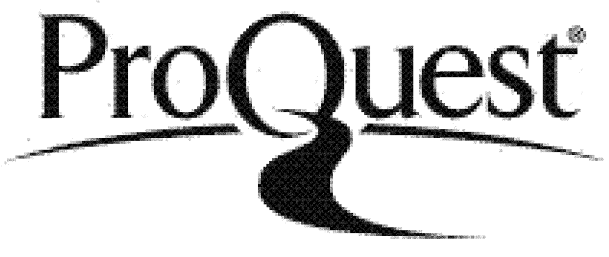
A STUDY INTO THE ROLE OF INSULIN AND INSULIN-LIKE GROWTH FACTOR I (IGF-I) IN RAT EMBRYONIC DEVELOPMENT

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

Elizabeth Asenath Cowley BSc (London)
Department of Anatomy
University of Leicester

December 1992



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ABSTRACT

Insulin, and the structurally related insulin-like growth factors (IGFs), are peptide growth factors believed to play a role in embryonic development. In addition to factors produced by the embryo, certain maternally derived growth factors may also be important during development. These are likely to act upon the extraembryonic membranes which surround the embryo throughout gestation, before they, or their breakdown products, are transported to the developing embryo.

This thesis examines the processing of both insulin and insulin-like growth factor I (IGF-I) by the visceral yolk sac, an extraembryonic membrane in the rat. Both radiolabelled and fluorescently labelled ligands have been examined in 17.5 day yolk sacs, and their cultured equivalent. It appears that both factors are digested by this tissue very rapidly, which may involve receptor-mediated pinocytosis or surface digestion.

Further, the role of the IGF-I receptor during rat embryonic development has been examined using a monoclonal antibody reported to block this receptor. When this antibody was applied to rat embryos cultured from 9.5 to 11.5 days of gestation, it resulted in growth retardation plus an associated increase in morphological abnormalities. These effects were largely reversed by the addition of an excess of IGF-I to the culture medium in the presence of this antibody, while the addition of insulin or IGF-II had no effect.

In conclusion, receptors mediating insulin and IGF-I uptake appear to be present on the surface of the rat visceral yolk sac. The growth inhibition seen in the presence of the antibody also implicates that IGF-I plays a role in the normal development of post-implantation rat embryos.

ACKNOWLEDGEMENTS

Firstly, I should like to thank the supervisor of this project, Dr Margaret Pratten, for all her help.

I would also like to thank the technical staff of Leicester University Department of Anatomy for all their help and assistance throughout my project. In particular, my deepest thanks goes to Mr Timothy Jefferson and Mr Simon Byrne, without whose patience, friendship and skills this thesis would never have been completed. In addition, I would like to thank Ms Dianne Meecham for help with the photography, and Mr Chris Rigel D'Lacey for assistance on the confocal microscope. In particular, my sincerest thanks go to Mrs Judy McWilliam for all her help, support and encouragement.

I also wish to acknowledge the Departmental Heads, Dr David Pallot, and latterly Dr Amir Gulamhusein, for their support, and to thank Dr Marjorie England for proof reading, encouragement and friendship.

I would like to take this opportunity to thank the secretarial staff, both past and present, again for their friendship and efficiency, while my gratitude must also be extended to my fellow research workers, namely Mrs A. Shipman, Ms L. Sheahan, Mr N. Bright and Mr A. Hubbard for their help.

Finally, I should like to take this opportunity to thank Mr Paul Linsdell, without whose love and understanding none of this work would have been completed, or worthwhile.

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

Preface

A series of peptide factors, termed growth factors, have been implicated as important mediators of mammalian development. Throughout embryonic and fetal development, there are two possible sources from which these growth factors may be derived: the embryonic tissue itself, or alternatively, from the mother. This thesis describes work undertaken to investigate the role of two of these growth factors (insulin and the closely related insulin-like growth factor I) in rat development.

The Rat Visceral Yolk Sac

Introduction

During pregnancy, the nutritional requirements of the rat conceptus are met by two different processes: histiotrophic and haemotrophic nutrition. The embryo is sustained by histiotrophic nutrition from fertilisation, until the formation of the chorio-allantoic placenta. This process is defined as the intracellular breakdown of maternal macromolecules by the fetal membranes, from sources such as the decidua, uterine secretions and blood or its transudate. In the later stages of development, the rat's nutritional requirements are met by the chorioallantoic placenta, which develops during day 11. This process is called haemotrophic nutrition, and consists of the interchange of soluble materials between the fetal and maternal circulations across the placental barrier.

Histiotrophic nutrition occurs in all mammalian embryos, although the tissues responsible vary from species to species. In man, the syncytiotrophoblast (a layer of multinucleate cytoplasm which lies between the cell mass of the embryo and the maternal tissue) is responsible for the uptake of histiotroph from the maternal blood. In rats and other rodents however, the visceral yolk sac has been shown to be the principal site of histiotrophic nutrition. Briefly, the material is taken up by pinocytosis into the endoderm of the visceral yolk sac where it is broken down in the vacuolar system by hydrolytic enzymes. From here, the soluble digestion products pass by diffusion into the yolk sac blood circulation and thence to the embryo.

In addition to the nutritional requirements it fulfils, the rat yolk sac functions significantly up until term in the transport of other proteins essential for development. While complete pre-implantation development can occur in relatively simple nutrient media, following implantation the macromolecular requirements for embryonic growth rapidly increase. *In vitro*, the requirements following implantation also include a series of proteins referred to as "growth factors".

A brief discussion of the early development of the fetal membranes in the rat (reviewed Steven and Morriss, 1975), followed by an introduction to the fate of exogenous proteins, are included here to facilitate understanding of the events involved in the processing of proteins by the conceptus and yolk sac.

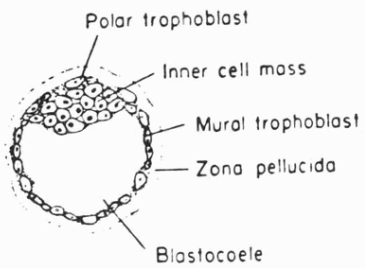

Fig. 1.1a Day 4
Pre-implantation blastocyst.

