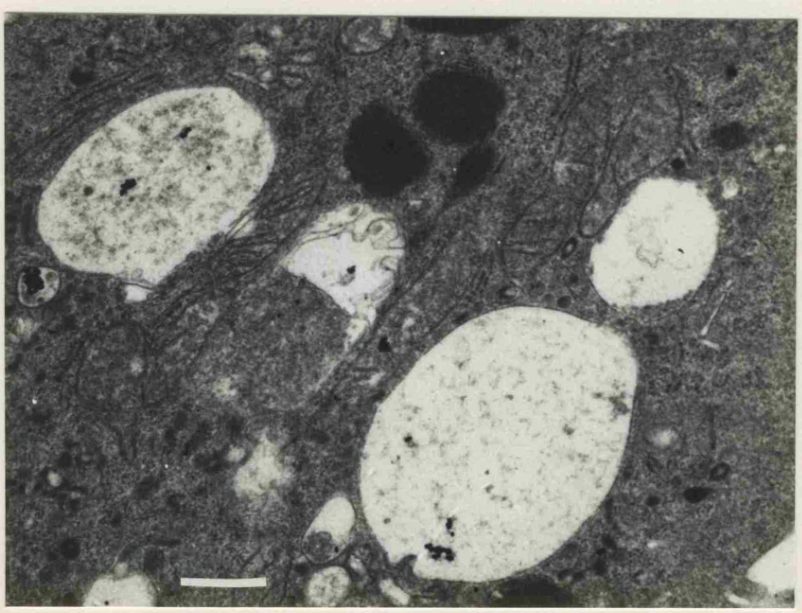


Plate 7.4 Uptake of human IgG-colloidal gold. After 1 hour incubation particles were seen in larger electron lucent vacuoles, secondary lysosomes and residual bodies deep in the cytoplasm and also in small electron dense vacuoles. Bar = 0.2 μm.

significantly less than at the apical surface. Again no IgG-colloidal gold was seen free within the cytoplasm in either tissue.

7.5.4 Discussion.

From these results we can say that both colloidal gold and IgG-colloidal gold are taken up in both tissues by endocytosis. However the clustering of IgG-colloidal gold and the greatly increased frequency of uptake of the IgG bound particles indicates a difference in the mechanism of uptake of the two colloids - colloidal gold being a marker of fluid phase pinocytosis and IgG-colloidal gold known to be taken up by specific receptor mediated pinocytosis. The micrographs also agree with the previous findings of Anderson et al. (1977) which showed that IgG binds only to receptors clustered in the coated regions of the plasma membrane.

The presence of IgG-colloidal gold and not colloidal gold alone at the basolateral surface indicates that some IgG appears to be transported across the cell. However the observation of IgG-colloidal gold also in the secondary lysosomes indicates that some IgG is also subjected to lysosomal digestion, as was shown in section 7.3.1 and has been shown in the work of others with the use of both visible markers and also quantitatively assessed using ^{125}I-IgG (Rodewald 1973; Morris and Morris 1974,1976; Weisbecker et al. 1983). The majority of IgG-colloidal gold was observed in small vesicles deep in the cytoplasm, which are perhaps the vesicles involved in protein transport avoiding lysosomal digestion as described by Helenius (1983).

This electron microscopic study therefore confirms the results obtained in the uptake studies (section 7.3.1) and stresses the importance of coated vesicles for the transport of intact IgG and also indicates the difference in efficiency between fluid phase pinocytosis and specific receptor mediated pinocytosis.

SECTION 8

THE TRANSPORT OF AMINO ACIDS

8.1 Introduction

Amino acids are the basic building blocks of all proteins, playing a crucial role in all biological processes and are therefore an essential nutritional requirement of the developing embryo. Before the establishment of the chorioallantoic placenta in the rat, the visceral yolk sac is solely responsible for providing embryonic nutrition, and hence supplying amino acids for protein synthesis.

In previous experiments we have shown, using the 'giant' yolk sac system, that the visceral yolk sac is capable of breaking down large ^{125}I-labelled proteins to their TCA soluble subunits and transporting these products into the exocoelomic fluid.

Freeman et al. (1981) used the whole embryo culture technique (New 1973) and incubated the conceptuses for periods up to 12 hours in dialysed serum collected from rats previously injected with ^{3}H-leucine; they terminated culture at 11.5 days. They found that the labelled serum protein was taken up by the yolk sac tissue, broken down and the products of lysosomal digestion transported to the developing embryo. Also in the yolk sac and the embryo there was a gradual accumulation of new proteins containing ^{3}H-leucine, presumably due to protein synthesis. This elegant experiment demonstrates the ability of the yolk sac to degrade protein and its important role in providing the embryo with the amino acids essential for protein synthesis.

The yolk sac has been shown to be the site of synthesis of several proteins including albumin, pre-albumin, alpha fetoprotein, alpha

antitrypsin, transferrin and embryo specific α-globulin (Gitlin and Kitzes 1967 using chick yolk sac; Gitlin and Perricelli 1970 using human yolk sac). Also work on the mouse yolk sac has shown it to be the site of synthesis of several fetal mouse serum proteins, particularly alpha fetoprotein, the synthesis of which has been localised in the endodermal cells (Gitlin and Boesman 1967; Gustine and Zimmerman 1972; Dziadek and Adamson 1978). Janzen et al. (1982) also demonstrated the synthesis of transferrin by mouse visceral yolk sac.

Although the yolk sac can degrade maternal protein to form amino acids essential for de novo protein synthesis, it is also capable of the uptake of amino acids from the external environment. Deren et al. (1966) used rabbit yolk sac at various stages of gestation to study amino acid transport and accumulation. They found that ^{14}C-valine accumulated in pieces of rabbit yolk sac only after 20-22 days of gestation, to a concentration five times that in the incubation medium. Before this stage there was no accumulation only equilibration within the tissue. By re-forming the yolk sacs into sacs (tied with cotton) with the endodermal surface outermost in contact with the incubation medium, they studied transmembrane translocation of ^{14}C-valine. They found that the amino acid was transported from the incubation medium to the internal compartment against a significant concentration gradient and that the tissue concentration rose to seven times that in the external medium and four times that in the internal cavity.

Al-Alousi (1983) used the 'giant' yolk sac and 12.5 day conceptus to study the uptake of a complex mixture of all twenty amino acids labelled with ^{14}C. He demonstrated the accumulation of radioactivity with time in the exocoelomic fluid of both systems. However, further characterisation of the amino acids present in the fluid was difficult due to the complex nature of the amino acid mixture used. He also demonstrated that there was some incorporation of ^{14}C-amino acids from the culture medium, into TCA insoluble protein in the exocoelomic fluid of both the 'giant' yolk sac and 12.5 day yolk sac.

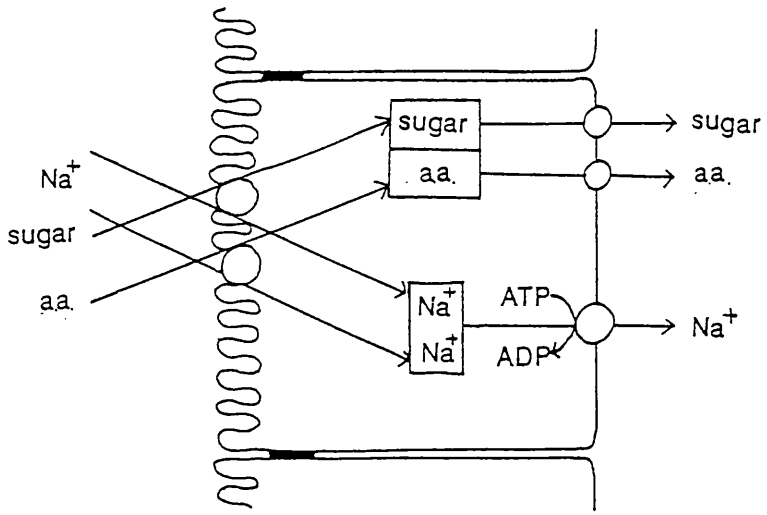
The mechanism of amino acid transport (and that of other organic solutes) is far from fully understood although the process has been observed in many cell types. The majority of detailed investigations have been carried out in a limited number of systems particularly pigeon erythrocytes, ascites cells and small intestine. However the evidence obtained in these systems, together with the observations made in other tissue types has led us to believe that the general characteristics of the processes involved in the transport of organic solutes are very similar in many cell types.

The mechanism of transport of organic solutes across a cell membrane has been thought for many years to be influenced by Na^+. However some of the first evidence was that of Christensen et al. (1952) in which the uptake of glycine or alanine by duck red blood cells was inhibited by removing some of the sodium in the incubation medium and replacing it with potassium. Further evidence of Na^+ involvement in organic solute transport in the small intestine of rats and toads, and

amino acid adsorption by intestine, thymus nucleii, kidney slices and brain slices, firmly established the importance of Na^+.

The transport of amino acids is an active process against an electrochemical gradient, however the process is not thought to be directly linked to an energy yielding mechanism. It has been suggested that transport occurs due to the concentration difference of Na^+ inside and outside the cell (Figure 8.1). As Na^+ enters the cell along its concentration gradient, amino acids are carried in as well complexed to the same carrier molecule (co-transport). The maintainence of the Na^+ gradient does however require energy and Na^+ entering the cell has then to be actively transported out again to maintain its concentration gradient. Once inside the cell, amino acids can be released from the carrier. This model has been called the sodium - gradient hypothesis (Crane 1965).

Figure 8.1 The Sodium-gradient hypothesis



From the kinetics of transport of various amino acids and competition between different amino acids for transport, it has been

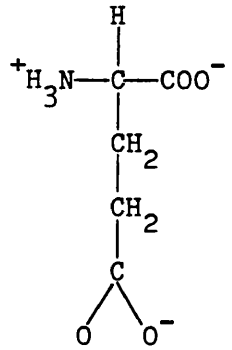
deduced that there are at least five different amino acid transport systems, each of which transports a group of closely related amino acids. Systems have been found for:

1. Small neutral amino acids,
2. Large neutral amino acids,
3. Basic amino acids,
4. Acidic amino acids,
5. Imino acid Proline.

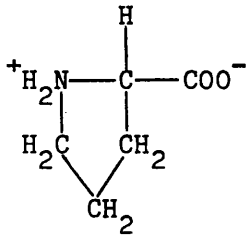
Each has been characterised by an optimum pH and by specific K_M values of the substrates. Inhibition is possible by certain amino acid analogues.

In view of the complex nature of the possible amino acid transport mechanisms this short study has been limited to the investigation of two amino acids, thought to be transported by two different mechanisms. The relative rates of uptake and incorporation of the two amino acids was studied.

(i) Glutamic acid (with an acidic side chain);



(ii) Proline (with a secondary amino group - an imino acid);



The uptake of ^{14}C-glutamic acid and ^{14}C-proline were studied using the 'giant' yolk sac system. The transmembrane transport of the amino acids and any tissue accumulation were studied as well as the accumulation of radiolabelled, newly synthesised proteins in the yolk sac tissue and the exocoelomic fluid.

8.2 Materials and Methods.

In this study the uptake, transport and accumulation of the ^{14}C-labelled amino acids glutamic acid and proline were investigated.

8.2.1 Culture of 'giant' yolk sacs.

'Giant' yolk sacs were cultured according to the method described in section 2.5. The 9.5 day embryos were explanted and cultured for 8 days in whole rat serum in an atmosphere of $20\%O_2;5\%CO_2;75\%N_2$. In each experiment 'giant' yolk sacs were cultured from the embryos explanted from two pregnant females. At 17.5 days the 'giant' yolk sacs were used for experimentation.

8.2.2 Preparation of incubation medium.

The incubation of 'giant' yolk sacs in ^{14}C-glutamic acid and ^{14}C-proline was carried out in Medium 199 not rat serum because although Medium 199 does contain amino acids, the concentration of each is at a pre-determined level (see Appendix C), unlike the variable levels in rat serum. ^{14}C-labelled glutamic acid and proline (both with the same specific activity - 280mCi/mM) were added individually to Medium 199 (containing 500IU/ml Penicillin and 500µg/ml Streptomycin) to a final concentration of 0.19µg/ml and 0.1µg/ml respectively. This was a trace amount to give an indication of amino acid uptake as there is a far greater concentration of unlabelled amino acids in the incubation medium (75µg/ml glutamic acid;40µg/ml proline).

8.2.3 Incubation of 'giant' yolk sacs.

At 17.5 days the culture bottles containing 'giant' yolk sacs were taken from the roller incubator and the culture serum removed. The yolk sacs were then washed as previously described and 7ml of fresh medium 199(containing antibiotics) was introduced. The incubations were gassed with $95\%O_2;5\%CO_2$ and allowed to equilibrate. At time zero the medium was removed and quickly replaced with warmed Medium 199 containing the ^{14}C-amino acid (either glutamic acid or proline). The incubation bottles were re-gassed with $95\%O_2;5\%CO_2$ and incubations carried out for up to 5 hours in a roller incubator at $37^{\circ}C$.

At the end of each incubation period the bottle or bottles (each containing 3 'giant' yolk sacs) were removed from the roller incubator and 2 x 0.5 ml samples of the incubation medium were taken and placed in 5 ml plastic scintillation tubes. The remaining medium was carefully removed and the 'giant' yolk sacs washed well in several changes of ice-cold 1% NaCl. The 'giant' yolk sacs were then removed from the incubation bottles as previously described (section 2.6). Each yolk sac was observed under the disection microscope to ensure that its structure was intact. The exocoelomic fluid was then collected using a syringe and fine needle and its volume noted. 2 x 0.5 ml samples were placed in 5ml plastic scintillation tubes. If the total volume of exocoelomic fluid obtained from a 'giant' yolk sac was less than 1 ml, the volume was noted and then it was made up to 1 ml with distilled water. The visceral yolk sac endoderm was dissected free from the amnion, ectoplacental cone and remains of the embryo and the inner surfaces washed again in ice-cold 1% NaCl. Each yolk sac was places in a plastic tube and the

total volume made up to 1.5 ml with distilled water. The 'giant' yolk sacs were then sonicated using an MSE Ultra sonicator for approximately 10 seconds until the tissue appeared completely solubilised. 2 x 0.5 ml samples were placed in 5ml plastic scintillation tubes and the remainder kept for protein estimation carried out using a modification of the technique of Lowry et al. (1951) (see Appendix B).

8.2.4 Liquid scintillation counting of samples.

To one of each duplicate sample (exocoelomic fluid, yolk sac tissue homogenate and incubation medium) 0.25 ml of rat serum and 0.25 ml of trichloroacetic acid (TCA) was added, the samples were mixed well and then centrifuged in an MSE Centaur 2 centrifuge for 30 minutes at 3000rpm. This caused the TCA insoluble protein fraction to precipitate and leave the TCA soluble fraction in the clear supernatant, which was decanted into a clean 5 ml plastic scintillation tube.

4.5 ml of Fisofluor '1' (Fisons Scientific Apparatus) was added to all samples (the original samples and those to which TCA had been added) and the samples extensively vortexed to ensure a good mix of the sample and scintillation fluid. The scintillation tubes were then placed in plastic scintillation vials and the ^{14}C in all samples measured three times on a Packard Tri-carb Liquid Scintillation Spectrometer. From the results mean values for the counts per minute in each sample were calculated.

In order for the results to give meaningful information it was necessary to determine the amount of quenching occurring in each sample,

as all the samples were of different composition.

8.2.5 Correction for quenching.

Figure 8.2 Energy transfer process.

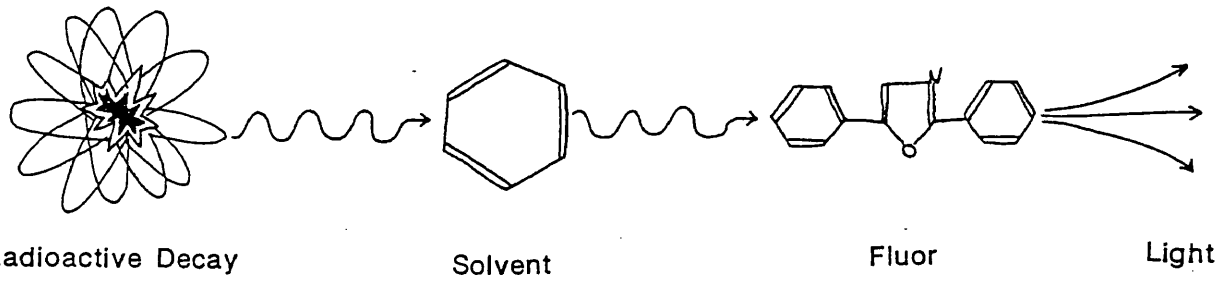


Figure 8.2 shows the scintillation energy transfer process. Ideally energy transfer would be perfectly efficient, however in reality energy losses (quenching) can be substantial and are dependent on a number of factors. Three main types of quenching occur:-

(i) Physical quenching due to a lack of intimate contact between the radioactive particle and the scintillation fluid or when photons of light generated by the process are absorbed within the vial.

(ii) Chemical quenching can be produced by any compound, the degree of quenching depending on the chemical composition and structure of the compound.

(iii) Colour quenching occurs after the fluorescence stage when light absorbing compounds can reduce the number of photons detected. As fluorescence occurs in the blue region of the spectrum the order of severity of colour quenching is:-

red > orange > yellow > green > blue.

Light detected after energy transfer can be described as counts per minute (CPM) and the number of actual radioactive particles decaying as disintegrations per minute (DPM). Therefore cpm is only a value of the apparent activity present after quenching has occured. To compare results in different samples it is necessary to find the true activity actually present in dpm, by evaluating the amount of quenching in each sample.

One such method is to 'spike' each sample with a known amount of activity and calculate the efficiency from the cpm measured.

$$\text{Efficiency} = \frac{\text{Activity measured (cpm)}}{\text{Activity added (dpm)}}$$

This was carried out by adding a range of activities to samples identical to those used in the experiment (ie 0.5ml exocoelomic fluid, 0.5ml yolk sac homogenate and 0.5ml Medium 199 both with and without the addition of TCA). The mean efficiency found in each case are shown in Table 8.1.

Table 8.1 Efficiency of scintillation counting in different media.

	Medium 199	Medium 199 + TCA	Fluid	Fluid + TCA	Tissue	Tissue + TCA
Mean % efficiency +/- SE	59.0 +/- 0.7	66.6 +/- 0.5	67.6 +/- 0.3	67.7 +/- 0.4	70.0 +/- 0.3	67.2 +/- 0.3

The individual results of all samples in cpm were therefore amended using the above efficiencies to give dpm, and therefore meaningful comparisons of the results could be made.

8.2.6 Calculating rates of uptake and accumulation.

All results were expressed as dpm/mg yolk sac protein. The amount of amino acid uptake and transport to the exocoelomic fluid was calculated from the sum of the TCA soluble radioactivity present in the tissue and in the exocoelomic fluid. Also the amount of new protein synthesised was calculated from the difference between TCA soluble radioactivity and total radioactivity in each sample (exocoelomic fluid, tissue and medium). An overall value for the processes of amino acid uptake, transport and protein synthesis was obtained by summing the total radioactivity in the exoceolomic fluid and tissue and the TCA insoluble radioactivity in the incubation medium.

8.3 Results.

Table 8.2 shows the results of the uptake of ^{14}C-proline and ^{14}C-glutamic acid expressed in DPM/mg protein after various incubation times. Results have been expressed as TCA soluble and TCA insoluble radioactivity in the different compartments and also as overall amounts. Obviously any TCA soluble radioactivity (amino acids) which is taken up by the tissue and released back into the incubation medium, cannot be distinguished from the labelled amino acid already in the medium as a substrate. From the results in table 8.1, plots have been constructed to illustrate any difference in the uptake and processing of the two amino acids.

Figure 8.3 shows the overall uptake (DPM/mg protein) of ^{14}C-glutamic acid and ^{14}C-proline, calculated from the total radioactivity in the exocoelomic fluid, the total tissue associated radioactivity and the TCA insoluble radioactivity in the incubation medium. It can be seen that the uptake of both amino acids appears linear with time and that the uptake of ^{14}C-proline appears to be greater than that of ^{14}C-glutamic acid, the mean rate of uptake of ^{14}C-proline being 1263 DPM/mg protein/hour and the mean rate of uptake of ^{14}C-glutamic acid being 733 DPM/mg protein/hour.

Figure 8.4 shows the transport of both amino acids (calculated from the tissue associated TCA soluble radioactivity and the TCA soluble radioactivity in the exocoelomic fluid). Also plotted is the amount of this total which is tissue associated. It is clear with both amino acids that the amount of TCA soluble tissue associated radioactivity is quite

Table 8.2 **The uptake and processing of ^{14}C-labelled amino acids by the giant yolk sac.**

Amino Acid	Time (Hours)	Disintigrations per minute / milligram tissue protein: Tissue Sols.	GYSF Sols.	**Total Sols.**	Tissue Insols.	GYSF Insols.	Medium Insols.	**Total Insols.**	**TOTAL ^{14}C-LABELLED AMINO ACID UPTAKE**
Glutamic Acid	1	120.0 +/- 4.1	627.7 +/- 30.0	**747.7 +/- 25.9**	61.1 +/- 2.2	319.1 +/- 15.3	2470.3 +/- 92.0	**2850.5 +/- 105.0**	**3598.2 +/- 131.0**
	2.5	122.5 +/- 10.9	1008.1 +/- 27.2	**1130.6 +/- 38.1**	138.1 +/- 12.3	672.1 +/- 18.3	3180.3 +/- 382.0	**3990.5 +/- 211.1**	**5121.1 +/- 312.9**
	5	129.0 +/- 2.5	1464.0 +/- 66.6	**1593.0 +/- 65.7**	196.7 +/- 3.9	714.5 +/- 32.5	4002.9 +/- 164.6	**4914.1 +/- 166.0**	**6507.1 +/- 182.0**
Proline	1	144.8 +/- 13.0	1163.7 +/- 107.3	**1308.5 +/- 94.0**	96.5 +/- 8.7	334.0 +/- 30.1	2612.1 +/- 104.5	**3042.6 +/- 126.7**	**4351.1 +/- 220.9**
	2.5	158.0 +/- 9.2	2653.0 +/- 175.6	**2811.0 +/- 166.0**	224.3 +/- 13.6	1006.3 +/- 66.6	3039.3 +/- 132.8	**4269.9 +/- 101.6**	**7080.9 +/- 153.1**
	5	160.3 +/- 2.5	3622.0 +/- 24.0	**3782.3 +/- 26.5**	418.4 +/- 6.6	1450.8 +/- 11.9	4177.2 +/- 132.1	**6046.4 +/- 177.0**	**9827.8 +/- 189.4**

Figure 8.3 **Uptake of ^{14}C-glutamic acid and ^{14}C-proline in the 'giant' yolk sac system.**

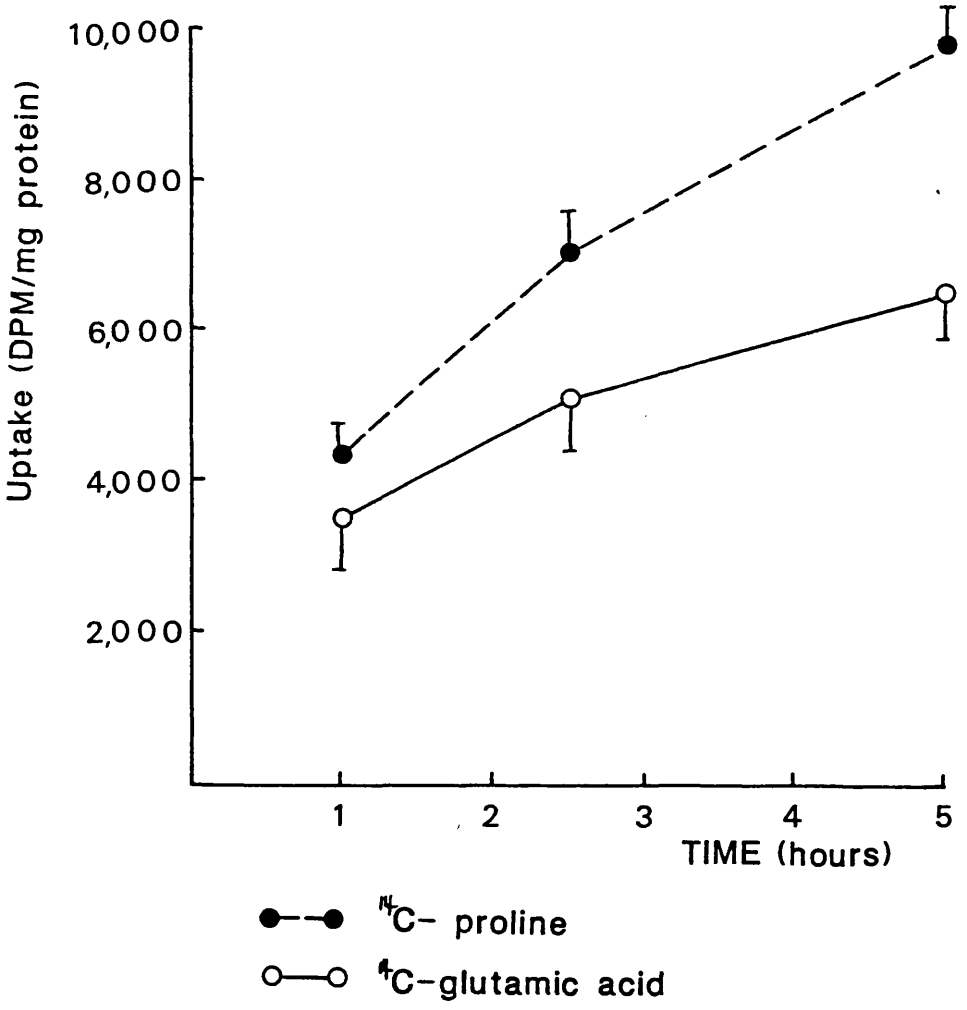
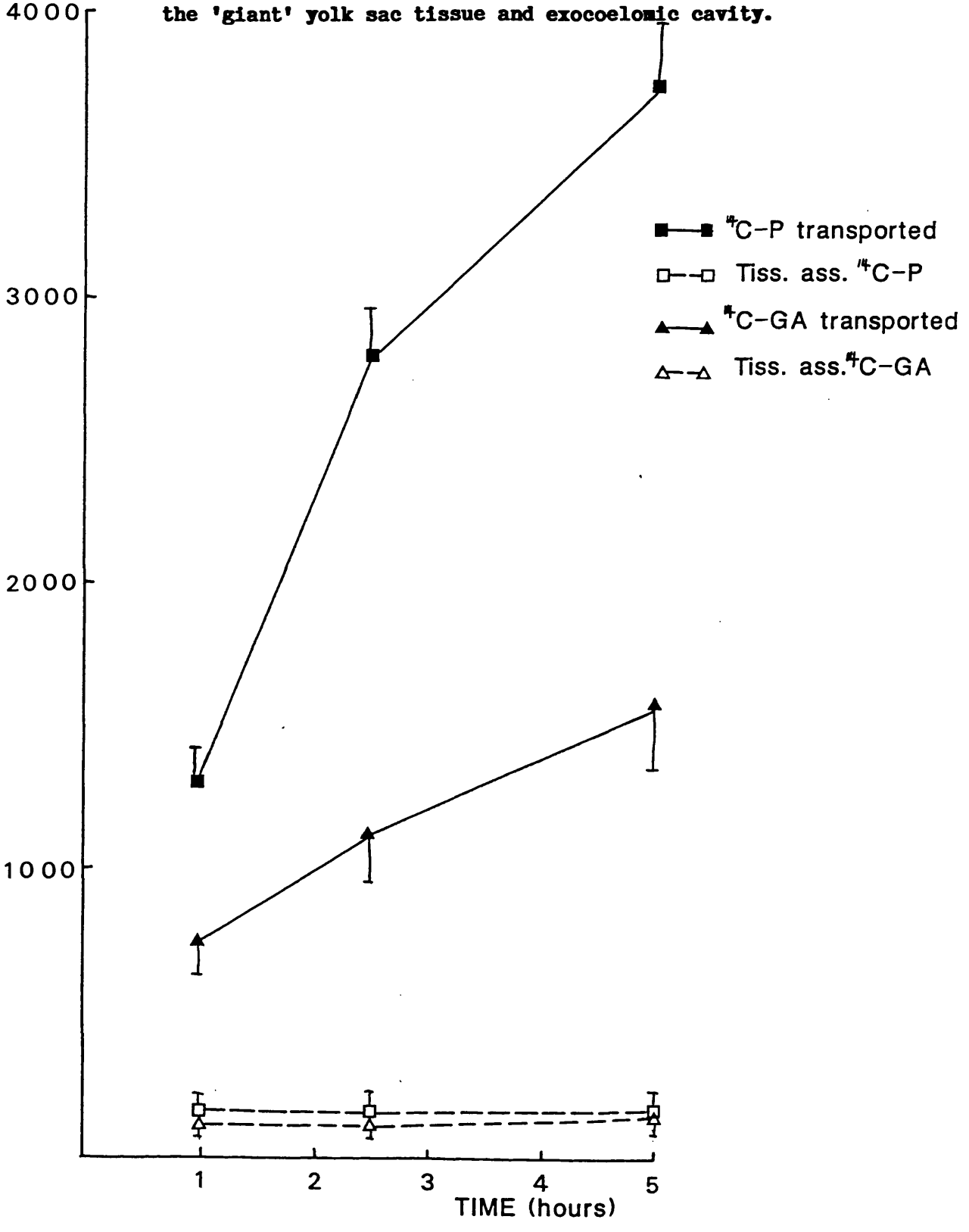


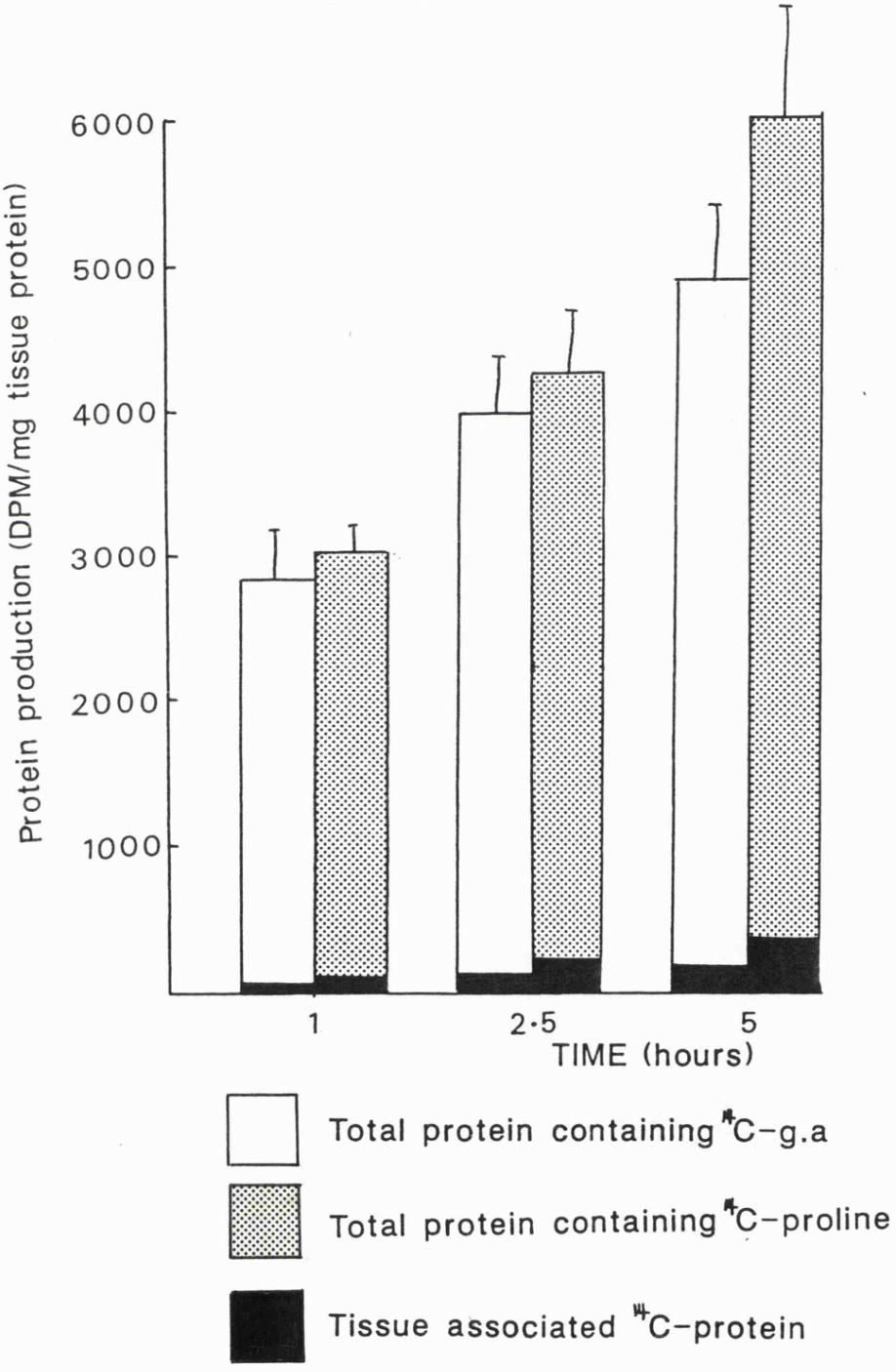
Figure 8.4 **Transport of ^{14}C-glutamic acid and ^{14}C-proline into the 'giant' yolk sac tissue and exocoelomic cavity.**



a small fraction and that the level stays fairly constant throughout incubation. Once again the transport of ^{14}C-proline seems to occur at a faster rate than the transport of ^{14}C-glutamic acid.

Figure 8.5 shows in the form of a histogram, the amount of new protein production incorporating the labelled amino acids (calculated from the TCA insoluble radioactivity accumulating in the tissue, the exocoelomic fluid and released back into the incubation medium). Again it can be seen that the tissue associated protein is only a small fraction of the total but that the levels continue to increase throughout incubation. The amount of TCA insoluble labelled protein synthesised and transported into the exocoelomic fluid and that synthesised and then transported back into the incubation medium, are shown (Table 8.2), for both ^{14}C-proline and ^{14}C-glutamic acid. The figures indicate that with both amino acids the amount transported back into the incubation medium is much greater than the amount transported into the exocoelomic fluid. However when the volumes of the two compartments (7ml incubation medium and approx. 1ml exocoelomic fluid) are taken into account, in terms of concentration (DPM/mg protein/ml) the level in the exocoelomic fluid is maintained higher in the exocoelomic fluid throughout incubation.

Figure 8.5 **The incorporation of ^{14}C-glutamic acid and ^{14}C-proline into new protein production by the 'giant' yolk sac.**



8.3 Discussion.

The depth to which the complex process of amino acid uptake could be investigated was unavoidably restricted and time permitted the study of only two ^{14}C-labelled amino acids. Glutamic acid and proline were chosen because they belong to different structural types (acidic amino acids and imino acids respectively) and are thought to be taken up by different transport mechanisms.

The two amino acids (of identical specific activity) were added individually to incubations in trace amounts, to give an indication of amino acid uptake rather than a finite figure. Both were present unlabelled in much larger yet slightly different amounts in the incubation medium (See Appendix C for a full list of the components of Medium 199). Previous experiments have involved the uptake of mixtures of radiolabelled amino acids and the results have proved complex to assess.

From the results presented here (Table 8.2) it is clear that the 'giant' yolk sac is capable of the uptake of both amino acids followed by both transport into the exocoelom and also the incorporation of the labelled amino acids in new protein. The total ^{14}C-proline uptake appears to be greater than the total uptake of ^{14}C-glutamic acid. As the amount (in molar terms) of ^{14}C-glutamic acid in the incubation medium is greater than that of ^{14}C-proline this shows a real difference in the way the two amino acids are handled. However it is important to bear in mind that other amino acids, present in varying concentrations, may be entering the cells via the same route as one of the labelled amino

acids.

In such a situation the concentration of unlabelled amino acid is far greater than the concentration labelled and there is likely to be a saturation of the transport mechanism. Competition for transport will exist between the labelled and unlabelled amino acids.

It was also possible to measure the amount of new protein incorporating the two ^{14}C-labelled amino acids, which was produced by the yolk sac and either incorporated into the yolk sac structure or released into the exocoelom for transport to the embryo. As has previously been described the visceral yolk sac is known to synthesise several proteins important for embryonic growth and development. These proteins are transported to the embryo either through the exocoelomic fluid or via the vitelline circulation.

A future experiment could be constructed to study the uptake of a range of ^{14}C-labelled amino acids thought to be transported by different transport mechanisms, in an environment containing no competing unlabelled amino acids. In the absence of such competition a comparison of the different rates of uptake, transport and incorporation into protein could be investigated.

SECTION 9

THE GROWTH SUPPORTING PROPERTIES OF 'GIANT' YOLK SAC EXOCOELOMIC FLUID

9.1 Introduction.

As described in detail in the General Introduction (section 1.7), the nutritional demands of the early rat embryo before the establishment of the chorioallantoic placenta, are met solely by histiotrophic nutrition. This involves the breakdown of maternally supplied macromolecules in the fetal membranes (Freeman et al. 1981) and includes the passage of amino acids to the embryo for rebuilding into new protein (Freeman and Lloyd, 1983). The products of histiotrophic nutrition may pass directly to the embryo in the yolk sac blood circulation or via the exocoelomic fluid.

Whole serum has for many years proved an ideal medium for supporting normal embryonic growth. It is a complex medium and the exact nutritional requirements of a developing embryo are as yet, largely unknown. However steps have been made to elucidate embryonic growth requirements by many methods including:-

(i) Characterising the constituents of serum before and after embryo culture.

(ii) Studying the uptake of specific factors.

(iii) Studying the growth of embryos after the removal of certain factors.

(i) Analysis of serum before and after embryo culture.

Various specific substances have been found to be depleted from serum after embryo culture. Two examples are detailed below.

Sanyal (1980) measured glucose levels in serum after culture of rat embryos following the New technique (New 1978). He found levels fell from 172mg/100ml to 86mg/100ml in the first 24 hours and to 34mg/100ml in the second 24 hours. This indicated the importance of glucose to the developing embryo. Other authors have observed a similar reduction in the level of glucose post culture (Gunberg, 1976; Cockroft, 1976).

Therefore substances which are present in relatively large amounts in serum and which are depleted during embryonic growth, can be studied in this way as their depletion is easy to assess accurately. However many other factors are present in very low concentrations in serum and although depleted during embryonic growth are not easily quantified.

Priscott et al.(1983) used gradient polyacrylamide gel electrophoresis to identify 19 serum proteins of which 4 were significantly depleted following the culture of 10 day rat embryos. Klein et al.(1978) also recorded protein depletion - a protein of molecular weight 125,000 was specifically depleted. As the amount of protein utilised by a developing embryo is very small, compared to that present in the serum, any reduction in a particular protein is difficult to assess. Some workers have therefore used a process of repeatedly culturing embryos in the same serum to highlight any protein depletion (Priscott et al. 1983; Al-Alousi 1983; Brooke 1985). However such repeated culture increases the risk of embryonic development being affected by a build-up of toxins, such as lactate. To overcome this the serum is extensively dialysed between cultures.

(ii) The study of the uptake of specific factors.

Various macromolecules have been observed to be taken up by the visceral yolk sac and transported intact to the embryo, either directly in the yolk sac circulation or via the exocoelomic fluid (Brambell and Halliday 1956; Weisbecker et al. 1983). Huxham and Beck (1981) showed 11 serum proteins including IgG and transferrin to be transported intact into the exocoelomic fluid in 11.5 day conceptuses.

It is also possible that other serum constituents such as the low molecular weight growth factors are severely depleted during embryonic development, as they are only present in small quantities in serum. Growth factors are defined as growth stimulating substances that are not nutrients and are active at very low concentrations. As the majority of growth factors stimulate DNA synthesis and hence protein synthesis *in vitro*, they are essential for embryonic growth and development. Andrews (BSc. Thesis 1986) showed intact ^{125}I-EGF passed across the visceral yolk sac into the exocoelomic fluid.

(iii) Embryonic growth after the removal of certain factors from culture serum.

Various techniques have been used to deplete serum of certain factors thought to be essential; growth of embryos in such serum has been studied. Generally the methods used, remove a group of proteins rather than one specific factor. Sterile immersible millipore filters can remove substances below 10,000 or 30,000 daltons (depending on the size of filter). It has been found that the retenate, following the use

of a 30,000 dalton filter, supports very little embryonic growth (Andrews 1986).

Dialysis may also be used to deplete serum of essential low molecular weight factors. However the process gives rather variable results, depending on the length of time dialysis was carried out for and also the pressure under which the serum was placed in the dialysis tube (ie a large volume of fluid exerts more pressure on the tubing than a smaller volume on the same tubing. High pressure can cause the pore size of the dialysis tubing to increase). Serum rigorously dialysed for 4 days supports very little embryonic growth, even after glucose and vitamins have been replaced (Calvert 1985). However a significant improvement in embryonic growth was recorded when the dialysed serum was supplemented with various growth factors, including EGF and insulin.

As was previously mentioned the repeated growth of embryos in serum causes the depletion of certain essential factors. Each successive batch of embryos showing significantly inferior growth and development until the serum does not support any growth and is said to be 'exhausted'.