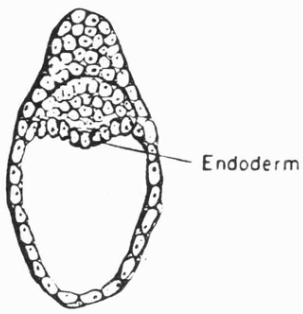

Fig. 1.1b Day 5
Implanting blastocyst.
Polar trophoblast cells proliferate and the inner cell mass differentiates into ectoderm and endoderm.

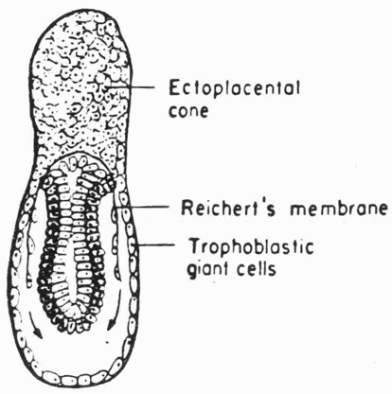

Fig. 1.1c Day 6
Inversion of the germ layers occurs.
Origin of Reichert's membrane.

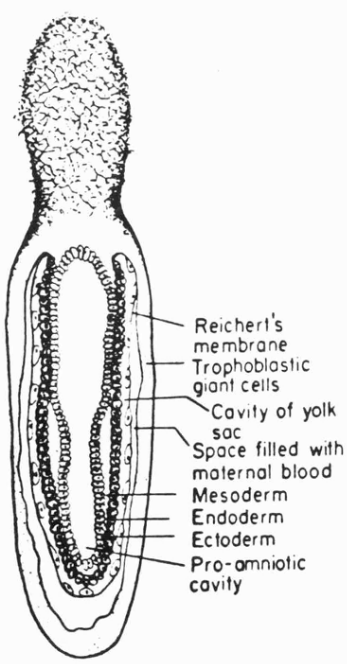

Fig 1.1d Day 6
Early appearance of mesoderm.

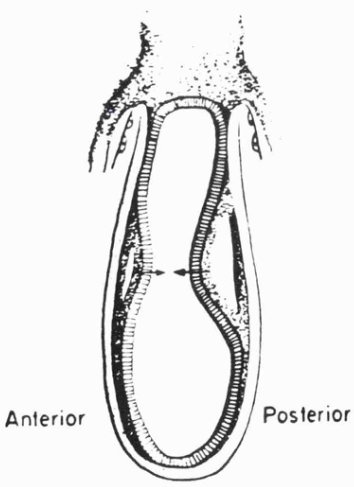

Fig. 1.1e Day 8-9
Increasing number of mesoderm cells produce cavity at posterior end of embryo.

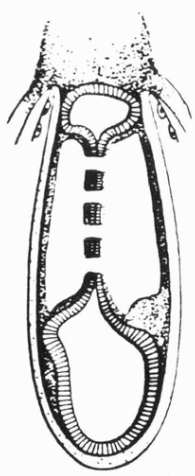

Fig 1.1f Day 8-9
Increasing cavity size pushes layer of ectoderm and endoderm into lumen of cylinder.

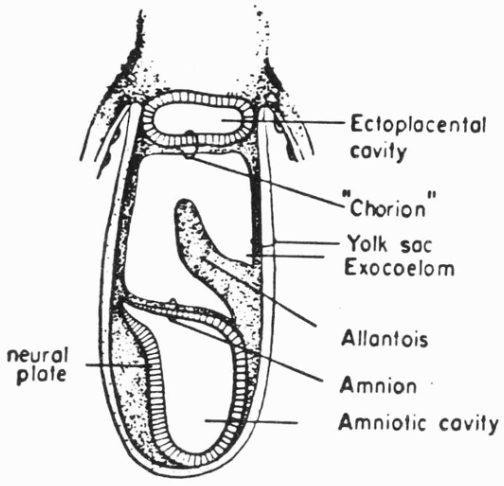

Fig. 1.1g Day 8-9
Formation of amnion and chorion.

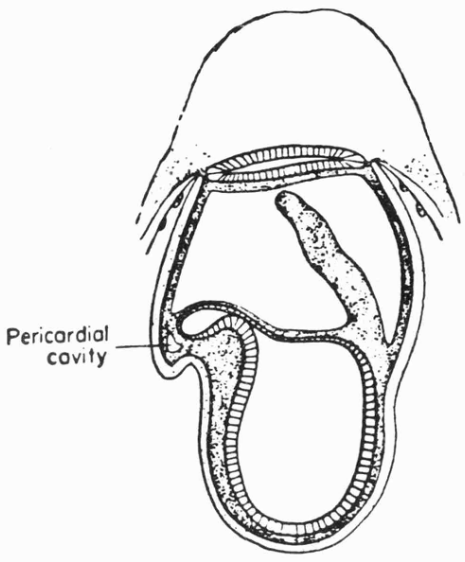

Fig 1.1h Day 10
Allantois fuses with the chorion, and ectoplacental cone disappears, forming the allantoic placenta.

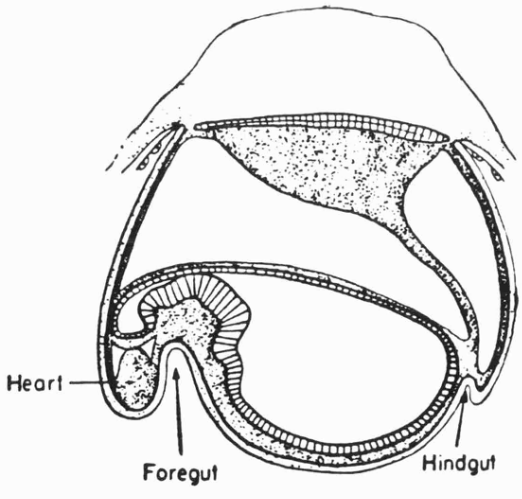

Fig 1.1i Day 10
In the embryo, fore and hind gut are produced by "reversal".