Most workers have therefore concentrated on the requirements of the embryo or the whole conceptus and only recently have the requirements of the yolk sac been considered. Gale (1986) showed the growth of 'giant' yolk sacs in 'exhausted', dialysed serum was inferior to their growth in dialysed, whole serum. She concluded that this was due to the depletion of specific factors in the serum and therefore the requirements for normal yolk sac growth are to some extent similar to

the requirements for normal embryonic growth.

Some essential factors may be stored in the yolk sac and released later either into the exocoelom or directly to the embryonic circulation. Lambson (1966) demonstrated that the yolk sac took up ferritin and stored it in large heterogenous vacuoles until near to term - it was then passed to the embryo.

The yolk sac has also been shown to synthesise a variety of proteins including alphafetoprotein, transferrin, albumin, prealbumin, alpha-antitrypsin, embryo specific alpha-globulin and conalbumin (Gitlin and Kitzes 1967; Gitlin and Perricelli 1970; Dziadek and Adamson 1978; Janzen et al. 1982;). General protein synthesis has been demonstrated in the 11.5 day yolk sac using ^{3}H-labelled serum proteins which were degraded in the yolk sac and then rebuilt into new yolk sac and embryonic proteins (Freeman et al. 1981). Also in this thesis (section 8) ^{14}C-amino acids were shown to be incorporated into new protein by the 17.5 day 'giant' yolk sac.

In the culture of 'giant' yolk sacs as described in section 2.5, the embryo develops normally from 9.5 to 11.5 days and has a well developed plexus of yolk sac blood vessels. However at about 12 days the embryo dies and the yolk sac circulation is no longer functional. The products of histiotrophic nutrition normally transported to the developing embryo either directly in the yolk sac circulation or via the exocoelomic fluid will accumulate in the exocoelomic fluid. Obviously some products will be used to meet the nutritional requirements of the

yolk sac. Therefore exocoelomic fluid obtained from 'giant' yolk sacs should be an excellent source of the products of histiotrophic nutrition and give an insight into the factors essential for embryonic growth and development. In fact the exocoelomic fluid may prove to be an ideal supplement to a synthetic medium providing specific trophic factors for embryonic growth.

In this section the potential of the exocoelomic fluid for supporting the growth of 9.5 day rat embryos for 48 hours is assessed.

9.2 Materials and Methods.

In this series of experiments the growth supporting properties of various media was assessed by their ability to support the growth and development of 9.5 day rat embryos over a 48 hour period, using the whole embryo culture technique (New,Coppola and Terry 1973).

9.2.1 Preparation of solutions.

A detailed list of the incubation media used is given in section 9.2.2, however before the media could be made up various constituent solutions were prepared as follows:-

(i) Whole rat serum.

Whole rat serum was collected and prepared as outlined in detail in section 2.3.

(ii) Hank's Balanced Salt Solution (BSS).

Hank's BSS was prepared as outlined in Appendix A. This solution was used to dilute the rat serum (see section 9.2.2) and also as a base for the protein containing solution below (iii).

(iii) Bovine Serum Albumin Solution (BSA).

BSA was used in this experiment as a source of bulk protein. A solution was made of 200mg BSA/ml in Hank's BSS. The BSA containing

solution was made in 20ml plastic 'universal' bottles, which were rotated at 37°C to help the protein to dissolve. After the BSA had dissolved the phenol red in the BSS indicated that the solution had become very acidic (yellow). The solution was therefore dialysed against balanced salt solution for 2 days to correct the pH. Antibiotics (100IU/ml penicillin ; 100µg/ml streptomycin) were added to prevent infection and the solution was stored at -20°C ready for use.

(iv) Exocoelomic fluid.

As described in section 2.7 the exocoelomic fluid was routinely collected from ''giant'' yolk sacs used in the morphological studies. The fluid was centrifuged at 3000rpm to remove cell debris, antibiotics added and the fluid stored at -20°C ready for use. A protein assay was carried out on all samples following a modified version of the technique of Lowry et al. (1951).

9.2.2 Preparation of culture media.

The following media were prepared:-

(i) 50% rat serum; 50% Hanks BSS

(ii) 50mg/ml BSA in Hank's BSS

(iii) Exocoelomic fluid

(iv) 75% Exocoelomic fluid; 25% BSA in Hank's BSS(200mg/ml)

Media (i), (ii) and (iv) all had a final protein content of approximately 50mg/ml.

9.2.3 Specific tests carried out on culture media.

Prior to use various assessments of each media were made:-

(i) A protein assay was carried out following the modified technique of Lowry et al.(1951) - see Appendix B.

(ii) A glucose assay was carried out using a standard assay kit.

(iii) The pH of each medium was measured and corrected if necessary to the accepted physiological level.

(iv) The osmolality of each medium was measured. The normal physiological range for embryo culture is 285-320 mOsmol.

Following the above assessments, glucose (100mg/ml in Hank's BSS) and vitamins (MEMS Essential Vitamins - see Appendix F) were added to the levels normally associated with rat serum.

Having prepared the culture media, 5ml of each was introduced to 60ml culture bottles and the bottles equilibrated at 37^{o}C in a roller incubator.

9.2.4 Explantation of 9.5 day embryos.

9.5 day rat embryos were explanted from 2 pregnant females as outlined in section 2.4, and were carefully checked to ensure none were damaged. They were then randomly allocated to incubation bottles containing the different media using a pasteur pipette (1 embryo/ml medium).

9.2.5 Whole embryo culture.

The embryos were then cultured for 48 hours in a roller incubator following the method of New et al. (1973). The incubator was maintained at 37°C and rotated at 30-40 revolutions/minute. Gas mixtures were introduced to the culture bottles and allowed to equilibrate for 2 minutes:-

(i) At start of culture - $5\%O_2;5\%CO_2;90\%N_2$

(ii) After 24 hours - $20\%O_2;5\%CO_2;75\%N_2$

(iii) After 44 hours - $40\%O_2;5\%CO_2;55\%N_2$

The series of experiments was carried out three times mean values of all the assessed factors calculated +/- SE.

9.2.6 'Harvesting' embryos after 48 hours culture.

At the end of the 48 hour culture period the bottles were removed from the incubator and the 11.5 day conceptuses were transferred to a petri dish containing Hank's BSS and rinsed thoroughly. The embryos were then observed using a dissecting microscope and their development assessed.

9.2.7 Assessment of embryonic development.

Although not used in this experiment,a full morphological scoring system was developed by Brown and Fabro(1981) for routine assessment of embryonic growth at 11.5 days. It involves the observation of the stage of development of 13 features easily observed and rapidly developing at this stage. The technique is suitable for use when assessing large numbers of embryos yet sensitive enough to detect minor abnormalities. The stage of development of each feature is given a score, which when added together give an overall morphological score for that embryo. Plate 9.1 shows a typical 11.5 day embryo within its yolk sac. The yolk sac diameter and crown rump length are measured using an eyepiece graticule in the dissecting microscope. Also the total protein of each embryo is measured using a modification of the technique of Lowry et al. (1951). A full discussion of the development of the embryo between 9.5 and 11.5 days is not given here, however details can be found discussed at length by Brown and Fabro (1981).

For the purposes of this comparative experiment embryonic growth and development was assessed by observing the presence or absence of five features;any specific abnormalities were noted. Also the number of somites, the yolk sac diameter and the embryo protein content were recorded.

The factors observed were as follows:-

(i) The presence of a beating heart,

(ii) The presence of a full plexus of yolk sac vessels,

(iii) A fused allantois,

Plate 9.1 11.5 day rat embryo within a well vascularised yolk sac. Bar = 2 mm

(iv) The neural tube closed along its length,

(v) The presence of forelimb buds,

Details of the development of the five features mentioned above will be outlined:-

(i) The two endocardial heart tubes fuse along the mid-line to form a single tube which begins to beat at 10.5 days. Differential growth of this tube forms an S-shaped configuration which subsequently becomes convoluted. At approximately 11.5 days the three chambers are visible (bulbus cordis, atrium communis and ventriculus communis). This further develops to form a fourth chamber.

(ii) The yolk sac is served by vitelline vessels reaching it via the yolk stalk. Initially blood islands appear in the mesoderm at approximately 10.5 days. These blood islands form in coronae around the ectoplacental cone. Anastomoses are formed between the islands producing the vitelline vessels within the yolk stalk and those of the yolk sac. Eventually a full plexus of vessels is formed. The vitelline artery and vein develop in the yolk stalk and blood flows from the aorta, through the vitelline artery, circulates in the yolk sac plexus and returns to the sinus venosus in the vitelline vein. Initially the artery and the vein travel together but can eventually be seen as two distinct structures.

(iii) On day 9.5 the mesenchymal allantoic bud develops and starts to grow across the exocoelom to fuse with the chorionic ectoderm on day 10. The ectoplacental cavity then collapses and blood islands developing in the allantois, coalesce to form the umbilical

vessels. The cells invade the chorion to form the chorionic placenta. Initially the umbilical and vitelline arteries share a common aortic origin but on day 11 they separate, the umbilical artery originating more caudally.

(iv) Neurulation is the term given to the formation of the neural plate, neural folds, neural groove and ultimately the neural tube which make up the central nervous system. The neural plate develops following induction of the embryonic ectoderm by the developing notochord. Cranially this forms the brain and caudally the spinal cord. As the notochord elongates the plate broadens and folds to give a neural groove. The edges of the neural folds close around the neural groove and meet to form a neural tube which sinks below the surface. Closure begins at the mid-point and proceeds cranially and caudally leaving the tube continuous with the amniotic cavity via anterior and posterior neuropores. At approximately 11.5 days the posterior neuropore closes.

(v) A distinct elevation appears on the mesonephric ridge (Wolffian Crest) at the level of somites 9-13 on approximately day 11. These swellings develop into distinct fore limb buds at approximately day 11.5. Further development results in the buds becoming paddle shaped and an apical ectodermal thickening develops on the apical ridge.

9.3 Results.

The results of this series of experiments using four different culture media are summarised in Table 9.1

Table 9.1.

	Control 50% rat serum 50% Hank's BSS (n = 15)	BSA in BSS (n = 15)	Exo. Fluid (n = 15)	25% BSA in BSS 75% Exo. Fluid (n = 15)
Beating Heart	+	-	-	+
Yolk sac circulation	+	-	-	Rudimentary circulation - blood islands
Fused allantois	+	-	-	+
Closed neural tube	+	-	-	-
Fore limb buds	+	-	-	-
Mean yolk sac diameter (mm)	4.01+/-0.09	-	-	2.20+/-0.12
Mean somite number	24.60+/-0.36	-	-	11.25+/-0.42
Mean protein content (mg)	188.0+/-7.9	-	-	47.5+/-2.3

+ and - show presence or absence of feature.

The embryos cultured in the control media of rat serum diluted 50:50 with Hank's BSS (final protein content - 43.8 mg/ml) grew normally having a mean somite number of 24.6 +/- 0.36. These embryos did not differ significantly from those routinely cultured to 11.5 days in 100% whole rat serum.

The embryos grown for 48 hours in a solution of BSA in Hank's BSS (50mg/ml) showed no significant growth. They appeared just as they had at 9.5 days. A protein estimation was not carried out on the embryonic tissue as levels would have been too low to measure accurately.

The embryos cultured for 48 hours in exocoelomic fluid again showed no real growth. However the egg cylinders did appear to have expanded slightly.

The results of the greatest interest are embryos which were cultured in the medium containing both exocoelomic fluid and a source of bulk protein - significant growth was achieved. The yolk sacs had expanded and become spherical. A rudimentary circulation was developing in the yolk sacs and blood islands had formed. The heart of the embryos was observed to be beating but was still an S-shaped tube, the chambers having not yet differentiated. In all cases the allantois was seen to have fused to the ectoplacental cone. The embryos were mainly ventrally convex and in all cases the neural folds had formed but the neural tube open along its length. The embryos had an average of 11 somites. All these factors along with the protein content of the embryo and its yolk sac diameter, show the developmental stage of the embryos cultured in exocoelomic fluid supplemented with a source of bulk protein, to be that

usually reached after 24 hours in rat serum - ie the 10.5 day stage. No abnormal development was seen, although at this stage such abnormalities would be difficult to identify.

9.4 Discussion.

In this series of experiments it has been shown that normal embryonic growth from 9.5 - 11.5 days to the 25 somite stage, can be achieved in 50% rat serum;50% Hank's BSS with a total protein content of 43.8mg/ml. This correlates well with results previously obtained (Stubbs - personal communication). These embryos serve as controls for those grown in the other culture media.

No significant growth was obtained when embryos were cultured in a simple solution of BSA as a source of bulk protein, with vitamins and glucose supplemented to the levels normally associated with rat serum. However in view of the complexity of early embryonic growth this is not surprising - it would be unlikely to achieve normal embryonic growth on a single protein despite the ability of the conceptus to break down protein to amino acids and reform new proteins (Freeman et al. 1981).

Normal embryonic growth is known to require many factors normally present in serum. These factors fall into classes. Firstly those having a nutritional role such as amino acids and lipids. Others may transport low molecular weight nutrients (eg transferrin carries iron). In addition serum contains the non-nutrient hormones and growth factors, the provision of which is now thought to be very important.

No significant growth was obtained by using 100% exocoelomic fluid as a culture medium. Again this is not surprising because although the exocoelomic fluid is thought to contain some of the non-nutrient growth factors essential for embryonic growth, its total protein content was

found to be only 5.27 mg/ml +/- 0.15. Obviously during embryonic growth the yolk sac system is dynamic and protein and amino acids are continually being supplied to meet the requirements of the developing embryo.

The important results are obtained when both exocoelomic fluid and a source of bulk protein are used in combination as a culture medium. The embryonic growth obtained over a 48 hour period is significant although by no means perfect when compared to the control embryos. However this result does support the view that specific factors essential for embryonic growth are present in exocoelomic fluid.

After this preliminary series of experiments further modifications were carried out in an attempt to achieve a greater level of embryonic growth and development.

I assumed that many of the essential trophic factors were present in the exocolomic fluid of the 'giant' yolk sac, but concluded that perhaps the less than perfect embryonic growth was attributed to the fact that the concentration of these factors was too low. To overcome this two further experiments were carried out.

The concentration of exocoelomic fluid was increased by freeze drying the fluid and then redissolving it in half the original volume of double distilled waster. This was the maximum concentration that could be used without raising the osmolality of the culture medium above the acceptable physiological range (285 - 320mOs.). As a control, rat serum was also freeze dried and reconstituted. This process had no effect on

its ability to support normal embryonic growth from 9.5 - 11.5 days. The concentrated exocoelomic fluid was incorporated into a culture medium again with BSA a source of bulk protein. The experiment was repeated several times and on each occation no improvement of embryonic growth was achieved, despite an obvious increase in the concentration of factors present in the exocoelomic fluid.

The second experiment involved frequent changing of the culture medium as an alternative method of providing more of the factors contained in exocoelomic fluid. 9.5 day rat embryos were cultured in 25% BSA in BSS; 75% exocoelomic fluid as before, but the medium was replaced every 6 hours over the 48 hour period. Control yolk sacs grown in 50% rat serum;50% Hank's BSS were also subjected to this continual changing of medium and were not significantly affected. However there was no significant improvement in the growth of embryos in the experimental medium.

These two additional experiments seemed to indicate that perhaps some additional factors essential for embryonic growth had either already been removed from the exocoelomic fluid in the course of the 'giant' yolk sac culture, or were just not present in the fluid. It is possible that some factors are transported directly to the embryo as required and otherwise either not taken up by the yolk sac or taken up and then stored within the yolk sac.

The culture period was also extended by a further 24 hours , to see if further embryonic growth could be achieved. This was found not to be the case. There was no further growth beyond the 11 somite stage.

The experiments could be continued and the subject further investigated perhaps by using an alternative source of bulk protein such as casein. Preliminary results (Pratten - personal communication) indicate that embryonic growth is greater when casein rather than BSA is used as a source of bulk protein to supplement various serum fractions. Also it is possible that various specific factors known to be essential for embryonic growth could be added to the system either individually or in combination (eg IgG, insulin, EGF, transferrin).

In summary these results show a very significant increase in embryonic growth when exocoelomic fluid is incorporated into a culture medium. As expected the growth was not perfect but did indicate the presence of some essential non-nutrient factors, the concentration of which obviously could not be compared to that supplied to a normal developing embryo within a functioning visceral yolk sac *in vivo*.

It is therefore important to analyse the exocoelomic fluid to identify its components in comparison with the components of rat serum. Hopefully it may eventually be possible to identify the trophic factors essential for embryonic growth which are supplied before the establishment of the chorioallantoic placenta, by histiotrophic nutriton across the visceral yolk sac.

SECTION 10

GENERAL CONCLUSIONS

10.1 General Conclusions.

1. In this thesis I have described the culture of the rat visceral yolk sac over a prolonged period. I have characterised the external morphology and ultra-structure of the cultured yolk sac and assessed its functional activity by studying the uptake, processing and transport of macromolecules, in view of its known association with early embryonic nutrition. Before the establishment of the chorioallantoic placenta in the rat, the visceral yolk sac serves as the sole organ of embryonic nutrition, and continues to function throughout gestation.

2. Rat conceptuses explanted at 9.5 days can be maintained in organ culture over a long period and although the embryo dies at approximately 12 days and is partially autolysed within its amnion, the yolk sac continues to grow and can reach a diameter in excess of 2cm at 17.5 days. The closed yolk sac vesicle formed, which has been called the 'giant' yolk sac, is physiologically intact and maintains the exocoelomic fluid separately from the culture medium.

3. The 'giant' yolk sac system has been modified and improved by the removal of the embryonic tissue from egg cylinders explanted at 9.5 days. The wound made during this procedure quickly heals and a closed yolk sac vesicle develops which is free from embryonic effects. Although these 'giant' yolk sacs are slightly smaller than those grown with the embryo, they are easy to handle, provide adequate yolk sac tissue and exocoelomic fluid for analysis and enable the visceral yolk sac to be studied in isolation.

4. Considering the length of time that the 'giant' yolk sac is maintained in organ culture and the fact that for the majority of this time it does not contain an actively developing embryo, as is the case *in vivo*, the morphological similarities between the two systems are remarkable. It would be naive to expect tissues in such different environments to be identical and the differences noted seem logical. For example in the *in vivo* yolk sac which is supporting the growth and development of the embryo, adequate stores of the carbohydrate glycogen are essential. In the 'giant' yolk sac system there are not the same nutritional demands and hence large deposits of glycogen are unnecessary.

5. The main morphological difference observed between 'giant' yolk sac tissue and *in vivo* yolk sac tissue was an increase in the vacuolar compartment in the 'giant' yolk sac. This could be due again to the lack of a developing embryo. Removal (or death) of the embryo means that the nutritional requirements of the system are significantly lower than those of the conceptus *in vivo*. The processed histiotroph is utilised only by the growth of the yolk sac itself. This could result in an accumulation of the excess internalised macromolecules within the lysosomal system of the endoderm cells. Morphometric analysis of the vacuolar system in endoderm cells from 'giant' yolk sacs and *in vivo* yolk sacs showed the 'giant' yolk sac to have a significantly larger vacuolar volume.

6. ^{125}I-Polyvinylpyrrolidone (PVP) is an inert, non-digestible macromolecule used as a marker for fluid phase pinocytosis. The basal rate of pinocytosis in the 'giant' yolk sac was compared with that of the *in vivo* yolk sac. The technique of Williams et al. (1975a) was used to measure ^{125}I-PVP uptake, the rate of uptake being expressed as an Endocytic Index. The measured rate of uptake of ^{125}I-PVP by the 'giant' yolk sac (basal pinocytic rate) indicated that the system was functionally active. When incubations were carried out in Medium 199 the EI of 'giant' yolk sac tissue was less than that *in vivo*, but in the more physiological whole rat serum, the two systems appeared to be functioning almost identically.

The *in vivo* yolk sacs responded quickly to medium composition, the rate of pinocytosis decreasing with an increased concentration of serum protein. Medium composition had little effect on the rate of uptake of ^{125}I-PVP by 'giant' yolk sac.

The uptake of ^{125}I-PVP by the 'giant' yolk sac was studied throughout the culture period and was shown to decrease with increasing gestational age.

Throughout all the experiments there was no significant leakage of ^{125}I-PVP into the exocoelom, which demonstrates the physiological integrity of the system. Also very little ^{125}I-PVP was released from the yolk sac tissue when pre-loaded tissue was re-incubated in fresh medium.

In all experiments the results obtained using 'giant' yolk sacs

cultured after removal of the embryonic tissue were not significantly different from those cultured with the embryonic tissue.

7. Bovine serum albumin (BSA) is a protein which is taken up by adsorptive pinocytosis and broken down in the lysosomes. As the products of its digestion cannot be utilised by the yolk sac (or embryo) it is an ideal macromolecule for studying uptake, processing and release. The uptake of ^{125}I-BSA was studied in serum-free Medium 199 and whole rat serum. In both incubation conditions the EI of ^{125}I-BSA was found to be higher than that of ^{125}I-PVP. In Medium 199 the uptake of ^{125}I-BSA was much greater with *in vivo* tissue than 'giant' yolk sac tissue, but in the more physiological whole rat serum uptake in both systems was similar.

In the *in vivo* yolk sac incubated in serum, there is competition for binding sites between labelled BSA and unlabelled serum proteins and hence a reduction in the rate of uptake of ^{125}I-BSA. No such competition was observed in the 'giant' yolk sac system. If uptake is occuring by adsorptive pinocytosis this is difficult to explain. We could postulate that the lower nutritional demands of the 'giant' yolk sac has led to an overloading of the lysosomal system and may have resulted in a non-reversible down regulation of the rate of endocytosis.

In the *in vivo* yolk sac the rate of uptake of ^{125}I-BSA and its digestion are in equilibrium. In the 'giant' yolk sac the tissue associated radioactivity increases throughout incubation and is a

greater proportion of the final total, thus indicating that the digestion of the protein is rate limiting and not its uptake.

Throughout incubation the concentration of iodotyrosine (ng/mg tissue protein/ml) in the exocoelom is maintained at a higher level than the concentration in the incubation medium, indicating a directional transfer of iodotyrosine.

The amount of intact ^{125}I-BSA released back into the incubation medium was found to be very small in both tissues.

8. In the rat the visceral yolk sac is an important route for the passage of passive immunity, from the mother to embryo. In both *in vivo* yolk sacs and GYS ^{125}I-IgG is taken up and processed with greater efficiency than either ^{125}I-PVP or ^{125}I-BSA. 'Giant' yolk sacs cultured by both methods function similarly. ^{125}I-IgG differs from the other macromolecules studied in that a large proportion of it is taken up by the tissue and released intact. This means that the EI's calculated for all three tissue types are greatly underestimated as it is impossible to distinguish between ^{125}I-IgG released and that already present in the medium.

In a separate series of experiments yolk sacs pre-loaded with ^{125}I-IgG, over a 3 hour period, were re-incubated in fresh medium and the release of intact ^{125}I-IgG and its digestion products monitored. With 'giant' yolk sacs approximately half of the total released over a 3 hour period was intact ^{125}I-IgG and with *in vivo* yolk sacs approximately 3/4 was intact ^{125}I-IgG. This difference may

be because release into the medium, from *in vivo* yolk sacs can be from both apical and basal surfaces whereas release from the physiologically intact 'giant' yolk sac is from the apical surface only.

By extrapolation of the results of uptake and release experiments an estimate of a closer approximation to the true rate of uptake of ^{125}I-IgG was calculated. Also it was concluded that intact ^{125}I-IgG taken up and then released by the 'giant' yolk sac was maintained at a higher concentration in the exocoelom than in the medium, indicating the preferential transport of IgG into the exocoelom.

An electron microscopic study revealed that both colloidal gold and IgG-colloidal gold were taken up by endocytosis in 'giant' yolk sac and *in vivo* yolk sac tissue. However the uptake of IgG-colloidal gold was seen to be much more efficient - the particles observed with greater frequency and in large clusters. Also IgG-colloidal gold was observed at the basal cell surfaces in both tissue types indicating some IgG transport.

9. The uptake of two ^{14}C-labelled amino acids was studied using 'giant' yolk sacs. ^{14}C-glutamic acid and ^{14}C-proline were chosen as they are thought to be transported by different mechanisms. They were each shown to be taken up and transported into the exocoelom and also incorporated into new protein (found both in yolk sac tissue and exocoelomic fluid). From this preliminary study it appeared that ^{14}C-proline was taken up and processed at a greater rate than ^{14}C-glutamic acid. However it is important to note that other

non-labelled amino acids, present in varying concentrations, may enter the tissue via the same route as ^{14}C-proline or ^{14}C-glutamic acid; such competition may have biased the results. Future experiments on the uptake of individual ^{14}C-amino acids should be carried out in an environment free from competing unlabelled amino acids.

10. In the rat the visceral yolk sac is the sole organ responsible for histiotrophic nutrition before the establishment of the chorioallantoic placenta. Therefore exocoelomic fluid collected from 'giant' yolk sacs should contain many of the factors essential for normal embryonic development. The growth supporting properties of this fluid were studied by assessing the growth and development of 9.5 day embryos over a 48 hour period. Normal development was achieved in 50% rat serum;50% Hank's BSS (50mg serum protein/ml). There was no growth in a simple solution of BSA (50mg/ml) as a source of bulk protein and no significant growth in a solution of 100% exocoelomic fluid. However in a solution containing 75% exocoelomic fluid;25% BSA solution (50mg protein/ml) embryonic growth was significant although not perfect. This indicates that some of the factors essential for normal embryonic growth are present in exocoelomic fluid and analysis of this fluid, perhaps by electrophoresis may lead to their identification.

11. In summary the 'giant' yolk sac system has proved a very useful and viable model for studying the ultrastructure and functional activity of the rat visceral yolk sac, cultured in isolation over a prolonged period of time. The system is free from the complexities of the maternal system, the chorioallantoic placenta and an actively developing embryo and yet maintains a physiological integrity, previously only found *in vivo*.

SECTION 11

APPENDIX

Appendix A.

Hank's Balanced Salt Solution (BSS).

Hank's BSS cannot be used as a culture medium but has been used successfully to dilute whole rat serum up to 50% and does not affect the growth supporting properties of the serum (Huxham and Beck 1985).

Salt	Concentration (g/l)
$CaCl_2.2H_2O$	0.1855
KCl	0.40
KH_2PO_4	0.60
$MgSO_4.7H_2O$	0.20
NaCl	8.00
$NaHCO_3$	0.70
Na_2HPO_4	0.04575
D-glucose	1.00
Sodium phenol red	0.017

$CaCl_2.2H_2O$ was dissolved in 50 ml of distilled water and all the other reagents dissolved in 950 ml of distilled water. The two solutions were sterilised by auto clave separately to avoid precipitation of the calcium salts. After cooling the two solutions were combined and stored at -4^oC ready for use.

Appendix B.

Modifications of the Lowry technique for protein assessment.

The total protein content of yolk sacs / embryos was assessed using various modifications of the technique developed by Lowry et al. (1951). In all methods the stock solutions and reagents used were identical.

Stock Solutions.

(a)	1M NaOH	40g/l distilled water
(b)	3M HCl	3.67ml conc. HCl made up to 11ml with distilled water.
(c)	Sodium carbonate	2g anhydrous Na_2CO_3 per 100ml distilled water.
(d)	Copper sulphate	1g $CuSO_4.5H_2O$ per 100ml distilled water.
(e)	Sodium tartrate	2g $(CH(OH).COONa)_2.2H_2O$ per 100ml distilled water.
(f)	Bovine serum albumin	(i) 5mg BSA /ml 1M NaOH (ii) 1mg BSA /ml distilled water
(g)	Folin Ciocalteu's reagent	Stock solution (store at $-4^{\circ}C$)

Reagents.

(i)	Folin A (prepare daily)	100ml (c) 1ml (d) 1ml (e)
(ii)	Folin B (use immediately)	Dilute (g) 50:50 with water.

Method for assessing the protein content of giant yolk sacs.

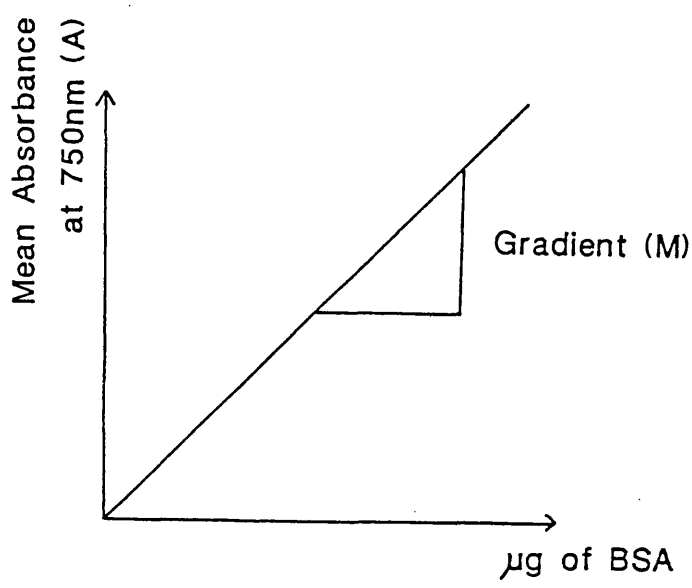
1. Each yolk sac was placed in 2.5ml of 1M NaOH in a plastic tube, which was sealed, whirlimixed and incubated at 37°C until the tissue dissolved (approx. 2 hours). The tube was whirlimixed frequently throughout incubation.
2. 0.2ml 1M NaOH and 0.25ml distilled water was pipetted in duplicate into plastic tubes. 0.05ml of sample was also added to each tube.
3. Standards were prepared as below in duplicate.

Tube number	1mg/ml BSA (ml)	Distilled water (ml)	1M NaOH (ml)
1	0.00	0.25	0.25
2	0.05	0.20	0.25
3	0.10	0.15	0.25
4	0.15	0.10	0.25
5	0.20	0.05	0.25
6	0.25	0.00	0.25

4. 2.5ml of Folin A was added to each tube and left at room temperature for 20 minutes.
5. 0.25ml of Folin B was added to each tube whilst whirlimixing. The tubes were left at room temperature for 45 minutes.
6. The absorbance at 750nm was read on a spectrophotometer (Cecil CE292 digital ultraviolet spectrophotometer) using 0.00ug/ml BSA standard as the zero.
7. This is a colorimetric analysis of the protein content and a graph plotted using the standards of mean absorbance at 750nm / protein

content (ug) is linear. The gradient of this graph can be used to calculate the protein content of each yolk sac (See figure 11.1 below)

Figure 11.1



Micrograms of protein per sample $= \frac{A}{M}$

Micrograms of protein per yolk sac $= \frac{A}{M} \times 50$ (As yolk sac dissolved in 2.5 ml H_2O.)

Method for assessing the protein content of in vivo yolk sacs at 17.5 days gestation.

The method used to assess the protein content of in vivo yolk sacs at 17.5 days gestation was identical to that described above except that in step 1. each yolk sac was dissolved in 5ml 1M NaOH to compensate for the greater amount of tissue in these yolk sacs. Therefore to calculate the actual amount of protein in each yolk sac the amount per sample has to be multiplied by 100.

Method for assessing the protein content of embryos/yolk sacs at 11.5 days gestation.

1. The embryo or yolk sac was dissolved in 1ml 1M NaOH by incubating at 37°C for approximately 2 hours.
2. Standards were prepared. 0, 10, 20, 30, 40, 50ul of the stock BSA soluton (5mg/ml 1M NaOH) were pippetted in duplicate and made up to 1ml with 1M NaOH.

Standards and samples were treated identically from this stage.

3. 0.145ml 3M HCl was added and the contents whirlimixed.
4. Two 0.5ml alliquots were pipetted into plastic tubes.
5. 2.5ml of Folin A was added to each tube and left at room temperature for 20 minutes.
6. 0.25ml of Folin B was added to each tube whilst whirlimixing. The tubes were left at room temperature for 45 minutes.
7. The absorbance at 750nm was read on a spectrophotometer using 0ug/ml BSA standard as the zero.
8. A graph was plotted of the mean absorbance of the standards / protein content (ug) which was linear. The gradient of the graph was used to calculate the protein content of each sample as described above.

Appendix C.

Medium 199

Medium 199 (Gibco, U.K.), contains amino acids, vitamins, inorganic salts and other compounds. Like Hank's BSS it can be used to dilute whole rat serum to produce a medium which supports normal embryonic growth and development. Brooke (1985) showed that 20% whole rat serum supplement was the minimum essential for normal embryonic growth.

Amino Acid	Concentration (mg/L)
L-alanine	25
L-arginine HCl	70
L-aspartic acid	30
L-cysteine HCl	0.1
L-cystine	20
L-glutamic acid	75
L-glutamine	100
L-glycine	50
L-histidine HCl.H_2O	22
L-hydroxyproline	10
L-isoleucine	20
L-leucine	60
L-lysine HCl	70
L-methionine	15
L-phenylalanine	25
L-proline	40
L-serine	25
L-threonine	30
L-tryptophan	10
L-tyrosine	40
L-valine	25

Vitamins	Concentration (mg/L)
p-aminobenzoic acid	0.05
ascorbic acid	0.05
D-biotin	0.01
calciferol	0.1
D-Ca-panthothenate	0.01
cholesterol	0.1
choline chloride	0.5
folic acid	0.01
i-inositol	0.05
menadione	0.01
nicotinamide	0.025

Vitamins	Concentration (mg/L)
nicotinic acid	0.025
pyridoxal HCl	0.025
pyridoxine HCl	0.025
riboflavin	0.01
thiamine HCl	0.01
DL-Tocopherol phosphate	0.01
Tween 80	5.0
Vitamin A	0.1

Inorganic salts and other compounds	Concentration (mg/L)
As for Hank's BSS (see Appendix A) plus:	
Adenine HCl.$2H_2O$	12.1
AMP	0.2
ATP	10.0
Deoxyribose	0.5
Dextrose	1000.0
L-glutathione	0.05
Guanine HCl.H_2O	0.33
Hypoxanthine	0.30
Ribose	0.5
Sodium acetate	83.0
Sodium phenol red	17.0
Thymine	0.30
Uracil	0.30
Xanthine	0.30

Appendix D

^{125}I labelling of tracer proteins

A modification of the Chloramine-T method (Greenwood and Hunter 1963).

Stock solutions.

A. Phosphate buffer (pH 8.0)

0.05M Na_2HPO_4 - 7.098 g/L

0.05M KH_2PO_4 - 6.804 g/L

B. Chloramine-T

1 mg/ml solution in phosphate buffer (A).

C. Sodium metabisulphate

2 mg/ml solution in phosphate buffer (A).

D. Formaldehyde

10% solution in phosphate buffer (A).

E. Sodium chloride (for dialysis).

1% solution of NaCl in distilled water.

Method

The detailed method outlined here is that used for the labelling of BSA. In the case of IgG labelling the quantities used were scaled down as a smaller amount of protein substrate was available.

The whole labelling process was carried out in a fume cupboard behind lead shielding.

1. Dissolve 20mg BSA in 9ml of 0.05M phosphate buffer (A), in a glass bijou bottle.

2. Place bijou bottle in ice bath on magnetic stirrer and stir gently throughout.
3. Add 1mCi ^{125}I and stir for 2 minutes.
4. Add 4ml Chloramine-T (B) and stir for 8 minutes.
5. Add 3ml of sodium metabisulphate (C) and stir for 2 minutes.
6. Add approximately 100mg of solid KI
7. Add an equal volume of 10% formaldehyde (D) and leave for 72 hours at 4^{o}C to denature the protein.
8. Soak dialysis tubing (Visking size 2 - 18/32") for 2-3 hours in distilled water. Place solution into dialysis tubing.
9. Dialyse against 1 % NaCl (E), changing the NaCl twice each day until the dialysate contains negligable amounts of radiation above background. Alliquot ^{125}I-labelled BSA solution into bijou bottles and store at -20^{o}C ready for use.

Efficiency of labelling.

A sample of labelled tracer solution before and after dialysis was analysed to assess the efficiency of the labelling process.

2x 1ml samples of a 1/20 dilution of each were placed in 3.5 ml plastic tubes and the amount of radioactivity in each measured using an LKB Ria Automatic gamma counter.

Table 11.1. Efficiency of labelling of BSA solution.

	Mean cpm/ml stock sol'n	% efficiency
Before dialysis	9.60×10^8	71.4%
After dialysis	6.86×10^8	

Table 11.1 shows the efficiency of labelling of ^{125}I-BSA to be approximately 70% using this method.

Table 11.2. Efficiency of labelling of IgG solution.

	Mean cpm/50ul stock sol'n	% efficiency
Before dialysis	4.3×10^9	67.4%
After dialysis	2.9×10^9	

Table 11.2 shows the efficiency of labelling of ^{125}I-IgG to be 67% using this method.

Assessment of % Trichloroacetic Acid soluble radioactivity present in solution after extensive dialysis.

A sample of the tracer solution was assessed immediately after dialysis and after each experiment to ensure that the amount of TCA soluble radioactivity was not above the acceptable limit before the start of the experiment. It is known that the level of TCA soluble radioactivity associated with a sample of ^{125}I-labelled protein can increase over a period of time, especially if subjected to repeated freezing and thawing.

4 x 1ml samples of a 1/20 dilution of the labelled BSA stock solution were placed in 3.5 ml plastic tubes and the amount of radioactivity in each measured on an LKB Ria Automatic gamma counter. The value obtained gave a measure of the total radioactivity (both TCA soluble and insoluble) in each sample.

To each sample 0.5 ml rat serum was added (to increase the protein content and hence aid precipitation) and 0.5 ml of 10% TCA. This caused a white precipitate of TCA insoluble whole protein.The tubes were well mixed and spun at 3000rpm for 30 min. in an MSE Centaur II centrifuge. The clear supernatent (approx. 1.2 ml) was decanted off into a clean 3.5ml plastic tube. The samples of supernatent containing any TCA soluble radioactivity (mainly in the form of free ^{125}I) were then re-measured on the gamma counter.

Table 11.3 Assessment of TCA soluble radioactivity in ^{125}I-BSA.

	Mean cpm/50ul stock sol'n	% TCA Solubles
Total radioactivity	32939600	
		0.52%
TCA Solubles	172800	

Initially therefore the % of TCA soluble radioactivity is approximately 0.5% (Table 11.3). This test was repeated with samples taken after each experiment from culture bottles incubated without any yolk sacs. The % of TCA soluble radioactivity was corrected for in each experiment.

The % TCA soluble radioactivity present in the ^{125}I-IgG solution was assessed using an identical method, but used a more dilute sample of stock solution.

Table 11.4 Assessment of TCA soluble radioactivity in ^{125}-IgG.

	Mean cpm/2.5ul stock sol'n	% TCA solubles
Total radioactivity	12004260	
		0.63%
TCA Solubles	75620	

Initially the % TCA solubles in the stock solution is 0.63%.

Appendix E

Microcomputer programme.