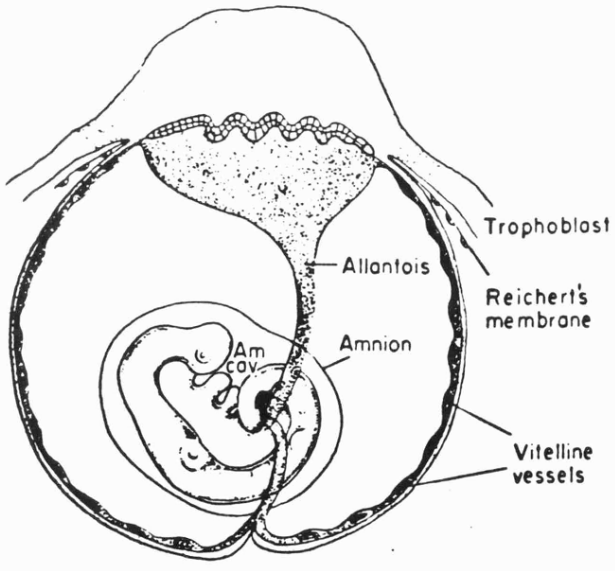

Fig 1.1j Day 11
Allantoic placenta continues to develop. Embryo has now turned and is dorsally convex.

Fig. 1.1 Development of the fetal membranes of the rat.

(Adapted from Steven and Morriss, 1975)

Development of the fetal membranes of the rat.

As the fertilised rat secondary oocyte passes through the oviduct, the earliest cleavage divisions take place, so that the embryo is at the late morula stage (12-16 cells) as it enters the uterus. A blastocyst is then formed as a fluid-filled space develops within the morula. This blastocyst consists of an outer layer of trophoblast cells within which are the inner cell mass, attached eccentrically to the polar trophoblast cells, and a central cavity, or blastocoele (Fig. 1.1a). While the trophoblast is eventually responsible for establishing and maintaining contact with the maternal tissues, it is the inner cell mass which gives rise to the embryo, amnion and allantois.

Prior to implantation, the blastocyst is surrounded by the zona pellucida, a translucent membrane which appears to prevent the outer surfaces of trophoblast cells from adhering to the epithelia lining the oviduct and uterus, and which also protects the early embryo from leucocytic attack. However, by day 4-5, this zona pellucida has been lost, allowing implantation to occur (Fig 1.1b). In the rat, the blastocyst attaches eccentrically, in a groove on the antimesometrial aspect of the uterine wall. The rodent yolk sac first appears as a bilaminar structure, consisting of trophoblast cells plus a single layer of endodermal cells (or hypoblast). These endoderm cells proliferate from the inner portion of the inner cell mass, while polar trophoblast cells divide to produce the ectoplacental cone at the embryonal pole of the elongated blastocyst. The mural trophoblast cells, however, do not divide, but their DNA content increases and they form primary trophoblastic giant cells (Fig. 1.1c). The inner cell mass continues to divide and the differentiation of cells into ectoderm and endoderm occurs at around day 5 *post coitum*. Further division of the inner cell mass results in the endoderm being forced into the blastocoele cavity where it produces an embryonic disc. The overall result of this invasion by endoderm cells into the blastocoele is an inversion of the germ layers, such that the endoderm layer covers the central ectoderm cells in which the primary embryonic cavity forms. This phenomenon of inversion, termed entypy, is a notable feature of rodent embryology, and results in early rodent development being more compact in comparison to other mammals.

On the 6th day, an extraembryonic membrane develops from an amorphous secretion along the inner wall of the trophoblast. This membrane was first described by Reichert in the guinea pig, and subsequently discovered to be present only in rodents and insectivores. Reichert's membrane is a thick basement membrane, arranged in a three dimensional network of 3-8nm thick cords, rich in collagen, which separates the trophoblastic giant cells from the endoderm cells which form the yolk sac epithelium (Inoue *et al*, 1983). At this stage of gestation, Reichert's membrane is the only structure which separates

the maternal blood, (present in capillaries in the decidual tissue and feeding into a space between the trophoblast and Reichert's membrane), from the yolk sac and embryonic endoderm (Fig. 1.1d). Because this membrane arises due to the inversion of the germ layers, this means that the chorion does not surround the embryo in rodents, as it does in other species. Reichert's membrane is present until the 18th day of gestation, when it ruptures and retracts to the margins of the placenta. Up until the 18th day, the surface area of Reichert's membrane increases dramatically, and the total protein content rises, while there is a parallel reduction in the collagen component. (Clark *et al*, 1975).

On the 8th day of gestation, the primitive streak forms and produces embryonic mesoderm, which migrates to lie between the ectoderm and endoderm. This is termed gastrulation (Fig. 1.1e). These mesoderm cells divide, so increasing in number until an annular cavity is formed around the cylinder, eventually resulting in a layer of mesoderm and ectoderm lying in the centre of the lumen of the cylinder. This layer of cells fuses before breaking down at around 9.5 days to produce two horizontal membranes (Fig 1.1f). These membranes are the amnion, which lies immediately above the embryo, separating the amniotic cavity from the exocoelom, and the chorion, which lies beneath the ectoplacental cone and separates the ectoplacental cavity from the exocoelom (Fig. 1.1g). Shortly after this (approximately day 10), the ectoplacental cavity disappears, so the number of embryonic cavities is reduced from three to two.

At around this time also, the allantois develops from extraembryonic mesoderm at the posterior end of the embryo. This grows across the exocoelom to fuse with the chorionic ectoderm (Figs. 1.1h; 1.1i). Blood vessels now develop in the allantois and invade the chorionic ectoderm, so that now only a thin layer of chorion separates the vessels from the maternal blood. This is the beginning of the chorio-allantoic placenta (Fig. 1.1j).

The Fate of Extracellular Proteins

Macromolecules present in the extracellular environment of embryonic tissue, or any other cell type, can be subjected to a number of fates. These can be considered in terms of extracellular and intracellular processing mechanisms.

i) Extracellular Processing

Extracellular molecules may initially be attacked by a variety of hydrolytic enzymes present either free in the extracellular fluid, or alternately bound to the surface of the cell. Such ectoenzymes, as they are termed, are associated with the plasma membrane and are arranged with the active domain

exposed at the extracellular surface (Kenny, 1989). Cell surface proteases are widely distributed throughout mammalian cell types, though they are thought to be particularly important at the brush border membranes of the intestine where they are associated with the final stages of digestion. In other locations, however, it may be possible that the extracellular digestion of protein plays an important role in many protein mediated responses by inactivating protein signals.

ii) Intracellular Processing

A large number of molecules are removed from the extracellular environment by internalisation into neighbouring cells, where they are subsequently processed. The most common mechanism for uptake of molecules too large to diffuse across the plasma membrane is pinocytosis, a universal process found in all eukaryotic cells (reviewed Pratten *et al*, 1980). During pinocytosis, small volumes of extracellular fluid are internalised due to the invagination of an area of the cell membrane. Molecules may either be taken up non-selectively, as molecules present in solution will be taken up via fluid-phase pinocytosis, while those attached non-specifically to the cell surface will enter via adsorptive pinocytosis when the plasma membrane invaginates. Molecules may also be taken into the cell in a more specific manner via binding to specific cell surface receptors (receptor mediated pinocytosis). A wide variety of essential molecules are taken up via receptor-mediated pinocytosis, such as the nutrients low density lipoprotein (LDL) (reviewed Goldstein *et al*, 1985) and transferrin, certain growth factors and hormones, in addition to foreign viruses and antigens (reviewed Smythe and Warren, 1991). In many cases, for example EGF, once the ligand has bound to a specific cell surface receptor, the complex formed then becomes clustered in specialized areas of the cell membrane, termed coated pits, prior to invagination of the plasma membrane. In other cases, for example transferrin and LDL, the receptors are already clustered in coated pits and are internalised irrespective of whether they are associated with ligand (Trowbridge, 1991). The principal component of this coat is the protein clathrin, which forms a lattice around the ligand-receptor complex, so that after invagination, a coated vesicle is produced. This coat is then removed before the receptor-ligand complex fuses with a population of organelles termed primary endosomes.

Attempts to quantify pinocytosis have included morphological studies of the number of vacuoles within a cell as visualised by light or electron microscopy, though interpretation of this type of data is far from easy due to

difficulties in precisely identifying the membrane-bounded vesicles when seen in section. A much more efficient technique for quantifying pinocytosis involves the detection of a macromolecule after it has been taken up by a cell. This principle may involve chemical detection or the use of a radioactive or fluorescently labelled marker. Non-biodegradable markers such as [^{198}Au] colloidal gold and [^{125}I] PVP (polyvinylpyrrolidone) have proved useful indices of pinocytosis as cellular accumulation is proportional to their rate of capture, provided that their rate of exocytosis is comparatively low. It is important when measuring uptake to be sure that it is due to pinocytosis rather than mere binding to the cell surface, and certain criteria have been laid down to enable the two processes to be separated. Essentially, pinocytosis is inhibited at low temperature (Bowers, 1977) or in the presence of metabolic inhibitors, (Duncan and Lloyd, 1978) and also results in a progressive accumulation of substrate over a long time course. In contrast, binding to the surface of a cell is a rapidly saturable process, resulting in a plateau of substrate uptake with time. It will consequently be unaffected by lowering the temperature or the addition of metabolic inhibitors because it is not an active, energy consuming process.

The fate of material internalised by pinocytosis is variable. Essentially, material may either by transported intact, or undergo intracellular digestion.

i) Transport

In polarised cells such as epithelia, certain receptors and their ligands are directed from endosomes to the surface of the cell opposite that at which they entered. This process is termed transcytosis (reviewed Mostov and Simister 1985), and the best documented examples are the transport of intact immunoglobulins which results in the transfer of immunity from mother to young. Immunoglobulin G (IgG) present in the mother's milk of a variety of species binds to IgG receptors present on the surface of cells in the neonatal proximal small intestine, where the pH is 6. Following uptake via pinocytosis and subsequent transfer to endosomes, the ligand-receptor complex is transported intact across the cell where the IgG is released into the blood, where the pH is approximately 7.5. It is thought that the IgG receptor then recycles back the surface of the intestine to bind IgG once more.

ii) Lysosomal Digestion

The remainder of molecules channelled into the cell undergo intracellular digestion of some sort. In some cases (eg asiasoglycoprotein),

ligands dissociate from the receptor within the endosome from which the ligand then passes through a series of endosome fusions leading ultimately to fusion with lysosomes. The receptor is then recycled back to the cell membrane (reviewed Rodman *et al*, 1990). In other cases, eg EGF, both the ligand and the receptor are channelled to the lysosomes for digestion. In either case, the final result is the production of low molecular weight products within the lysosomes. These organelles are a morphologically heterogeneous system of organelles containing a variety of hydrolytic enzymes (reviewed de Duve, 1983). Lysosomes are principally characterised by their acidic pH, maintained by an ATPase associated proton pump. The action of these enzymes results in breakdown products which pass out of the lysosome and which may subsequently be used for cellular metabolic processes, while any substance which cannot be further broken down remains within the lysosome as a residual body.

iii) Non-lysosomal Digestion

Any substances taken into the cell and not channelled to the lysosomes must somehow be digested via a non-lysosomal pathway. One possibility is that certain enzymes are not active at the acidic pH of the lysosomes, so that a major site of digestion is within the less acidic endosomes. This route has certainly been proposed to be responsible for a proportion of insulin degradation in rat hepatocytes (Hamel *et al*, 1988) and cultured simian kidney cells, where an insulin-degrading enzyme (IDE) is expressed and is probably present within endosomes (Kuo *et al*, 1991). Another possible route of non-lysosomal digestion is via enzymes present within the cytosol, and despite the obvious lack of a mechanism by which cytosolic enzymes could reach ligand present within endosomes, this route cannot be excluded.

Endocytic Index

Whether uptake of material is results in lysosomal or non-lysosomal degradation, the production of digestion products is an added complication when it comes to calculating the pinocytic rate. In such cases, it must be remembered that the tissue level of the substance and the release of digestion products must be summed to get the true rate. In the past, accurate comparisons between pinocytic rates as reported in the literature were impossible to make due to the use of non-comparable units. This fact led eventually to the development of the Endocytic Index as a concept. Proposed by Williams *et al* (1975a), the Endocytic Index is defined as the volume of

culture medium (in μl) whose contained substrate is captured per milligram of cell protein per hour. Thus, using this index allows direct comparison of uptake rates between different substrates and different cell types.