To aid the process of calculating the uptake of ^{125}I-BSA in 17.5 day *in vivo* yolk sac tissue, it was possible to use a microcomputer programme. A programme kindly donated by the Biochemical Research Unit, University of Keele, was adapted for use on an 'apple' microcomputer. A copy of the programme follows:

```
LIST 1,500
  5 *FX6,0
  7 MODE 3
  8 VDU 19,0,4,0,0,0
 10 CLS
 20 DIM E(30),H(30),I(30),J(30),K(30),L(30),S(30),T(30),P(30)
 50 PRINT TAB(8);"Margy's Protein Program":PRINT
 60 INPUT "Expt.number = ";V$:PRINT
 70 INPUT "Background in CPM = ";A:PRINT
 80 INPUT "Percent solubles in prep = ";B:PRINT
 90 INPUT "Counting time medium totals, secs = ";C:PRINT
100 PRINT "Counting time medium solubles, ";:INPUT TAB(29)"secs = ";Z:PRINT
110 INPUT "YS Counting time, secs = ";D:PRINT
120 INPUT TAB(0,16);"No. of points in plot = ";G
122 PRINT:IF G>30 THEN PRINT TAB(0,18);G; " is too many points - maximum = 30":GOTO 120
130 REM ABOUT TO ENTER A SERIES OF VALUES ACCORING TO SIZE OF G
140 CLS:PRINT "Please enter the following:-":PRINT
142 PRINT " INC.   MEDIUM   ACID    COUNTS  PROTEIN"
144 PRINT " TIME   COUNTS   SOL.    IN YS   ":PRINT
149 C1=16:T1=6
150 FOR I=1 TO G
151 IF I<C1 THEN 155
152 FOR J=1 TO 15:PRINT TAB(0,J+5);"                                                ":NEXT J
153 C1=G+1:T1=6
155 PRINT TAB(0,T1);I
160 INPUT TAB(2,T1);Z$:H(I)=VAL(Z$):IF H(I)=0 THEN PRINT CHR$(7):GOTO 160
165 INPUT TAB(7,T1);Z$:I(I)=VAL(Z$):IF I(I)=0 THEN PRINT CHR$(7):GOTO 165
170 INPUT TAB(16,T1);Z$:J(I)=VAL(Z$):IF J(I)=0 THEN PRINT CHR$(7):GOTO 170
175 INPUT TAB(24,T1);Z$:K(I)=VAL(Z$):IF K(I)=0 THEN PRINT CHR$(7):GOTO 175
180 INPUT TAB(34,T1);Z$:L(I)=VAL(Z$):IF L(I)=0 THEN PRINT CHR$(7):GOTO 180
185 T1=T1+1
190 NEXT I
200 REM CALCULATION AND PRINT-OUT BEGIN HERE
210 FOR X=1 TO G
220 M=((I(X)*60)/C)-A
230 N=((J(X)*60)/Z)-A
240 O=N-(M*B/100)
250 Q=((K(X)*60/D)-A)*5
260 P=(M-N)+O/2
270 F=((10*O)+Q)*1000
280 E(X)=(Q*1000)/(L(X)*P)
290 S(X)=F/(L(X)*P)
300 T(X)=S(X)-E(X)
310 NEXT X
390 CLS:VDU2
400 PRINT TAB(0,0);"EXPERIMENT NUMBER ";V$:PRINT
410 PRINT TAB(0,3);"INC.    PROT.   MLITRES/  SOLUB.  TOTAL":PRINT "(HRS.)  IN YS.   MG.YS
    UPTAKE":PRINT
420 FOR I=1 TO G
422 PRINT TAB(1);H(I);TAB(8);L(I);
423 @%=&20209:PRINT TAB(17);E(I);TAB(26);T(I);TAB(34);S(I)
424 @%=&10
425 NEXT I
430 FOR I=1 TO 5:VDU(10):NEXT I
450 PROCVARIABLES
455 PROCCORRELATION
460 PROCREGRESSION
465 @%=&20309
470 PRINT "CORRELATION COEFFICIENT, R = ";R:PRINT
475 PRINT "SLOPE OF GRAPH (Y ON X)    = ";SLOPE1:PRINT
480 PRINT "INTERCEPT OF X ON Y AXIS   = ";I1:PRINT
497 FOR I=1 TO 10:VDU(10):NEXT I
499 @%=10:VDU3
500 PRINT:INPUT "ANOTHER RUN Y/N":Q$
```

```
LIST 501,1000
510 IF Q$="Y" THEN CLEAR:RUN
520 MODE 7
550 CLEAR:END
795 REM STATISTICAL CALCULATIONS BEGIN HERE
800 DEFPROCVARIABLES
810 X1=0:X2=0:Y1=0:Y2=0:S1=0:MX=0:MY=0
820 FOR I=1 TO G
825 REM SUM X, SUM Y
830 X1=X1+H(I):Y1=Y1+S(I)
835 REM SUM X SQUARED, SUM Y SQUARED
840 X2=X2+(H(I)*H(I)):Y2=Y2+(S(I)*S(I))
845 REM SUM X TIMES Y
850 S1=S1+(H(I)*S(I))
855 NEXT I
860 MX=X1/G:MY=Y1/G
870 ENDPROC
895 REM NEXT PROCEDURE CALCULATES R - THE COEFFICIENT OF CORRELATION
900 DEF PROCCORRELATION
910 R1=X2-((X1^2)/G):R2=Y2-((Y1^2)/G)
920 R3=S1-((X1*Y1)/G)
930 R=R3/SQR(R1*R2)
940 ENDPROC
945 REM PROCEDURE FOR REGRESSION
950 DEF PROCREGRESSION
955 REM SLOPE1 IS REGRESSION OF Y ON X
960 SLOPE1=R3/R1
962 I1=MY+SLOPE1*0-SLOPE1*MX
969 REM SLOPE2 IS REGRESSION OF X ON Y
970 SLOPE2=R3/R2
000 ENDPROC
```

Appendix F

Eagle's Minimum Essential Medium - Vitamins.

Originally 8 vitamins were found essential for growth and survival of mammalian cells in culture. Cockroft (1979) has since found only 4 of these to be necessary for the normal growth and development of rat embryos between 9.5 and 11.5 days gestation.

This vitamin supplement was obtained as a liquid concentrate from Flow Laboratories (Irvine, U.K.) and was stored at -20^{o}C until required. In section 9 the culture media used for the assessment of the growth supporting properties of giant yolk sac exocoelomic fluid, were supplemented with 10ul of MEM vitamins per ml of culture medium.

Vitamin	Concentration (mg/ml)
* D-Ca Pantothenate	0.1
Choline Chloride	0.1
Folic Acid	0.1
* I-inositol	0.2
* Nicotinamide	0.1
Pyridoxal HCl	0.1
* Riboflavin	0.01
Thiamin HCl	0.1

* Vitamins found essential for embryonic growth by Cockroft (1979).

SECTION 12

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The giant yolk sac: a model for studying early placental transport

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INTRODUCTION

During the early stages of development, from implantation to the onset of chorioallantoic placental function, the nutritional requirements of the mammalian embryo are met by histiotrophic nutrition. In the rat, the visceral layer of the yolk sac endoderm is largely responsible for this function. It has the capacity to pinocytose macromolecules from its surrounding environment, which in this species is a transudate of maternal serum (Merker & Villegas, 1970). Within the endodermal cells, macromolecules are transferred to lysosomes, digested, and the products passed to the developing embryo (Beck, Lloyd & Griffiths, 1967; Freeman, Beck & Lloyd, 1981).

Strips of rat visceral yolk sac have in the past been grown on solid media for up to fourteen days. Under the culture conditions used organotypic differentiation and pinocytic activity were maintained (Sorokin & Padykula, 1960). This technique, although useful for investigating *in vitro* differentiation of the visceral yolk sac, has limited applications in a study of its functional activity as there is no separation between the naturally occurring fluid compartments on the inside and outside of this membrane. In order to investigate this, the method first developed by Al-Alousi (1983) in which the whole yolk sac can be cultured intact over a long period is required.

To achieve this objective, the well known technique of rat whole embryo culture developed by New (New, Coppola & Terry, 1973) has been utilised in the present study. Usually, this method is employed to grow rat embryos within their surrounding membrane of visceral yolk sac over a 48 hours period between the head process and the 25 somite stages of development. By slightly modifying the procedure, the culture period can be extended by up to 14 days. With the modified technique used here, the embryo grows normally for 48 hours but soon after dies and is partially autolysed. Its surrounding visceral yolk sac continues to grow intact and reaches a diameter in excess of 2 cm.

We have called the yolk sac vesicle obtained after the extended culture, a 'giant yolk sac'. Its development is shown in Figure 1. The ectoplacental cone is surrounded by a smooth area of yolk sac, around which a wide region is thrown into complex folds (Fig. 2). The folding gradually decreases towards the pole opposite to the ectoplacental cone. The giant yolk sac vesicle is distended by the fluid contained in the extraembryonic coelom. Within it lies the amnion which is of variable size and contains an autolysed embryo.

The giant yolk sac vesicle appears to be a useful model for studying the function

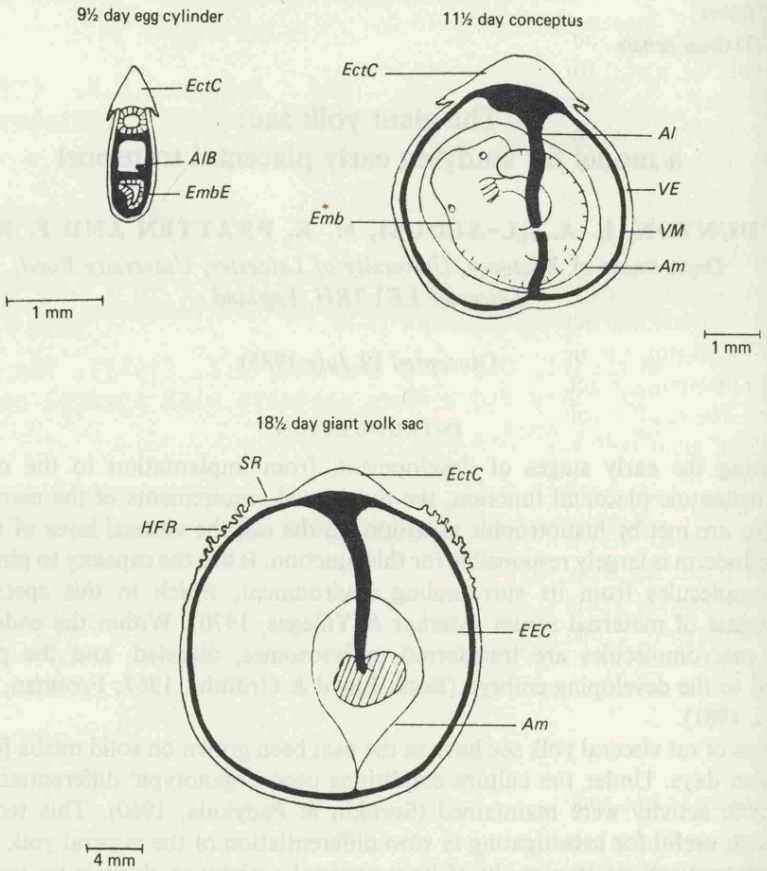
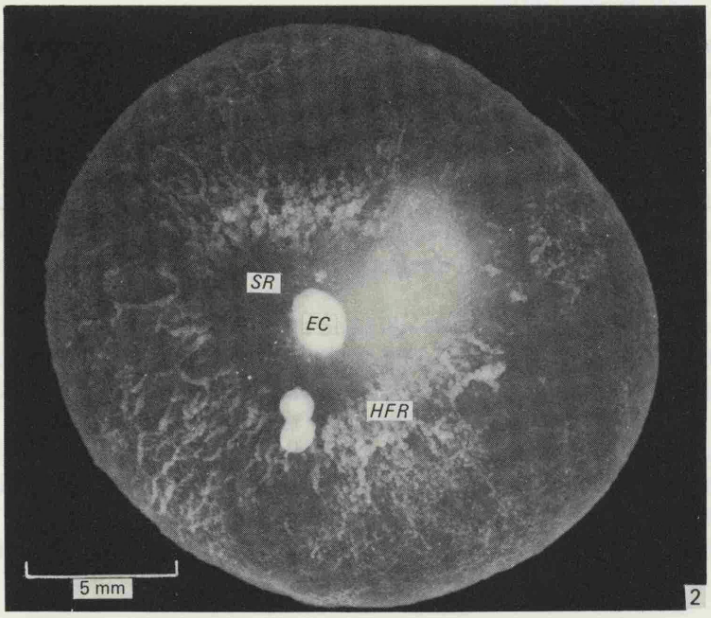


Fig. 1. Development of the giant yolk sac from the 9½ day egg cylinder stage showing the allantois (*Al*), allantoic bud (*AlB*), amnion (*Am*), ectoplacental cone (*EctC*), embryo (*Emb*), embryonic endoderm (*EmbE*), extra-embryonic coelom (*EEC*), highly folded region (*HFR*), smooth region (*SR*), visceral yolk sac endoderm (*VE*) and visceral yolk sac mesoderm (*VM*).



of visceral yolk sac epithelium over a protracted period of time. In particular it should be useful for studying the passage of materials from the maternal to the embryonic compartments in early development.

In this publication the giant yolk sac culture technique is outlined first and then the fine structure of the giant yolk sac is described in comparison with that of *in vivo* yolk sacs at the same age. Also, it is demonstrated that the giant yolk sac forms a physiologically intact cell membrane during the culture period. Using lanthanum nitrate it is shown that tight junctions form a barrier and prevent transepithelial movement by passive diffusion between the cells.

The functional activity of giant yolk sac endodermal cells has been investigated by measuring the uptake of radiolabelled polyvinylpyrrolidone and, additionally, acid phosphatase activity in the giant yolk sac has been compared with that found *in vivo*. The relative volumes of the vacuolar system in the visceral yolk sac endodermal cells of giant yolk sacs have been compared morphometrically with the vacuolar volume of the endodermal cells in 18½ day *in vivo* yolk sacs.

Finally, it is shown that the fluid from within the extraembryonic coelom of the giant yolk sac has the ability to support the growth of 9½ day rat embryos. The experiment has been carried out using the giant yolk sac fluid alone as well as with the addition of a source of bulk protein. It is shown that some of the factors essential for embryonic growth are present in the fluid contained within the giant yolk sac vesicle.

MATERIALS AND METHODS

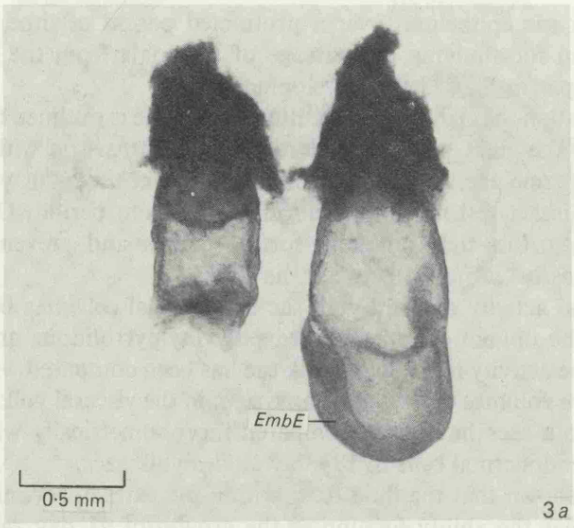
Culture of giant yolk sacs

Female Wistar rats were killed when 9½ days pregnant. The embryos were quickly explanted and cultured, following the technique developed by New *et al.* (1973), in immediately centrifuged heat-inactivated rat serum, in sealed culture bottles. A maximum of three embryos were placed in each bottle, in 3 ml of culture serum, and these were rotated in a roller incubator at 37 °C. The bottles were gassed with a mixture of 5% O_2; 5% CO_2; 90% N_2 at the start of culture and, every 24 hours thereafter, with a mixture of 20% O_2; 5% CO_2; 75% N_2. The serum was changed at intervals of 48 hours, the first and second changes at 1·5 ml/conceptus and the third at 2 ml/conceptus. The culture period was usually nine days and after this time the giant yolk sacs had a diameter of approximately 2 cm.

At the end of the culture period, the giant yolk sacs were washed three times in Hanks balanced salt solution and transferred to a small petri dish for examination under a dissecting microscope.

Giant yolk sacs were also cultured after prior removal of embryonic tissues. Conceptuses were explanted at 9½ days and Reichert's membrane removed as usual. The conceptuses were then steadied with a pair of fine forceps and the lower pole of the egg cylinder which contains all the embryonic tissue was gently cut away with a scalpel, removing as little as possible of the visceral yolk sac (Fig. 3*a*). The remainder of the conceptus (made up predominantly of the visceral yolk sac) was

Fig. 2. An 18½ day giant yolk sac showing the ectoplacental cone (*EC*), the smooth region (*SR*) and the highly folded region (*HRF*) which was used in morphological studies. Note that the rugose nature of the giant yolk sac decreases towards the pole opposite the ectoplacental cone.



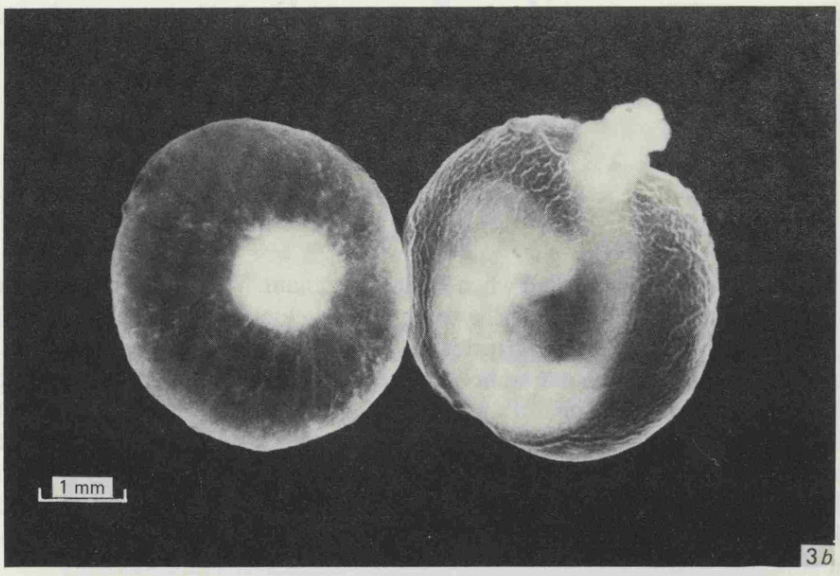


Fig. 3(*a–b*). (*a*) A 9½ day embryo before and after the removal of the embryonic tissues (*EmbE*). (*b*) A normal 11½ day conceptus after 48 hours culture in whole rat serum (right) and a 13½ day yolk sac from which the embryonic endoderm was removed at 9½ days (left).

then placed in Hanks balanced salt solution at 37 °C in the roller incubator for three hours, to allow healing to progress before being placed in serum and cultured as described above (Fig. 3*b*).

Yolk sacs were removed from rats at 17½ or 18½ days of gestation as controls for each experiment.

Collection of extraembryonic coelomic fluid and preparation of tissue for electron microscopy

The fluid from within the extraembryonic coelom (giant yolk sac fluid) of each giant yolk sac was removed using a 1 ml syringe with a fine needle and was pooled and centrifuged to remove cell debris. Antibiotics (penicillin 5000 I.U./ml, streptomycin 5000 μg/ml) were added to the fluid and it was stored at −20 °C, ready for use. Measurements were carried out to determine the protein content (Lowry, Rosebrough, Farr & Randall, 1951), glucose levels and osmolality of the fluid.

After the removal of the fluid, the whole yolk sac was fixed immediately in 3 % glutaraldehyde in 0·1 M phosphate buffer for two hours at 4 °C. Secondary fixation, of the rugose area of the yolk sac surrounding the ectoplacental cone (Fig. 1), was carried out with 2 % osmium tetroxide for one hour. The pieces of tissue were then dehydrated and embedded in Araldite. Pale gold/silver sections were stained with 10 % uranyl acetate and lead citrate (Reynolds, 1963). Sections were then examined using a JEOL 100S electron microscope.

Control yolk sacs were removed from 18½ day pregnant females, immediately fixed in 3 % glutaraldehyde and processed as outlined above.

Permeability of the giant yolk sac

In order to investigate the permeability of the preparations, Day 18 giant yolk sacs were incubated for 5 hours in medium containing 2 μg/ml ^{125}I-labelled polyvinylpyrrolidone and subsequently washed in 1 % sodium chloride. A sample of the giant yolk sac fluid was taken as previously described and the amount of radioactive label in the fluid and in a sample of the culture medium was measured on an LKB 1271 RIAGAMMA Automatic gamma counter.

Electron microscopy of the giant yolk sac endoderm cells showed the presence of tight junctions between the cells. Lanthanum nitrate was employed to test the integrity of this functional barrier. The method used was that of Lesseps (1967) which is a modification of the method developed by Doggenweiler & Frank (1965). Yolk sacs were fixed in a solution made up of 1 gm lanthanum nitrate, 1 gm potassium permanganate, 20 ml veronal acetate buffer and 6 ml Zetterquist salt solution. The pH was corrected to 7·8 using 0·1 M HCl and the solution made up to 100 ml. After fixation for one hour, the giant yolk sacs were washed in Tyrode's solution, dehydrated and embedded in Araldite; thin sections were cut and examined on the JEOL 100 S electron microscope.

Uptake of colloidal gold

The endocytic activity of the giant yolk sac was studied by incubation in a medium containing gold particles. The preparation of the colloidal gold was carried out according to the technique used by Horrisberger & Rosset (1977) as modified by Huxham & Beck (1981).

Intact giant yolk sacs were washed in warm Hanks balanced salt solution at 37 °C and incubated in 4 ml gold solution in Hanks balanced salt solution made up so that 10 ml solution contained approximately 20×10^{12} colloidal gold particles. The incubations were carried out for between 5 and 60 minutes and the bottles gassed with 20 % O_2; 5 % CO_2; 75 % N_2. At the end of the incubations, the giant yolk sacs were washed in Hanks balanced salt solution and prepared for electron microscopy as previously described.

The accumulation of gold particles was observed using a JEOL 100S electron microscope.

Morphometric analysis

In this study, sections from the highly folded part of the giant yolk sac were compared with those from an identical area of control yolk sacs at the same age (Fig. 1). A comparison between the volume of the vacuolar compartment in the giant yolk sac endoderm and that in control yolk sacs was made.

The Delesse principle states that the volume fraction of a component in a tissue is equal to the measured area fraction occupied by that component (Weibel, 1969). Thus,

$$\frac{V}{V_1} = \frac{A}{A_1},$$

where V and V_1 are the component volume and the tissue volume, A and A_1 are the component area and the tissue area.

Using a simple point counting method with the aid of a M.O.P. AMO2 quantitative analyser and Apple Computer, the volume of the vacuolar compartment, as a percentage of the total cytoplasmic volume was compared between the giant yolk sac and control 18½ day yolk sacs. In this experiment, three pregnant rats were used in each experimental group, two yolk sacs being used for each animal and two ribbons of sections cut from each tissue block.