Considerable work has been carried out on the rat yolk sac to investigate pinocytosis. Initially, [^{125}I]PVP was used as a marker, and an Endocytic Index of 1.71μl/mg protein/hour calculated (Williams *et al*, 1975b). In contrast, [^{125}I]dBSA (denatured bovine serum albumin) has a much higher rate, between 11.06 and 80.85μl/mg protein/hour, the high variability between batches being due to the protein being denatured to differing extents (Livesey and Williams, 1982). It was concluded that [^{125}I]PVP enters via fluid-phase pinocytosis, while [^{125}I]dBSA enters by adsorption to the cell membrane. It is generally assumed that an Endocytic Index of approximately 2.0μl/mg protein/hour is that due to fluid-phase uptake alone, thus any larger figure than this implies that some form of binding is taking place.

The role of the yolk sac

The rat visceral yolk sac is an actively pinocytosing tissue (reviewed Lloyd, 1990). Work carried out by Williams *et al* (1971) using yolk sacs removed from 17.5 day pregnant rats which had previously been injected with [^{125}I]BSA has demonstrated that the rat visceral yolk sac is capable of endocytosis and the subsequent breakdown of materials. Analysis of the culture medium revealed that labelled protein was undergoing digestion to produce amino acids, such as [^{125}I]iodotyrosine (Livesey and Williams, 1979). This is the principal digestion product, and cannot be subsequently reincorporated into protein. The results of differential centrifugation also carried out identified [^{125}I]BSA in the lysosomal fraction, pointing to this as the site of digestion of exogenous proteins. Freeman and Lloyd (1980) demonstrated uptake of [^{125}I]PVP by the rat yolk sac, with no transfer of radioactivity to the developing embryo. PVP cannot be digested by lysosomal enzymes, so no transfer of lysosomal breakdown products to the embryo could occur.

Further to this, it was possible to demonstrate *de novo* synthesis from amino acids released from lysosomes following pinocytosis (Freeman *et al*, 1981; Freeman and Lloyd, 1981). Here, [^{3}H]labelled rat serum proteins were included in the culture medium, which were degraded into amino acids including [^{3}H]leucine, detectable in both the yolk sac and the embryo, in the form of newly synthesised protein.

In addition to its nutritional role, the yolk sac is also involved in the passive immunity conferred on the fetus by the transport of immunoglobulins from the maternal to fetal circulation. This process requires that the immunoglobulins be transferred without degradation. Brambell (1966)

proposed that this occurred by the adsorption of immunoglobulins to a number of highly specific receptors on the inner face of pinocytic vesicles so that the proteins are protected from proteolytic attack when the vesicles fuse with lysosomes. It now seems likely that the proteins are selectively protected by receptor-mediated endocytosis on the surface of the cell, into coated vesicles (Wild, 1975). Indeed, Huxham and Beck (1981) have verified that IgG transport across the endoderm of 11.5 day rat visceral yolk sacs cultured *in vitro* occurs in this fashion, while other work has demonstrated breakdown of transported IgG by yolk sac lysosomes (Weisbecker *et al*, 1983).

The development of rat embryo culture techniques

The role of the yolk sac as a mediator of protein transport extends across the period of organogenesis. At this stage the embryo is highly susceptible to insult from teratogenic agents. Earlier in gestation, exposure to potent teratogens may result in the death of the embryo, but during organogenesis it may result in the production of major malformations.

This period is therefore of great interest, and over the past twenty years techniques have been developed which allow the embryo and its surrounding yolk sac to be removed from the mother and successfully grown in a defined culture medium. While teratogens may be added to the medium and any adverse effects observed, the system also has many advantages in the study of normal embryonic development and may be used to investigate the roles of nutritional factors, vitamins and growth factors.

A technique was originally devised for culturing rat embryos from the ninth day of gestation in homologous sera (New, 1966), in which embryos are explanted from the mother, the decidua removed, and the Reichert's membrane torn open. This work later led to the more successful technique of rat whole embryo culture developed by New *et al*, (1973), in which embryos are placed in glass bottles containing a serum-based culture medium and continually rotated at 37°C. Continuous improvements to this system resulted in a highly successful technique, reflected in the observation that over 90% of embryos explanted at 9.5 days gestation develop a blood circulation and continue growth and differentiation over 48 hours, so that they are indistinguishable from 11.5 day embryos *in vivo* (New *et al* 1976a; b). Thus, possible teratogens may be examined for insult to the embryo (New, 1976). The *in vitro* development of the yolk sac and embryo was found to resemble that seen *in vivo* most closely when embryos were cultured in rat serum diluted 50:50 with some medium, such as Hank's or Waymouth's, and in the presence of a high oxygen concentration (Ellington and New, 1980). Embryos can also be successfully explanted earlier in development, from 7.5 or 8.5 days (Buckley *et*

al, 1978). Depending upon when the embryos are removed, development can be supported up to the stage of digit formation on the limbs (the 15th day in the rat), although embryos which are removed from the mother after the formation of the chorioallantoic placenta do show a reduced rate of growth in culture as there can be no flow of culture medium equivalent to that of the maternal blood circulation in the placenta (Cockroft, 1976). Embryos explanted on day 11 and cultured for 24 hours have been reported to compare well with their *in vivo* counterparts in terms of morphological criteria, though there is some suggestion of a reduction in yolk sac function (Andrews *et al*, 1992), which may play a role in the reduced rate of development seen in culture.

Culture of rat yolk sacs

The development of *in vitro* culture techniques for the rat visceral yolk sac has allowed an in depth study of its function. Sorokin and Padykula (1960) reported that fragments of yolk sac explanted on the 13th day of gestation grew on solid media for up to 14 days, and maintained a morphology similar to those *in vivo*. Such explanted fragments actively pinocytose substances, and began to accumulate glycogen on or around day 15, as occurs *in vivo*. However, this accumulation was slower in the cultured yolk sacs, and morphological differences were also recorded with regard to their microvilli. *In vivo*, microvilli are at their largest between days 13-15, after which they become less conspicuous. However, *in vitro*, microvilli retain their height throughout culture, suggesting that the endodermal cells are highly pinocytic.

As previously discussed, Williams *et al* (1975a;b), developed a technique in which rat yolk sacs explanted from the mother at 17.5 days gestation can be cultured in shaker flasks for up to 12 hours. This system was used to investigate the uptake of [^{125}I]-labelled BSA and PVP, demonstrating differential rates of uptake by pinocytosis.

In both these culture systems, however, the yolk sac is open and there can be no distinction between the fluid compartments in and outside of the membrane. One system which has overcome this disadvantage arose as a modification of the rat whole embryo technique developed by New *et al* (1973). In this, Al-Alousi extended the culture system by up to 14 days (Al-Alousi, 1983), so that the embryo dies and becomes partly autolysed. However, the yolk sac continues to grow intact, and Al-Alousi suggested that the morphology was similar to those of *in vivo* yolk sacs of the same age.

This technique was further modified by Dunton (Dunton *et al*, 1986), by removing the embryonic pole from the egg cylinder when it is explanted at 9.5 days gestation. The yolk sac now develops to the same extent as before, but now without the embryo. The principal advantages of this system are that it is

possible to examine the fluid within the yolk sac (the exocoelomic fluid) and so investigate uptake and transport, while uptake is not complicated by embryonic metabolism (Pratten *et al*, 1987). One of the first proteins examined using this system was bovine serum albumin, a non-specific protein, found to be taken up by pinocytosis of the yolk sac in a fashion similar to that seen in *in vivo* yolk sacs of the same age (Dunton *et al*, 1988).

Growth Factors

Introduction

As mentioned earlier, the requirements for embryonic growth include not only adequate nutrition, but also a series of less clearly defined substances, termed growth factors. Growth factors are peptide molecules which are mitogenic, and whose presence has been shown in serum (Gospodarowicz and Moran, 1976).

Serum contains many factors which are essential for the proliferation of animal cells in culture, (Smith 1981; Gospodarowicz and Moran, 1976). These include amino acids, lipids, vitamins, various trace elements, mediators of cell attachment such as fibronectin, and molecules responsible for the transport of low molecular weight nutrients, such as the iron carrier transferrin. In addition, it also contains growth factors whose effects are not so clearly defined, though whose importance is not questioned. Such factors were defined by Gospodarowicz and Moran (1976) as "growth-stimulating substances that are not nutrients". They went on to define nutrients as "substances that are used within the cell as metabolic substrates or cofactors". Smith (1981) considered that the most important role of serum is to provide such growth factors and hormones. This view is supported up to a point by the ability of defined serum free medium to grow an established rat pituitary cell line (Hayashi and Sato, 1976). This medium contained physiological concentrations of four hormones - triiodothyronine (T3), thyrotrophin-releasing hormone, the biologically active peptide of parathyroid hormone, and a partially purified somatomedin preparation from serum - plus transferrin.

The growth of cells in culture could be limited by a lack of any of the required nutritional or non-nutritional factors mentioned above. In order to study growth factors it is, therefore, vital that all the nutritional requirements of the system being studied are satisfied, leaving the growth factor under investigation as the sole limiting factor. Such an essential scientific requirement may appear obvious, but is, in fact, very difficult to achieve in practice. One commonly used technique is serum starvation, when the medium is saturated with known nutrients, but contains a growth-limiting concentration of serum.

However, this may result in the limitation of several growth factors plus other necessary nutrients or unknown factors not yet clearly defined. For example, in a serum-starved system, other factors separate from, but essential for, growth factor function, may appear to possess some mitogenic activity because they allow the cells to respond to growth factors already present in the serum. Conversely, a growth factor present may appear to possess no activity because the cells lack the factors essential for the growth factor to produce its effect.

Another approach used to produce a medium in which only growth factors are limiting, is to use plasma, as this has been reported to have little growth-promoting activity in comparison to serum, though it contains similar levels of nutrients and other required substances (Balk, 1971). Cells may be kept alive in plasma supplemented with serum or by the addition of growth factors. To study growth factors individually, they must first be isolated. Growth factors having a physiological significance will tend to be active at very low concentrations, (Gospodarowicz and Moran, 1976). For example, epidermal growth factor is mitogenic for a variety of fibroblast cells at around 4ng/ml (Smith, 1981), which has meant that the isolation of growth factors from serum has proved very difficult. Instead, such factors are more readily isolated from tissues or cultured cells which express them; for example, nerve growth factor (NGF) and epidermal growth factor, (EGF) have both been isolated from the male mouse submaxillary gland (Cohen, 1960: Cohen, 1962 respectively), while a basic fibroblast growth factor (FGF) has been isolated from bovine pituitary and brain (Gospodarowicz, 1975).

To demonstrate the physiological significance of any particular growth factor, a mitogenic response must be recorded *in vivo*. This could be achieved in a fashion similar to that used to test putative hormones, ie. the source of the hormone is removed, and deficiency symptoms observed, which are then relieved by the administration of exogenous hormone. This approach has proved much more difficult in the case of growth factors as several organs may secrete or store the substance, and so these factors can only be evaluated *in vitro*. Gospodarowicz and Moran (1976) have suggested the following hierarchy of criteria to be considered when evaluating a putative growth factor: Does the substance:

i) induce the initiation of DNA synthesis?

ii) induce one cycle of division in confluent cells?

iii) induce several division cycles in sparse as well as confluent cultures?

iv) induce clonal growth (from a single cell to a monolayer)?

Growth factor superfamilies

The recent advances in molecular biology techniques have resulted in the determination of the amino acid and nucleotide sequences of many growth factors. Based on the homologies revealed, it now appears that growth factors may be grouped into various superfamilies. Although the number of known growth factors is constantly expanding, the main superfamilies and their most important members are characterised in Table 1.1 (reviewed in Heldin and Westermark, 1984; Mercola and Stiles, 1988).

In addition to the growth factors mentioned in the table, which are all mitogenic, nerve growth factor (NGF) was also isolated by Cohen and Levi-Montalcini (1956). Although termed a growth factor, NGF is not mitogenic *in vivo* or *in vitro*; instead it appears to play a role in maintaining the survival of sympathetic and some embryonic sensory neurons, as well as having a possible role as a chemotactic agent for axon growth.