Electron micrographs were taken using a JEOL 100 S electron microscope at 80 kV and a magnification of ×7000. The micrographs were a random sample of whole endodermal cells and a cumulative mean plot was constructed to ensure a large enough sample of cells had been used from each yolk sac. The minimum sample size was taken to be the value at which the plot reached and remained within 10% of the final cumulative mean (Williams, 1977).

Endocytic Index

A further method of investigating pinocytosis was used which involved the direct measurement of radiolabelled substrate captured by the endodermal cells (Williams, Kidston, Beck & Lloyd, 1975); ^{125}I-labelled polyvinylpyrrolidone (PVP), a non-degradable macromolecule, was used.

Whole 17½ and 18½ day giant yolk sacs were incubated at 37 °C in bottles containing fresh heat-inactivated whole rat serum. A mixture of 95% O_2; 5% CO_2 was found to induce an optimum rate of pinocytosis. At zero time, 2 μg/ml PVP was added to each culture bottle; the cultures were regassed and sealed. Over a period of five hours, samples of the culture medium and yolk sacs were taken and the amount of radioactive label in each measured on an LKB 1271 RIAGAMMA automatic gamma counter. Protein estimations were carried out on the yolk sacs and the rate of uptake of PVP (Endocytic Index) was calculated.

Control yolk sacs were also incubated under the same conditions and a comparison between the Endocytic Index for PVP in the giant yolk sac and in the control sac was made.

Histochemical and biochemical methods for demonstrating acid phosphatase activity

Giant yolk sacs and 18½ day control yolk sacs were obtained as previously described. Tissue pieces were fixed at 4 °C for 4 hours in 10% formaldehyde in sucrose (Straus, 1964) and stored in 30% sucrose. The technique used to demonstrate acid phosphatase was that of Barka & Anderson (1962). This is a simultaneous coupling technique using sodium α-naphthyl phosphate as the substrate and diazonium pararosanilin as a coupling agent. The tissue was stained in bulk for acid phosphatase, embedded in paraffin wax, sectioned and then counterstained with 1% methyl green.

Control incubations using giant yolk sacs and 18½ day *in vivo* yolk sacs were performed in a working solution without sodium α-naphthyl phosphate.

Biochemical demonstration of total acid phosphatase was carried out according to the method of Barrett (1972) – a modification of that of Fiske & Subbarow (1925). In this method, the amount of inorganic phosphate released from the substrate (glycerol-2-phosphate) was measured and used to indicate acid phosphatase activity.

The ability of giant yolk sac fluid to support the growth of 9½ day rat embryos

A series of experiments was carried out to assess the ability of giant yolk sac fluid to support embryonic growth and development both alone and in the presence of a bulk protein source. Previous work (N.H.C. Stubbs, personal communication), had shown that 9½ day rat embryos could be grown successfully for 48 hours in 50% rat serum in Hanks balanced salt solution. The protein content of whole rat serum is approximately 85 mg/ml and, therefore, a solution containing 50 mg/ml protein should have more than satisfied the bulk protein requirements of a developing embryo over the 48 hours period.

In these experiments, the source of bulk protein used was bovine serum albumin. Approximately 200 mg/ml bovine serum albumin was dissolved in Hanks balanced salt solution and the protein containing solution then dialysed against a balanced salt solution for two days to correct the pH. This solution was stored at −20 °C.

Nine and a half day egg cylinders were cultured for 48 hours in a control medium comprising 50% heat-activated rat serum and 50% Hanks balanced salt solution (final protein level, 42 mg/ml).

Nine and a half day egg cylinders were also cultured in giant yolk sac fluid (5 mg protein/ml), bovine serum albumin in Hanks balanced salt solution (50 mg protein/ml) and in a medium containing 75% giant yolk sac fluid and 25% bovine serum albumin in Hanks balanced salt solution (50 mg protein/ml).

Glucose and essential vitamins were supplemented in each experiment to the levels normally found in rat serum.

RESULTS

Structure of the giant yolk sac

After 9 days of culture the giant yolk sac had a mean diameter of 19·0 mm and a characteristic appearance (Fig. 2). The ectoplacental cone was surrounded by an area of yolk sac which appeared smooth when viewed under a dissecting microscope. Around this was a wide band of tissue which was thrown into complex folds. Its corrugated nature decreased towards the pole opposite the ectoplacental cone.

In those specimens in which the embryo had not been removed at explantation, the amniotic sac was spherical and always contained the remnants of a dead embryo

Table 1. *Comparison between the giant yolk sac and* 18½ *day conceptus*

	Giant yolk sac	18½ day conceptus
Yolk sac diameter (mm)	*19·00±0·36 $n = 36$	23·30±0·81 $n = 20$
Total protein concentration of yolk sac fluid (exocoelomic fluid) mg/ml	*5·27±0·15 $n = 12$	2·92±0·12 $n = 12$
Glucose concentration of yolk sac fluid (exocoelomic fluid) mg/ml	*0·18±0·02 $n = 10$	0·79±0·03 $n = 10$

Results represent mean ± S.E.M.
* Significantly different from 18·5 day conceptus, $P < 0·01$.

but the diameter of the amnion varied between 4·0 and 16·0 mm. The allantois was always well developed and was sometimes still connected to the ectoplacental cone, although on occasions this connection was severed.

All the observations described below were made on giant yolk sacs in which the embryonic tissues had not been removed at explantation. They therefore contained a dead embryo within an intact amnion. Yolk sacs from which embryonic tissue had een removed prior to explantation were smaller (about 1 cm in diameter and containing approximately 0·4 ml of fluid). Although detailed morphometric studies were not performed on these yolk sacs, it was demonstrated that their general structure, permeability, pinocytic activity and Endocytic Index were similar to those in which the dead embryo was allowed to remain *in situ*.

Comparisons between some basic features of the giant yolk sac and 18½ day control yolk sacs are given in Table 1. The results show that the protein content of giant yolk sac fluid was significantly higher than that of control yolk sac fluid, perhaps due to the absence of a live embryo utilising protein transported or secreted by the visceral yolk sac and also perhaps due to some soluble proteins diffusing from the dead embryo into the giant yolk sac fluid. The results also show the glucose levels in the giant yolk sac fluid to be significantly lower than in control yolk sac fluid. This may have been because glucose is transported to the fetal circulation via the chorioallantoic placenta *in vivo* and this could not take place in the giant yolk sac.

Electron microscopy of the giant yolk sac showed it to be composed of three cellular layers separated by two basement membranes:

(i) The outermost endodermal layer was made up of columnar epithelial cells (Fig. 4). At the free surface was a microvillous border and at the bases of some microvilli, invaginations formed with a fuzzy surface coat. These probably develop to form coated vesicles as later results using colloidal gold demonstrated.

In the apical cytoplasm there were numerous vacuoles. These varied in electron density, some very dark, others filled with flocculent material of varying electron density and others electron-lucent. The cytoplasm contained many well developed mitochondria, an extensive Golgi apparatus and granular endoplasmic reticulum, characteristic of an active protein producing cell. Typical junctional complexes were present composed of zonula occludens, zonula adherens and macula adherens. This layer of cells rested on the visceral basement membrane.

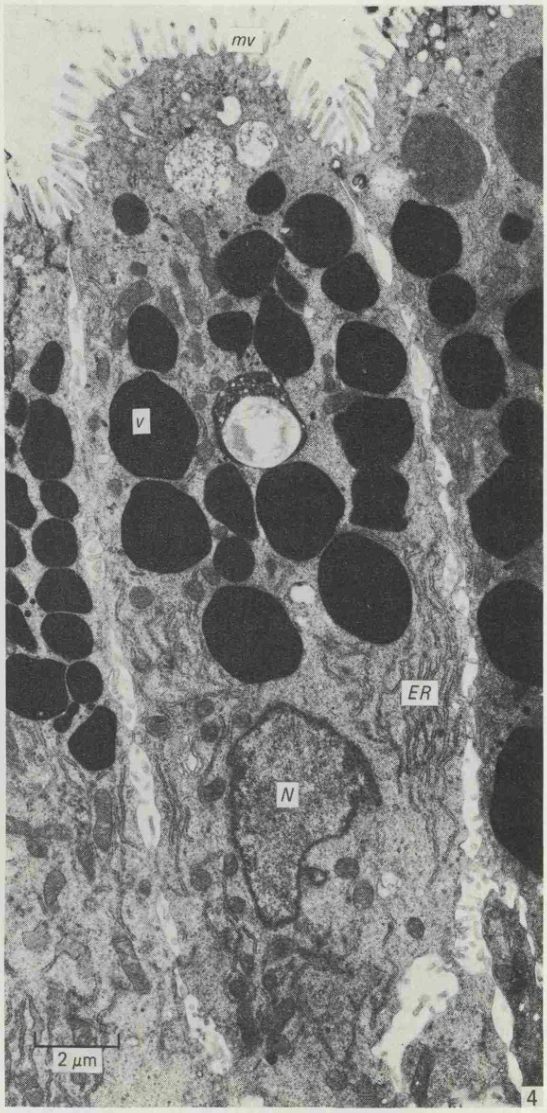


Fig. 4. Electron micrograph of an 18½ day giant yolk sac endoderm cell, showing typical features: a microvillous border (*mv*), a large vacuolar compartment with many electron-dense vacuoles (*v*), well developed granular endoplasmic reticulum (*ER*) and a basally situated nucleus (*N*).

(ii) The second layer consisted of scattered mesenchymal cells and collapsed fetal capillaries.

(iii) The third cell layer, which lined the exocoelom, consisted of flattened mesothelial cells and was separated from the second layer by the serosal basement membrane.

Control 18½ day yolk sac showed a generally similar morphology but exhibited certain differences (Fig. 5):

(*a*) In the endodermal cells of the 18½ day control yolk sacs glycogen deposits

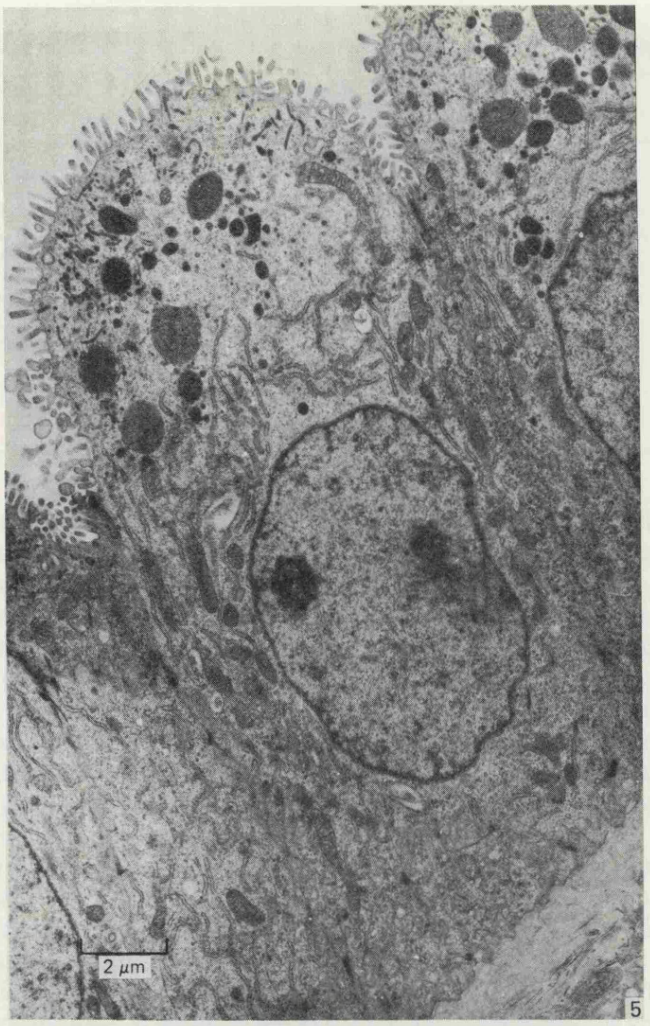


Fig. 5. Electron micrograph of an 18½ day control yolk sac endoderm cell. Note the reduced vacuolar volume in comparison to Figure 4.

were present in varying amounts. None were seen in the giant yolk sac endodermal cells.

(*b*) The serosal basement membrane was thicker in 18½ day control yolk sacs and contained more collagen fibres.

(*c*) The vacuolar compartment in the giant yolk sac endodermal cells and the individual vacuoles were larger than in the control yolk sacs.

(*d*) The 18½ day control yolk sacs had a well developed network of blood vessels. In the giant yolk sac the capillaries were collapsed.

Table 2. *Concentration of radioactivity (cpm/ml) found in the fluid within the extra-embryonic coelom of 18½ day giant yolk sacs after incubation in ^{125}I-labelled polyvinylpyrrolidone*

Incubation time	cpm/ml in yolk sac fluid	cpm/ml in medium
1 hour	81 ± 16 $n = 5$	200, 370
5 hours	170 ± 21 $n = 5$	211, 240

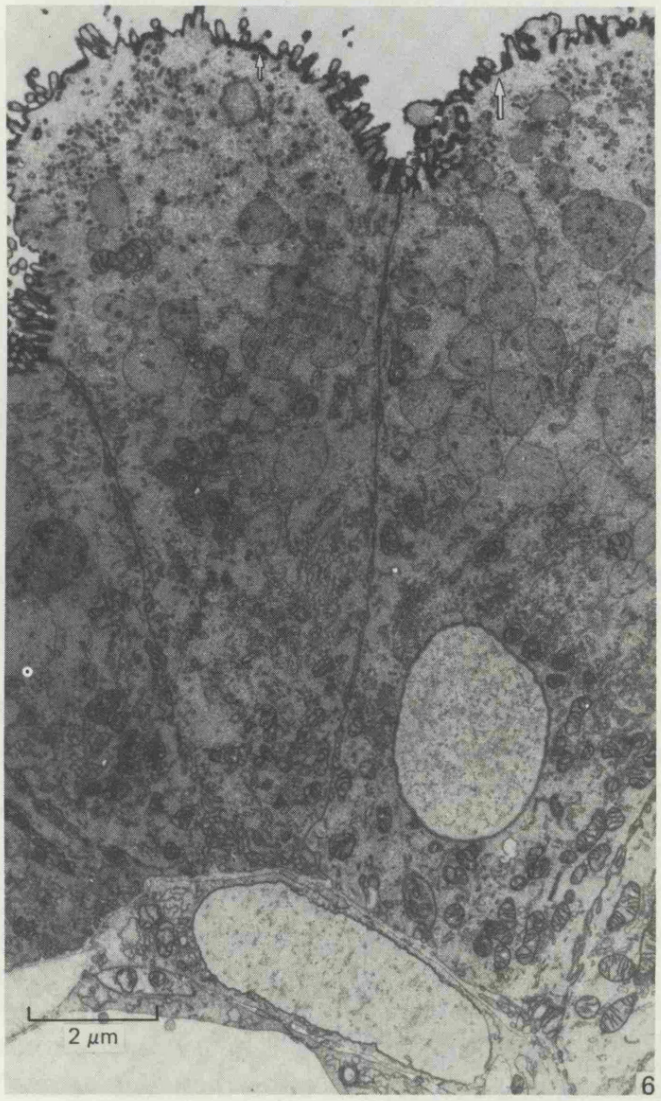


Fig. 6. An electron micrograph of 18½ day giant yolk sac endoderm cells after exposure to lanthanum nitrate. Note the continuous layer of lanthanum staining material (arrows) covering the microvillous border, to the level of the junctional complexes.

Permeability of the giant yolk sac

The fluid collected from giant yolk sacs previously incubated for five hours in the presence of ^{125}I-labelled PVP contained negligible amounts of radioactive label (Table 2).

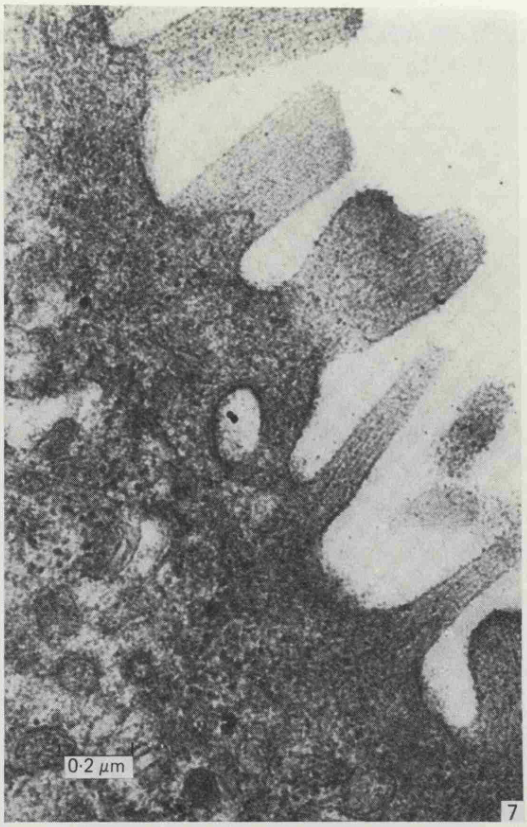


Fig. 7. Electron micrograph of the surface of a giant yolk sac endoderm cell showing the presence of 18 nm colloidal gold particles in a coated vesicle after incubation in colloidal gold for 5 minutes.

Table 3. *Results of morphometric analysis on the rat visceral yolk sac endoderm*

	Volume density of vacuolar compartment	
	In vivo	*In vitro*
Day 9½	15·82 ± 0·51	16·61 ± 0·59
Day 11½	10·68 ± 0·21	11·2 ± 0·64
Day 18½	6·65 ± 0·34	*23·75 ± 0·52

Each value is the mean ± S.E.M. for six embryos.
* Significantly different from *in vivo*, $P < 0·01$.
Results for day 9½ and day 11½ from Gupta, Gulamhusein & Beck (1982).

Sections of giant yolk sac stained with lanthanum showed a thick electron-dense layer of lanthanum-stained material external to the cell membrane on the apical surface (Fig. 6). This layer covered the convolutions of the microvillous border and invaginations in the cell surface to the level of the tight junctions. The lanthanum did not penetrate this junction and no lanthanum was observed in any membranous structures within the cell or in the cell cytoplasm.

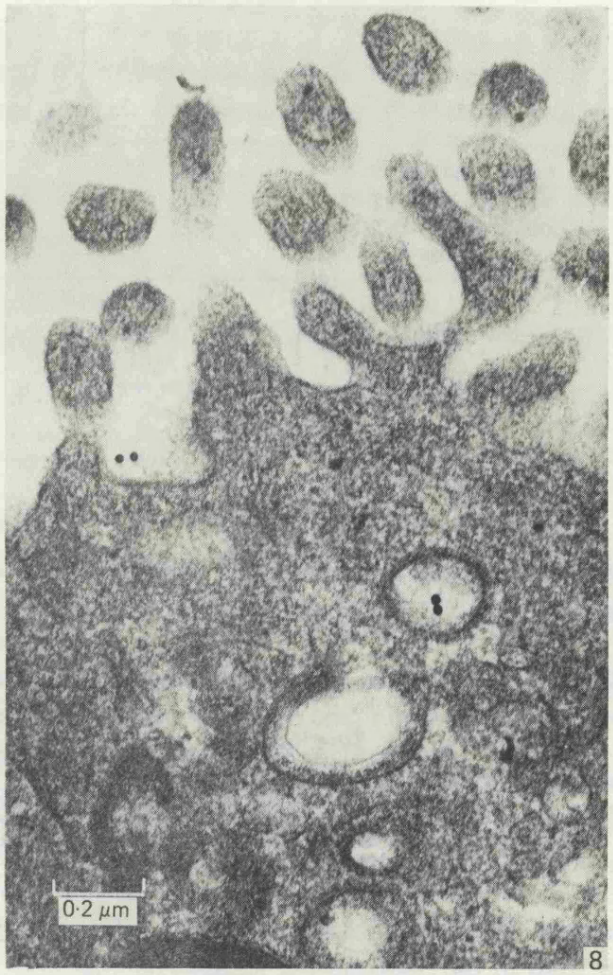


Fig. 8. Electron micrograph of the surface of a control yolk sac endoderm cell showing the presence of 18 nm colloidal gold particles in a coated pit and a coated vesicle after incubation in colloidal gold for 5 minutes.

Uptake of colloidal gold

After five minutes incubation, gold particles were seen at the surface in the small invaginations of the plasma membrane and also inside small vacuoles near the surface.

After ten minutes, some particles were also seen in the larger vacuoles deeper in the cytoplasm, some of which contained flocculent material.

After twenty minutes, some particles were observed in the large electron-dense vacuoles as well as those previously mentioned.

After a total incubation time of one hour, there was further accumulation of particles in the large electron-dense vacuoles and those containing flocculent material. Even after one hour's incubation with colloidal gold, there was no morphological evidence of cell damage and gold particles were never observed free in the cytoplasm or in any cell layer beneath the endoderm (Fig. 7).

The uptake of colloidal gold was found to be similar in control yolk sacs (Fig. 8).

Morphometric analysis of cell function

The results of the morphometric analysis of visceral yolk sac endoderm cells of the giant yolk sac and 18½ day control yolk sac are presented in Table 3.

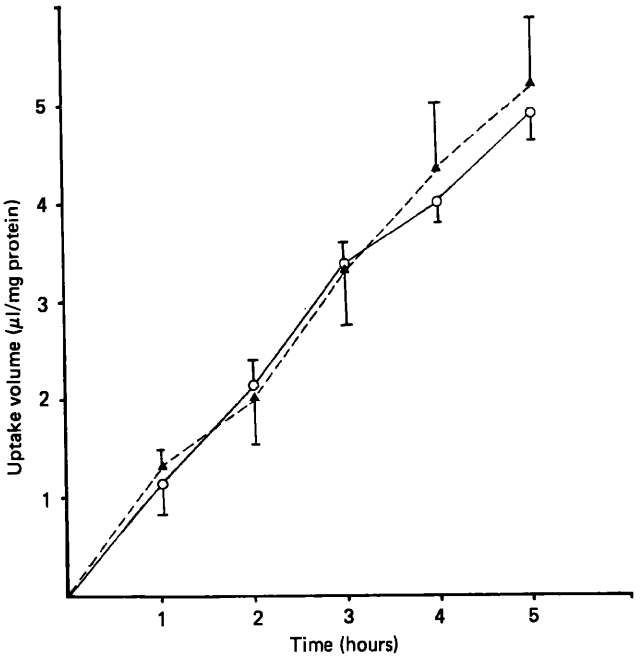


Fig. 9. Graph to show the rates of uptake of ^{125}I-PVP by 17½ day giant yolk sacs and control yolk sacs of the same age, incubated in whole rat serum, gassed with 95% O_2; 5% CO_2, over a period of 5 hours. Each point is a mean ± S.E.M. for 6 yolk sacs. ○—○, uptake of ^{125}I-PVP by giant yolk sacs; ▲—▲, uptake of ^{125}I-PVP by control yolk sacs.

The volume density of the vacuolar system in giant yolk sac endodermal cells was significantly greater than in the control yolk sacs.

Gupta, Gulamhusein & Beck (1982) found the volume density of the vacuolar system to be similar *in vivo* and *in vitro* at 9½ days and at 11½ days and they showed a fall in the size of the vacuolar volume between 9½ and 11½ days. The results of the present study, at 18½ days, showed a fall in the volume density of the vacuolar system *in vivo* but a rise in the volume density in the giant yolk sac.

Endocytic Index for polyvinylpyrrolidone

The uptake of PVP by the yolk sac was expressed as the volume of culture medium whose contained substrate was captured by unit quantity of yolk sac tissue (Williams *et al.* 1975). This volume (μl/mg tissue protein) was calculated from:

$$\frac{Y}{MP}$$

where Y is the total radioactivity (cpm) in the yolk sac, M is the radioactivity (cpm) per μl medium, and P is the protein content (mg) of the yolk sac.

The rate of uptake was called the Endocytic Index and was derived from the plot of uptake volume against incubation time. The units of the Endocytic Index were μl/mg yolk sac protein/hour incubation.

Figure 9 shows graphically that the rate of uptake of PVP by 17½ day giant yolk sacs was very similar to the rate of uptake in control yolk sacs, when incubations were carried out in 100% rat serum and gassed with 95% O_2; 5% CO_2. The Endocytic Index of the giant yolk sacs was 0·94 ± 0·014 (mean ± S.E.M.) and the Endocytic Index of control yolk sacs was 1·03 ± 0·019 (mean ± S.E.M.).

Table 4. *Specific activity of acid phosphatase in the giant yolk sac and* 18½ *day control yolk sac (sodium glycerophosphate as substrate)*

	Activity (nanomoles of glycerophosphate liberated/minute/mg protein)
Day 18½ (control)	61·88 ± 1·89
Day 18½ (giant yolk sac)	60·91 ± 1·31

Results represent mean ± S.E.M.

Acid phosphatase activity

(i) *Histochemical demonstration.* Both the 18½ day control and 18½ day giant yolk sacs showed a strong reaction for acid phosphatase in the supranuclear region of the endoderm cells.

(ii) *Biochemical demonstration.* The results are shown in Table 4, which expresses total acid phosphatase in the tissue homogenate. The two tissues showed no significant difference in total acid phosphatase using this method.

Table 5. *The ability of giant yolk sac fluid to support the growth of* 9½ *day embryos over a* 48 *hour culture period*

	Control 50% rat serum 50% Hanks balanced salt solution $n = 15$	Bovine serum albumin in Hanks balanced salt solution $n = 15$	Giant yolk sac fluid $n = 15$	25% Bovine serum albumin in balanced salt solution 75% giant yolk sac fluid $n = 15$
Beating heart	+	−	−	+
Yolk sac circulation	+	−	−	Rudimentary circulation – blood islands
Fused allantois	+	−	−	+
Closed neural tube	+	−	−	−
Fore limb buds	+	−	−	−
Mean yolk sac diameter (mm)	4·01 ± 0·09	−	−	2·20 ± 0·12
Mean somite number	24·60 ± 0·36	−	−	11·25 ± 0·42
Mean protein content (mg)	188·0 ± 7·9	−	−	47·5 ± 2·3

+/−, Presence or absence of feature.

The ability of giant yolk sac fluid to support the growth of 9½ *day rat embryos*

Table 5 shows that 9½ day embryos cultured for 48 hours in a solution containing a bulk protein (50 mg/ml bovine serum albumin in Hanks balanced salt solution) glucose and vitamins did not grow at all; 9½ day embryos cultured for the same period in giant yolk sac fluid with added glucose and vitamins showed no significant growth although the egg cylinder had expanded very slightly. However, when a

medium containing both a source of bulk protein and giant yolk sac fluid was used for culture, significant growth was achieved. The yolk sac had expanded and become spherical, some blood vessels were formed and blood islands present. The embryos were mainly convex ventrally and had a beating heart. In all cases the neural folds were formed but the neural tube was open along its length. The embryos had an average of 11 somites.

DISCUSSION

The present work describes a prolonged organotypic culture method for the rat visceral yolk sac. Using this technique, yolk sacs are grown routinely until they reach a diameter of approximately 2 cm and contain up to 1·0 ml of exocoelomic fluid. By removing the embryonic pole of the egg cylinder at the beginning of culture it is possible to grow the yolk sacs without a contained embryo. The vesicular nature of the cultured yolk sac makes it possible to study its functional activity; this aim cannot be achieved *in vivo*, neither is it possible by any other culture technique so far described (New *et al.* 1973; Sorokin & Padykula, 1960). The importance of the visceral yolk sac for the maintenance of the rat embryo between implantation and the establishment of a chorioallantoic placenta is well recognised (Beck & Lloyd, 1977) and, in this respect, the yolk sac performs the same functions as does the human syncytiotrophoblast from the time of implantation to the formation of the tertiary chorionic villi. Information concerning rat visceral yolk sac function is, therefore, of general interest in the study of mammalian development from gastrulation to the establishment of full chorioallantoic function. This period spans the development of the primitive streak and a major part of basic organogenesis.

Before generalising from observations made on the giant yolk sac model, it is necessary to establish whether it remains functionally similar to normal yolk sac. The work reported here would suggest that this is, indeed, the case. The well known pinocytic activity exhibited by rat visceral yolk sac epithelium can easily be demonstrated in the giant yolk sac model. This is shown by the colloidal gold uptake experiments. A precise estimation of the Endocytic Index (Williams *et al.* 1975) displayed by the cultured yolk sac, has been calculated with respect to ^{125}I-PVP. The results of this experiment show that, under the experimental conditions used, little difference exists between the endocytic index *in vivo* and *in vitro*. Passive diffusion of material from the culture medium to the interior of the giant yolk sac does not take place. This is illustrated by the inability of ^{125}I-PVP or lanthanum to enter the exocoelom in the experiments described here. The physiological separation of the exocoelomic cavity from the culture medium surrounding the giant yolk sac makes this a very useful model for investigating its transport functions.

The kinetics of active transport across the cells of the giant yolk sac clearly differ from those *in vivo*. In the latter state, a growing embryo is able to utilise and perhaps transform material coming to it across the yolk sac wall whilst, in the case of the giant yolk sac, such material will merely accumulate in the exocoelom. Additionally, the giant yolk sac will not accumulate substances which might have reached the exocoelom via a functional chorioallantoic placenta. The authors believe that these considerations explain the differences in the level of the total protein and glucose concentrations found in the giant yolk sac exocoelom from those present *in vivo*. Similar considerations might also explain the deviation of the volume density of the vacuolar system from the *in vivo* situation.

The standard technique of culturing post-implantation rat embryos *in vitro* (New

et al. 1973) involves growing them inside their visceral yolk sacs between 9·5 and 11·5 days of gestation in specially prepared rat serum. It has been observed (Al-Alousi, 1983) that serial passage of embryos through the same serum eventually exhausts its capacity to sustain embryonic growth, even after dialysis to remove toxins, followed by glucose and vitamin supplementation. Circumstantial evidence suggests that this is due to the exhaustion of specific trophic factors (Gulamhusein, Huxham & Faisal, 1984). Furthermore, it has been shown that certain macromolecules can cross the visceral yolk sac unchanged (Huxham & Beck, 1984). The suggestion that specific trophic molecules are implicated in the growth of the post-implantation embryo derives further support from the results presented here concerning the ability of the giant yolk sac fluid to support embryonic growth when supplemented with a non-specific source of protein (bovine serum albumin). The results obtained are dramatic and difficult to explain in any other way.

The giant yolk sac is easy to prepare and we believe that the model will be of use both in delineating the functional capacity of the extraembryonic membranes as well as more fundamentally in the investigation of the transport of an epithelial sheet.

SUMMARY

Nine and a half day rat embryos can be cultured for 48 hours in whole heat-inactivated rat serum using the roller culture method described by New, Coppola & Terry (1973). We have prolonged the culture period, usually by seven days. Although the embryo dies almost immediately during this extended culture period, the yolk sac continues to grow and reaches a diameter of approximately 2 cm; we have called this the giant yolk sac.

The morphology of the giant yolk sac is very similar to that of control yolk sacs (17½ or 18½ days *in vivo*), the main difference being the greatly enlarged vacuolar volume in the endodermal cells of the giant yolk sac, which have been studied morphometrically.

The pinocytic nature of the giant yolk sac has been demonstrated by its ability to take up colloidal gold. Its rate of uptake of ^{125}I-polyvinylpyrrolidone in whole serum gassed with 95% O_2; 5% CO_2 has been shown to be similar to the rate of uptake found in control yolk sacs under the same incubation conditions.

Acid phosphatase activity was found to be similar in the giant yolk sac and control yolk sacs using both histochemical and biochemical methods.

Giant yolk sacs without a contained dead embryo can be produced by removing the embryonic pole of the egg cylinder prior to incubation. They exhibit all the features detailed above.

Finally it is shown that the fluid from within the extra-embryonic coelom of the giant yolk sac has some capacity to support the growth and development of 9½ day rat embryos when a source of bulk protein is also provided.

This model, therefore, seems to be very useful for the study of transport in a placental system. Its full potential requires further study.

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THE GIANT YOLK SAC—A MODEL FOR STUDYING TRANSPORT ACROSS THE YOLK-SAC PLACENTA

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Introduction

The nutritional requirements of the post-implantation rodent embryo can be met solely by the visceral yolk sac until the chorioallantoic placenta develops (New, Coppola & Terry, 1973). These species are therefore uniquely suited for studying the needs of post-implantation embryos in their pre-placental period of development. We have developed a method of growing the rat visceral yolk sac as a closed vesicle over a prolonged culture period, having first removed the embryonic tissue by microsurgery or alternatively allowing it to die within its own amnion. We have called this system the giant yolk sac (Dunton, Al-Alousi, Pratten & Beck, 1986).

Experimental

Rat embryos were explanted at 9.5 days and cultured in whole heat-inactivated rat serum using a roller culture technique (New *et al.* 1973) for a period of 8 or 9 days in an atmosphere of O_2–CO_2–N_2, 20:5:75. The culture serum was changed every third day, the amount used being increased as the yolk sac grew. The morphology of the giant yolk sac has been shown to be similar to that of control yolk sacs of the same age.

Results and Discussion

The functional activity of the giant yolk sac has been assessed by investigating the rate of uptake of various ^{125}I-labelled macromolecules. Using a modification of the shaker flask method (Williams, Kidston, Beck & Lloyd, 1975), the rate of uptake of the non-digestible macromolecule polyvinyl-pyrrolidone (PVP) was found to be very similar in the giant yolk sac and in control yolk sacs, when incubated in rat serum in an atmosphere of O_2–CO_2, 95:5, over a 5-hour period. As uptake was linear with time, the results could be expressed in terms of the Endocytic Index (μl of fluid whose contained substrate was taken up per mg protein/hour). The Endocytic Index ($\pm$SEM) was found to be 1.03 ± 0.019 μl/mg protein/hr for the control yolk sac and 0.94 ± 0.014 μl/mg/hr for the giant yolk sac.

We have studied the growth-supporting properties of the fluid from within the giant yolk sac (giant yolk sac fluid) by culturing 9.5-day rat embryos for 48 hours in a series of media. Glucose and vitamins were added to each medium to the level normally associated with rat serum.

A medium containing 50% rat serum and 50% Hank's balanced salt solution (BSS) with a protein concentration of approximately 50 mg/ml supports the normal growth of embryos to the 25-somite stage. No embryonic growth occurred when embryos were cultured in a BSS containing protein in the form of bovine serum albumin at 50 mg/ml (Table 1). Neither was any growth achieved in a medium containing only giant yolk sac fluid. However, when giant yolk sac fluid and a source of protein were combined in a medium significant embryonic development was achieved (Table 1). The giant yolk sac fluid must therefore contain some factors essential for embryonic growth and development.

Conclusion

Thus we have a functional system ideally suited for studying transport across an epithelial sheet. The giant yolk sac is particularly useful as its continuous epithelium separates the internal cavity from the external culture medium. Also the fluid within the giant yolk sac is an excellent source of processed histiotroph, which has been shown to be essential for embryonic growth.

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Table 1. Growth of 9.5-day rat embryos in giant yolk sac fluid and other media

Measurement	BSA in BSS	Giant yolk sac fluid	25% BSA in BSS, 75% giant yolk sac fluid	50% rat serum, 50% BSS
Mean yolk-sac diameter (mm)	1.01 ± 0.13	0.97 ± 0.11	2.2 ± 0.12	4.01 ± 0.086
Mean somite no.	None	None	11.25 ± 0.42	24.6 ± 0.36
Mean protein content (mg)	—	—	48.48 ± 2.32	188 ± 7.9

BSA = Bovine serum albumin BSS = Hank's balanced salt solution
Values are means $\pm$ SEM for groups of 15 embryo cultures.

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Uptake and Digestion of ^{125}I-labelled Bovine Serum Albumin by the Rat Visceral Yolk Sac Cultured in Vitro as a Closed Vesicle

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INTRODUCTION

Prior to the functional establishment of a chorioallantoic placenta, the mammalian embryo is sustained by histiotrophic nutrition. This process involves the endocytosis of maternal macromolecules (histiotroph) from various local sources such as the decidua, uterine secretions, blood and serum transudate by cells of the extraembryonic membranes. The histiotroph is degraded in the vacuolar system of the cells which have interiorized it, and the onward transmission of the breakdown products enables the embryo to assemble its tissues under the control of its own genome (Steven, 1975; Beck, 1981; Mossman, 1987). In addition, the extraembryonic membranes also transmit a number of specific proteins in an unchanged form from mother to embryo; their passage is receptor-mediated and many of the polypeptides so transported serve as growth factors (Huxham and Beck, 1984, 1985). Even after the chorioallantoic placenta begins to function, histiotroph still plays a significant, though variable, part in the development of all mammalian embryos studied so far (Mossman, 1987).

The particular extraembryonic membranes which digest histiotroph vary with species. During human organogenesis the early postimplantation syncytiotrophoblast is the tissue responsible; it takes up material from the sluggishly circulating blood in the maternal blood lake which forms around the newly-implanted chorionic vesicle (Boyd and Hamilton, 1970). In the rat and other rodents the visceral layer of the yolk sac effects histiotrophic nutrition. This is shown by the fact that, up to the stage at which the chorioallantoic placenta begins to function, normal embryonic development in vitro occurs when the embryo and visceral yolk sac are explanted in homologous rat sera after removal of the other extraembryonic structures, that is, the trophoblast and parietal yolk sac (New, Coppola and Terry, 1973). During early development the rat visceral yolk sac comes to surround the embryo and amnion, forming a vesicle within which the embryo grows (see Steven, 1975, for topographical relationships). Histiotroph is absorbed from serum transudate which in vivo bathes the maternal aspect of the visceral yolk sac endoderm until 16.5 days of gestation. After this, uterine secretions provide the substrate absorbed by the yolk sac.

We have recently developed a method of culturing the visceral yolk sac for a protracted period as a closed vesicle: the so-called 'giant' yolk sac (Dunton et al, 1986). Essentially, the method consists of maintaining explanted 9.5-day rat embryos in whole embryo culture con-

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ditions for a period of eight days or more. The embryo itself dies at approximately 12.5 days and is partially autolysed, but the yolk sac continues to grow and reaches a diameter in excess of 2 cm. If necessary, the embryo can easily be removed by microdissection prior to yolk sac culture in order to eliminate the minor biochemical changes resulting from its retention and autolysis, but in the experiments described here this made no difference to the results obtained.

The method is useful because yolk sac function can be monitored over a protracted period and the products of histiotrophic nutrition accumulate within the cavity of the giant yolk sac and are separate from the surrounding culture medium. Furthermore, the picture is not complicated by embryonic metabolism, and the model can be used to study the physiology of histiotrophic nutrition in isolation. In a broader context, the giant yolk sac system can be used to study various parameters of transcytosis in epithelia; in rodents and lagomorphs passive immunity is mediated prenatally by the transport of immunoglobulin G across the visceral yolk sac, a process which may in some ways resemble that of the neonatal rat gut before 'closure' (Rodewald, 1973; Pett, 1977). However, differences in the local environment in terms of pH, venous and lymphatic drainage, and differences in brush border morphology and enzyme complement may profoundly influence the precise mechanisms of polarized transport involved.

In the present study we have monitored the uptake of ^{125}I-labelled formaldehyde-denatured bovine serum albumin (BSA) by the giant yolk sac and its subsequent digestion, as well as the release of both its digestive products and BSA which has not been broken down to its monomers. This substrate was chosen to study the pinocytic and digestive capacity of the giant yolk sac because it has been shown that formaldehyde denaturation of albumin renders it more susceptible to capture and breakdown by the cells of the yolk sac epithelium (Moore, Williams and Lloyd, 1977). The effect of the chelating agent ethyleneglycol-bis(aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), a known inhibitor of endocytosis (Duncan and Lloyd, 1978), is described.

MATERIALS AND METHODS

Preparation of ^{125}I-labelled bovine serum albumin

Bovine serum albumin (BSA) was radiolabelled with [^{125}I]iodide using a modification of the chloramine T method (Hunter, Greenwood and Glover, 1963), denatured with 10 per cent formaldehyde for 72 h, and then extensively dialysed against 1 per cent NaCl to remove any free iodide, as described by Moore, Williams and Lloyd (1977).

Method for quantifying the uptake of [^{125}I]BSA by 17.5-day yolk sacs

The method used for measuring the uptake of [^{125}I]BSA in the control yolk sacs explanted directly from a 17.5-day pregnant female was a modification of that of Williams et al (1975). Briefly, 60-ml bottles were prepared, each containing 10 ml of incubation medium (either medium 199 or whole rat serum), and were gassed with 95 per cent O_2, 5 per cent CO_2—a mixture previously shown to promote an optimum rate of endocytosis of ^{125}I-labelled polyvinylpyrrolidone (PVP) (Dunton, unpublished). The bottles were allowed to equilibrate at 37°C for 30 min, prior to the introduction of four 17.5-day yolk sacs explanted directly from the female. After a further period of equilibration 10 μg/ml [^{125}I]BSA were added to each incubation bottle and the bottles were regassed with 95 per cent O_2, 5 per cent CO_2. Incubations were carried out at 37°C for periods up to 5 h.

At the end of each incubation period yolk sacs were removed from the incubation bottles, washed three times in ice-cold 1 per cent NaCl to remove extracellular substrate, blotted, and

placed in 1 M NaOH. Samples of the dissolved yolk sac and the incubation medium were assayed for radioactivity, using an LKB RIA automatic gamma-counter. The digestion products released were also measured by quantifying the amount of trichloroacetic acid-(TCA-)soluble material in the culture medium. The protein content of each yolk sac was determined using a modification of the method of Lowry et al (1951).