Mechanism of growth factor action

Although the various growth factors act on a wide variety of cells, their roles can generally be described as the stimulation of cellular mitogenesis and the regulation of differentiation and morphogenesis. Growth factors, like hormones, appear to regulate gene expression within their target cells, and the initial step in this process is the binding of the growth factor to a specific cell surface receptor. Many growth factor receptors, though not all, possess an intrinsic ligand-sensitive protein tyrosine kinase activity (reviewed Yarden and Ullrich, 1988). Following binding of the ligand to the extracellular portion of the receptor, the intracellular receptor domain, which possesses the tyrosine kinase activity, undergoes phosphorylation. The receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin and insulin-like growth factor I (IGF-I) are known to possess intrinsic tyrosine kinase activity, while the IGF-II receptor has no such activity and the TGFβ and the HBGF receptors have not yet been characterised definitively.

Upon ligand binding to a tyrosine kinase-associated receptor, various cell surface events are initiated. These include the stimulation of pinocytosis and membrane-ruffling, along with other cytoskeletal and morphological changes, plus the stimulation of glucose and ion transport mechanisms.

GROWTH FACTOR FAMILIES

PLATELET DERIVED GROWTH FACTOR
PDGF-A
PDGF-B (sis product)
PDGF-AB
OSTEOSARCOMA-DERIVED GROWTH FACTOR
GLIOMA-DERIVED GROWTH FACTOR
TRANSFORMING FACTOR OF SIMIAN SARCOMA VIRUS (SSV)
FIBROBLAST-DERIVED GROWTH FACTOR (FDGF)

EPIDERMAL GROWTH FACTOR
EGF (urogastrone)
TRANSFORMING GROWTH FACTOR (TGFα)
VACCINIA GROWTH FACTOR (VGF)

INSULIN
INSULIN
INSULIN-LIKE GROWTH FACTOR I (Somatomedin C)
INSULIN-LIKE GROWTH FACTOR II
RELAXIN

TRANSFORMING GROWTH FACTOR ß
TGF-β1
TGF-β2
TGF-β1.2
INHIBIN A
ACTIVIN-A
ACTIVIN-AB
MULLERIAN INHIBITING SUBSTANCE

HEPARIN-BINDING GROWTH FACTORS
ACIDIC HBGF (acidic fibroblast growth factor)
BASIC HBGF (basic fibroblast growth factor)
PRODUCTS OF THE int-2, hst and KAPOSI'S SARCOMA PROTO-ONCOGENES

TABLE 1.
Growth Factor Superfamilies and their common members.

Intracellular changes also occur arising from the production of soluble intracellular second messengers. These include changes in glycolysis, polyamine synthesis and ribosomal S6 phosphorylation (reviewed Rozengurt, 1986). The second messenger involved may be a phosphoprotein, inositol phosphate, cyclic nucleotide, diacylglycerol, or a monovalent or divalent ion (reviewed Rozengurt, 1986). In the case of insulin, a transient activation of phospholipase C, together with a sustained increase in inositol phospholipid production, has been recorded in the rat epididymal fat pad (Farese *et al*, 1986), and in a cultured mouse myocyte cell line (Saltiel *et al*, 1986). It seems that stimulation of the insulin receptor results in the production of a novel glycosylated inositol derivative from the hydrolysis of a membrane linked phosphoinositol (Saltiel, 1990; Taylor, 1991). In some cases, changes in gene expression may be detectable within minutes of the formation of a growth factor-receptor complex. Later, increased macromolecular synthesis of protein, RNA and DNA may be observed 3-20 hours after ligand binding. In the case of insulin, stimulation of ion and hexose transport are also seen within minutes of the formation of the ligand-receptor complex, together with internalization of the receptor. Several hours after the initial insulin stimulus, increased protein, lipid and nucleic acid synthesis continues to occur, leading to cell growth (Rosen, 1987).

The various tyrosine-kinase associated receptors display certain differences in their mechanism of action. For example, some require the continuous presence of a ligand (for up to 8 hours) to produce a response in target cells (eg. the insulin and EGF receptors), while others need only a 30 minute exposure to induce DNA synthesis (eg. the PDGF receptor). PDGF differs from other growth factors in several other ways; unlike other mitogens, it can stimulate DNA synthesis and cell division in the absence of any other factors in NIH-3T3 fibroblasts (Rozengurt, 1986). It is also able to stimulate protein kinase C via an increased turnover of phosphotidylinositol and protein kinase A due to production of prostaglandin E (Shier and Durkin, 1982). Such activity is not seen with any other class of growth factor, and it may be a reflection of the PDGF receptor, which does not possess the cysteine-rich regions seen in other growth factor receptors. This may represent a tyrosine kinase receptor-associated subclass, which acts in a markedly different fashion to other growth factor receptors.

The roles of growth factors during development

Growth factors appear to be important mediators of cell-cell interactions. Being diffusible, they may be expected to affect the behaviour of many cells, and probably of more than one cell type. Although much remains unclear

about their precise roles in early development, due in particular to the difficulties associated with working with pre-implantation and early post-implantation embryos, their actions in controlling cell proliferation, and regulating differentiation are beginning to emerge, and they are a subject of increasing interest to developmental biologists. The major known effects of the growth factors families are briefly described below:

Heparin-binding Growth Factors (HBGF's): The family of HBGF's appear to play a role in early mesoderm development. Embryonal carcinoma-derived growth factors (ECDGF's) are HBGF-like molecules produced by mouse embryonal carcinoma (EC) cells, (Heath and Isacke, 1984), and which are mitogenic for mesoderm-like, but not ectoderm- or endoderm-like, derivatives of the EC cell line PC13. Slack and coworkers (1987) have also been able to demonstrate ECDGF and basic fibroblast growth factor (FGF) inducing mesoderm differentiation from animal pole ectoderm in Xenopus blastocysts. It also seems that HBGFs could be important inducers of angiogenesis during development, as a basic FGF-like factor has been discovered controlling angiogenesis in chicks (Risau, 1986). This suggests a possible role for this family as controllers of angiogenesis in tumours.

Platelet-derived Growth Factors (PDGF): PDGF is a potent mitogen for connective tissue cells, and is mitogenic for fibroblasts. PDGF activity can be detected in conditioned media from undifferentiated EC cell lines (Rizzino and Bowen-Pope, 1985), suggesting a role for the factor during early embryogenesis. Differentiation induced by retinoic acid reduces PDGF activity. The PDGF secreted from platelets is not identical to that secreted by EC lines, but instead is thought to play a role in wound healing when released at the site of an injury (reviewed Ross *et al*, 1986).