Uptake of [^{125}I]BSA by giant yolk sac (grown with and without the embryo)

Giant yolk sacs were grown in whole heat-inactivated rat serum as first described by Al-Alousi (1983) and also using the modified method after prior removal of the embryonic tissue (Dunton et al, 1986). The method for measurement of uptake in both types of giant yolk sac was identical. Three yolk sacs were cultured in rat serum (6 ml) from 9.5 to 17.5 days of gestation.

At 17.5 days the giant yolk sacs were washed with several changes of incubation medium. Fresh medium (either medium 199 or whole rat serum) was introduced to each bottle containing giant yolk sacs, and the bottles were gassed with 95 per cent O_2, 5 per cent CO_2. After 30 min of equilibration at 37°C, 10 μg/ml ^{125}I-labelled BSA were added to each incubation bottle; the bottles were regassed and incubated in a roller incubator for periods up to 5 h. At the end of each incubation period bottles were removed from the incubator, taking care not to disrupt the delicate yolk sac membranes.

Samples of the incubation medium were removed from each bottle for analysis of total and acid-soluble radioactivity. The remaining medium was decanted and the giant yolk sacs washed in several changes of ice-cold 1 per cent NaCl. The exocoelomic fluid was removed from each yolk sac, its volume measured, and the contained radioactivity, both total and TCA-soluble, determined. The visceral yolk sac was dissected from the amnion and the remains of the embryo (in the case of yolk sacs grown by the original method) and the inner yolk sac surfaces washed thoroughly with ice-cold 1 per cent NaCl; each yolk sac was dissolved in 1 M NaOH and samples were removed for the assessment of radioactivity and protein content.

Calculating the rate of uptake as an endocytic index

The concept of the endocytic index (EI) was first described by Williams et al (1975) and is a universal unit used to express the rate of uptake of a substrate by a particular tissue type as a clearance rate. Expressing the results in this way eliminates the effects of the size of the yolk sac, the slightly different amounts of radioactivity in the incubation medium, and the effect of decay on the specific radioactivity of the substrate. The units are μl of fluid whose contained substrate was captured/mg tissue protein/h, although in the case of an adsorptive substrate it does not imply that that volume of medium was captured.

17.5-day control yolk sac. As ^{125}I-labelled BSA is digested within the lysosomes, the majority of the ingested [^{125}I]BSA is released after digestion in the form of iodotyrosine and does not accumulate in the tissue. Therefore, uptake was calculated from the sum of the amount of radioactivity in the yolk sac tissue and the amount of digestion products released back into the culture medium.

Giant yolk sac. The giant yolk sac is maintained throughout incubation as a closed vesicle in which the exocoelomic fluid is separated from the incubation medium. This necessitates an additional factor in the calculation of EI; the uptake volume is calculated from the sum of tissue-associated radioactivity and also any digestion products released into the exocoelomic fluid and into the culture medium.

Quantifying the release of [^{125}I]BSA by giant yolk sacs and control yolk sacs

In order to assess the quantity of intact [^{125}I]BSA released from the yolk sac tissue both giant yolk sac and control 17.5-day yolk sacs were incubated as previously described in medium 199 containing 10 μg/ml formaldehyde-denatured [^{125}I]BSA in an atmosphere of 95 per cent O_2, 5 per cent CO_2 for 3 h. The yolk sacs were washed in warmed medium 199 before being reincubated in fresh medium 199 for a further 3 h. Samples of incubation medium were taken at various intervals and the amount of total and TCA-soluble radioactivity in each measured. At the end of incubation the total tissue-associated radioactivity in both control and giant yolk sacs was measured, as was the total and TCA-soluble radioactivity in the exocoelomic fluid of the giant yolk sacs.

Results were expressed as a percentage of the total radioactivity associated with the yolk sac tissue at the start of reincubation.

Inhibition of [^{125}I]BSA uptake by EGTA

Both giant yolk sacs and 17.5-day control yolk sacs were incubated exactly as described previously in three different incubation media: (1) medium 199; (2) medium 199 containing 5 mM EGTA; (3) medium 199 containing 5 mM EGTA and 5 mM $CaCl_2$. Incubation was carried out over a 5-h period and the rate of uptake in both tissues calculated in all three incubation conditions, as previously described.

RESULTS

Uptake and digestion of [^{125}I]BSA

Figure 1 shows the mean rate of uptake of [^{125}I]BSA of 34 giant yolk sacs cultured in whole rat serum for a period of up to 5 h compared with 46 control yolk sacs taken from 17.5-day preg-

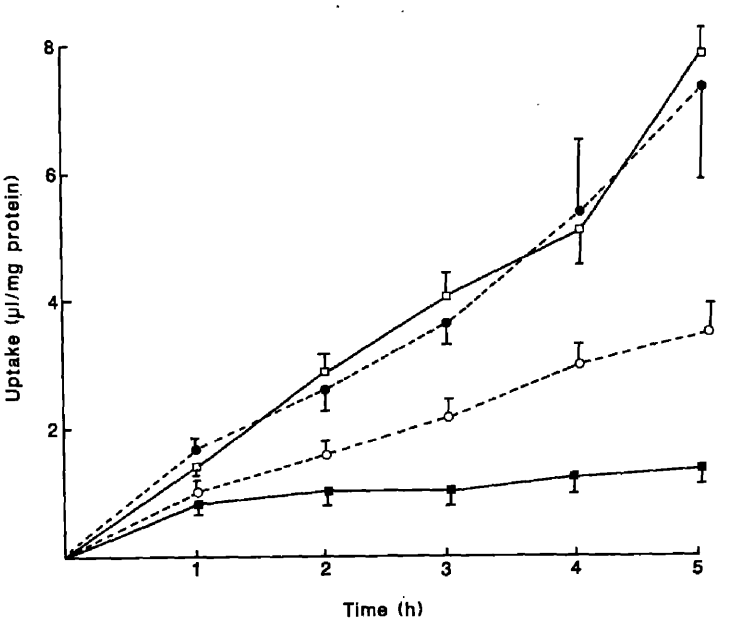

Figure 1. Uptake and digestion of ^{125}I-labelled formaldehyde-denatured bovine serum albumin by rat visceral yolk sacs. Each point represents the mean uptake for at least six yolk sacs ± s.e. Total uptake for yolk sacs obtained from the mother at 17.5 days' gestation (□——□) and yolk sacs cultured in vitro, 'giant' yolk sacs (●– – –●), are shown. The amount of radioactivity which becomes tissue-associated is also shown for control (■——■) and 'giant' (○– – –○) yolk sacs.

Table 1. The uptake of ^{125}I-labelled formaldehyde-denatured bovine serum albumin by 'giant' yolk sacs and control yolk sacs cultured in vitro[a]

	Endocytic index (μl/mg protein/h)		
		'giant' yolk sacs	
Culture conditions	control yolk sacs	with embryo	without embryo
Medium 199	28.4 ± 0.25 (n = 55)	1.48 ± 0.05 (n = 48)	1.55 ± 0.07 (n = 40)
Rat serum	1.34 ± 0.04 (n = 46)	1.41 ± 0.07 (n = 34)	1.44 ± 0.08 (n = 40)

[a]Results are expressed as mean endocytic index ± s.e.

nant animals. The figure shows that total uptake rate in the giant yolk system is similar to that obtained from control yolk sacs. However, over the five-hour period the tissue-associated (i.e., largely undegraded) radioactivity rises significantly above that found in the controls. Table 1 shows the endocytic indices obtained under various culture conditions. It confirms that there is no difference between the endocytic capacity of control and giant yolk sacs when they are cultured in homologous serum, and that the presence of an autolysing embryo does not affect the results. Interestingly, however, when control yolk sacs are cultured in medium 199 the endocytic index rises dramatically but fails to do so under similar conditions in the giant yolk sac. In this context it should be noted that explanted embryos between 9.5 and 11.5 days will survive for a period of hours in medium 199 but that normal growth will not take place unless at least 30 per cent homologous rat serum is present.

Figure 2a shows the relative amounts of radioactive digestion products (expressed as ng pro-

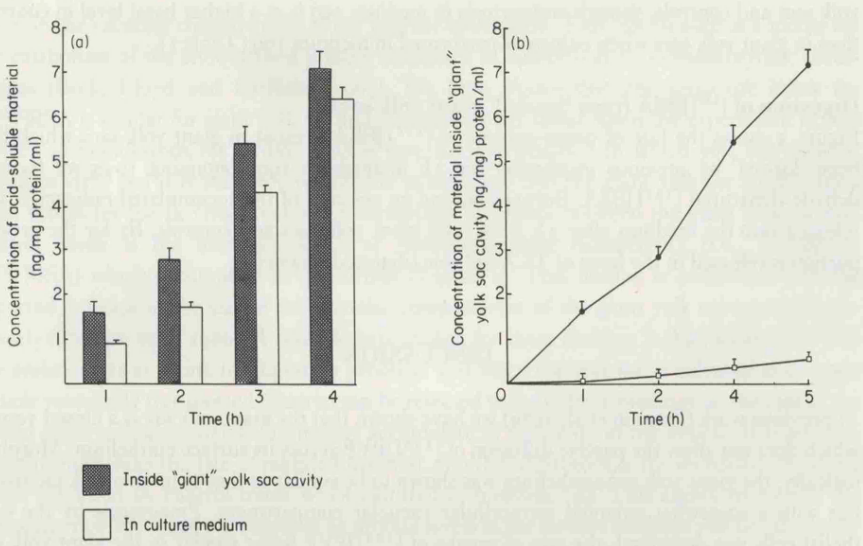

Figure 2. (a) The concentration of digestion products released to the inside and outside of 'giant' yolk sacs following incubation in the presence of ^{125}I-labelled formaldehyde-denatured bovine serum albumin. Results are expressed as the mean of at least six experiments ± s.e. (b) The relative concentration of intact ((□——□) ^{125}I-labelled bovine serum albumin and acid-soluble digestion products (●——●) inside the 'giant' yolk sac cavity. Results are given as the mean for at least six giant yolk sacs ± s.e.

Table 2. Uptake of ^{125}I-labelled formaldehyde-denatured bovine serum albumin in the presence or absence of chelating agent[a]

Culture conditions	Endocytic index (μl/mg protein/h)	
	control yolk sacs	'giant' yolk sacs
Medium 199	25.20 ± 1.40 ($n = 20$)	1.51 ± 0.08 ($n = 20$)
Medium 199 + 5 mM EGTA	1.61 ± 0.06 ($n = 24$)	0.21 ± 0.01 ($n = 21$)
Medium 199 + 5 mM EGTA + 5 mM $CaCl_2$	23.73 ± 1.24 ($n = 21$)	1.34 ± 0.05 ($n = 19$)

[a]Results are expressed as mean endocytic index ± s.e.

tein digested/mg yolk sac protein/ml) present on the inside and outside of the giant yolk sac during 5 h of incubation in medium 199 containing 10 μg/ml [^{125}I]BSA. It is apparent that the concentration of digestion products is greater inside the giant yolk sac than outside and remains so throughout the culture period. Figure 2b shows that the concentration of acid-soluble digestion products found inside the giant yolk sac cavity increases with time. The amount of intact ^{125}I-labelled bovine serum albumin remains at a very low level throughout the 5-h culture period.

Inhibition of endocytosis by EGTA

Table 2 shows that the pinocytosis of [^{125}I]BSA in medium 199 is greatly decreased (to less than 15 per cent of the levels observed in the absence of inhibitor) by 5 mM EGTA added to the culture medium. This effect is reversed by 5 mM $CaCl_2$. The effect is present in both giant yolk sacs and controls, though endocytosis in medium 199 is at a higher basal level in controls than in giant yolk sacs when culture is performed in medium 199 (Table 1).

Digestion of [^{125}I]BSA from 'loaded' giant yolk sacs

Figure 3 shows the fate of tissue-associated [^{125}I]BSA present in giant yolk sacs which had been 'loaded' by previous incubation for 3 h in medium 199 containing 10 μg/ml formaldehyde-denatured [^{125}I]BSA. Between 60 and 70 per cent of the accumulated radioactivity is released into the medium after 3 h from both giant yolk sacs and controls. By far the greater portion is released in the form of TCA-soluble (digested) material.

DISCUSSION

In previous work (Dunton et al, 1986) we have shown that the giant yolk sac is a closed vesicle which does not allow the passive diffusion of [^{125}I]PVP across its surface epithelium. Morphologically, the giant yolk sac epithelium was shown to be similar to that of in vivo yolk sac tissue but with a somewhat enlarged intracellular vacuolar compartment. Pinocytosis by the epithelial cells was described, the rate of uptake of [^{125}I]PVP being similar in the giant yolk sac and in control 17.5-day yolk sac. Acid phosphatase levels and location in giant yolk sacs and controls corresponded. Evidence for the specific transcellular passage of embryonic growth-promoting substances across the giant yolk sac surface epithelium was also presented.

In the present work we have investigated the fate of [^{125}I]BSA which in vivo is broken

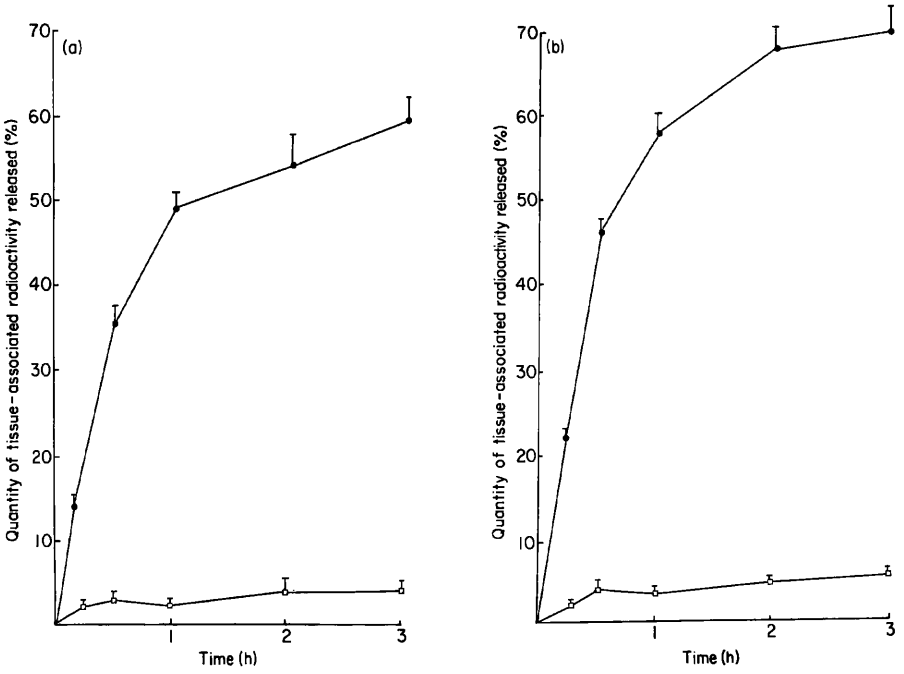

Figure 3. Release of radioactivity from yolk sacs preloaded with ^{125}I-labelled formaldehyde-denatured bovine serum albumin. (a) Release of trichloroacetic acid-insoluble (□——□) and -soluble (●——●) material from control yolk sacs. (b) Release of trichloroacetic acid-insoluble (□——□) and -soluble (●——●) material from cultured 'giant' yolk sacs. Results are given as the mean release from at least six yolk sacs ± s.e., and expressed as the percentage of the material originally associated with the tissue that had been released plotted against time of reincubation.

down in the vacuolar compartment of the yolk sac epithelium. This can be used as a model for the catabolism of the endocytosed protein contained in histiotroph by extraembryonic membranes (Beck, Lloyd and Griffiths, 1967). We have shown that the endocytic index for [^{125}I]BSA is similar for giant yolk sac and control yolk sac tissue when the experiment is performed in homologous rat serum. This is very like the situation in which yolk sac tissue is found in vivo, and it is therefore reasonable to conclude that the giant yolk sac constitutes a valid model for the in vivo situation. A significant difference between the giant yolk sac and control tissue is the increased levels of tissue-associated radioactivity (i.e., undigested [^{125}I]BSA) which accumulate in the former (Figure 1). This finding is consistent with the reported increase in the size of the vacuolar compartment of the giant yolk sac reported previously (Dunton et al, 1986). A possible explanation for these findings is that, whereas in vivo the embryo acts as a sink for digestion products and when the yolk sac is cultured as an open vesicle vectorially transported material can be released to the culture medium, in the case of the closed giant yolk sac material can merely accumulate in the cavity of the vesicle. It is possible that this influences the fate of ingested material. Another difference is the increased endocytic index exhibited by control tissue when cultured in medium 199. This ability to modify the endocytic rate apparently in response to protein levels in the culture medium was not shown by the giant yolk sac. This phenomenon was also observed when ^{125}I-labelled PVP was used as an endocytic marker.

The presence of EGTA in the culture medium caused a decrease in the rate of capture of ^{125}I-labelled BSA in both control and giant yolk sacs. This inhibitor has previously been

shown to inhibit both fluid phase and adsorptive pinocytosis (Duncan and Lloyd, 1978) and, therefore, this is good evidence that the substrate is internalized by the cells via a pinocytic route and is processed intracellularly. The effect of EGTA could be abrogated by addition of equimolar calcium.

Our experiments show that the half-life of formaldehyde-denatured [^{125}I]BSA in the giant yolk sac and in controls is in the region of one hour, and that some 70 to 80 per cent of the protein is broken down to TCA-soluble digestion products within three hours. Furthermore, we have been able to demonstrate that the TCA-soluble material accumulates within the cavity of the giant yolk sac. This constitutes evidence that the yolk sac epithelial cells have polarity not only with respect to their uptake of macromolecules (which takes place at the apical surface) but also with respect to their release of digestion products which appears to take place preferentially on the surfaces deep to the junctional complexes between the cells. A result of this nature can be obtained only from the giant yolk sac system; its general biological validity is based upon the demonstration that giant yolk sac function mirrors the function of control yolk sac tissue whenever the two can be compared directly.

SUMMARY

A system for culturing the rat visceral yolk sac in vitro as a closed vesicle—the 'giant' yolk sac—has been employed to investigate the vectorial nature of the uptake and digestion of exogenous protein substrates. Uptake of ^{125}I-labelled formaldehyde-denatured bovine serum albumin by such yolk sacs was found to be similar to that observed in yolk sacs removed directly from the mother at 17.5 days' gestation, provided that homologous serum was used as a culture medium. However, unlike the control yolk sacs, giant yolk sacs tended to accumulate substrate within the tissue with increasing culture time. The concentration of digestion products released to the inside of the closed vesicle was found to be greater than that released to the surrounding culture medium at time intervals up to five hours. Giant yolk sacs preloaded with ^{125}I-labelled bovine serum albumin were found to release material to the culture medium or the inside of the vesicle almost entirely in the acid-soluble (digested) form. This system is a useful model for studying the polar nature of epithelia, particularly those involved in the uptake and transport of nutritional and/or informational macromolecules.

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The 'Giant' Yolk Sac - An *In Vitro* Model for Studying Early Placental Transport. **Anne Dunton**

Abstract

In the rat, before the establishment of the chorioallantoic placenta, the nutritional requirements of the post-implantation embryo, are met solely by the visceral yolk sac and therefore a study of its structure and functions is essential to a full understanding of early embryonic nutrition.

A method has been developed for maintaining the rat visceral yolk sac in organ culture over a prolonged period, having first removed the embryo by microsurgery at 9.5 days or alternatively allowing it to die within its own amnion. The yolk sac continues to grow as a closed vesicle, and can reach a diameter of 2cm. The system has been called the 'giant' yolk sac.

The 'giant' yolk sac and *in vivo* yolk sac have been compared using various criteria.

A detailed morphological study was made, including a quantitative analysis of the vacuolar compartment. The endocytic capacity of both systems was studied using three different substrates; those used were ^{125}I-polyvinylpyrrolidone (PVP), a non-degradable macromolecule, taken up in the fluid phase and accumulated within the yolk sac tissue, ^{125}I-bovine serum albumin (BSA) taken up by adsorptive pinocytosis and digested within the lysosomes and ^{125}I-IgG (and colloidal gold-IgG) taken up with great efficiency by specific receptor mediated endocytosis. Also a preliminary study of ^{14}C-amino acid uptake was made.

In many instances the 'giant' yolk sac functioned very similarly to the *in vivo* yolk sac and therefore seems an ideal model for studying transport across an epithelial sheet. It is particularly useful as its continuous epithelium separates the exocoelom from the external culture medium.

The fluid maintained within the exocoelom of the 'giant' yolk sac should be an excellent source of processed histiotroph essential for embryonic nutrition during organogenesis. Experiments carried out indicate that some of the trophic factors necessary for growth are present in this fluid.