Epidermal Growth Factor (EGF): EGF, or a closely related factor, is detectable by the 8th day of gestation in chicken embryos, the 11th day in mouse embryos, and the 19th day in rat embryos (Rizzino, 1987). All members of the EGF family compete with EGF from the salivary gland, and bind to a tyrosine kinase transmembrane receptor, which is the product of the c-*erb* B proto-oncogene (Downward *et al*, 1984). EGF appears to play a role in the development of the lung and secondary palate, and in fact, the bioassay originally used by Cohen to monitor the purity of EGF involved its ability to trigger premature eyelid opening and incisor eruption in neonatal mice (Cohen, 1962). Analysis of EC lines has suggested that TGF-α is actually the primary component of fetal EGF, with TGF-α able to competitively inhibit EGF binding to membrane receptors

(Anzano *et al*, 1983). Indeed, partially purified TGF binds to two distinct cell surface components (Massague *et al*, 1982), one of which is believed to be the EGF receptor. Functional EGF/TGF-α receptors are detectable in the mouse embryo from the 11th day of gestation, so it may be that these EGF receptors are present in the embryo as stem cells differentiate.

Transforming Growth Factor-β (TGF-β): TGF-β, which does not bind to EGF/TGF-α receptors, may be able either to promote or inhibit cell differentiation or division. TGF-β itself is non-mitogenic, and was originally identified by its ability to induce anchorage-independent growth of NRK cells (rat fibroblast cells) in the presence of EGF or TGF-α. TGF-β induces chrondrogenesis and squamous differentiation of branchial epithelial cells, and also inhibits myogenesis and adipogenesis (reviewed Sporn *et al*, 1986). Its activity has been detected in the human placenta, and in mid to late gestation mouse and rat embryos. Another member of the family, Mullerian inhibiting substance (MIS), is produced by the developing testis, and induces the regression of the Mullerian duct.

TGF-β receptors have been detected on almost all cell types, although its precise role in early mammalian development is far from clear.

Insulin as a growth factor

The remaining growth factor family, and the one with which this thesis is concerned, is the insulin family, which comprises insulin itself, the insulin-like growth factors I and II, and relaxin. The majority of work carried out on insulin has concentrated on its role as a regulator of rapid anabolic responses. *In vivo*, its actions include the regulation of glycogen synthesis in the liver, fat synthesis in adipocytes, and the stimulation of amino acid and ion uptake in muscle (reviewed Denton *et al*, 1981; Kahn, 1985). More recently however, work has shown that insulin is a mitogen for most cell types in culture, and it appears that it has an important role to play as a growth factor and in embryonic and fetal development (reviewed Hill and Milner, 1985).

Insulin has been studied extensively since its discovery by Banting and Best in 1922, frequently being regarded as a model for the study of peptide hormones. In 1960, insulin became the first protein to be completely sequenced (Sanger, 1960), while twenty years later, human insulin became the first recombinant protein made available for clinical use (Goeddel *et al*, 1979). The enormous amount of work which has been carried out on insulin, as revealed by these milestones in biological chemistry, reflects the importance of insulin in human biology and medicine. While a total lack of insulin is incompatible with life, the best known disorder of insulin secretion, diabetes mellitis, is extremely

common, affecting between 1-2% of many populations, a figure which approaches 5% in the United States of America.

The role of insulin in fetal growth has also been extensively examined (reviewed Brinsmead *et al*, 1981), with evidence for an important role in development being most strongly suggested by the pathological conditions which arise as a result of an excess or deficiency of insulin in the fetal circulation. The best known example of this are the obese, overgrown infants born to diabetic mothers. This is most likely due to fetal hyperinsulinaemia as a result of fetal hyperglycaemia, which arises as a result of maternal hyperglycaemia. Conversely, virtual absence of insulin from the fetal circulation (hypoinsulinaemia) is commonly associated with severe fetal growth retardation, often typified by a decrease in muscle mass and underdevelopment of fatty tissues. Diabetic pregnancy is also associated with an increase in congenital malformations in offspring, estimated to be approximately four times that seen in the general population (Beard and Lowy, 1982).

Insulin acts as a mitogen for many different types of cells *in vitro* and is a necessary supplement to the media to achieve maximal growth of many more. Indeed, in the case of connective tissue cell types, supraphysiological (ie. greater than 1μM) concentrations are required for optimal replication. It seems likely that this is due to insulin acting via the type-1 IGF (IGF-I) receptor, as blocking the insulin receptor with anti-insulin receptor antibodies does not block the insulin-dependent uptake of [^{3}H]thymidine (King *et al*, 1980). Binding experiments using chick embryo fibroblasts have shown that both insulin and IGFs stimulate [^{3}H]thymidine incorporation when applied separately. When a concentration of each hormone which induced maximal incorporation was added together, their effects were non-additive, suggesting that insulin competes with IGFs for binding sites (Rechler *et al*, 1976).

At much lower insulin concentrations (less than 5nM), insulin acts as a mitogen for another series of cells, including rat hepatocytes and F9 embryonal carcinoma cells. F9 EC cells lack the IGF-I receptor (Nagarajan and Anderson, 1982), so it is likely that insulin is here acting via its own receptor.

Insulin is also believed to play an important role in early embryogenesis (Heyner *et al*, 1989; de Pablo *et al*, 1990). Insulin has been shown to stimulate meiotic maturation of Xenopus oocytes (El-Etr *et al*, 1979). It has also been found in the chick embryo before the appearance of the first pancreatic bud (de Pablo *et al*, 1982), although the source of synthesis of this insulin is unclear, and it may be derived from material stored in the fertilized egg rather than synthesised by the embryo. Its importance is also revealed by the further work of de Pablo *et al* (1985), who demonstrated that blocking the actions of insulin

with an antibody retards chick embryo development, while antibodies to EGF and NGF have no effect.

Insulin, and IGF-I, have been detected in mouse embryos from the eighth day of gestation (Spaventi *et al*, 1990) and both stimulate the proliferation of embryonic cells. Again, insulin is here performing a role prior to the formation of the embryonic pancreas, suggesting some paracrine or autocrine mode of action. Low insulin levels have also been reported to result in the retardation of growth and development of rat embryos explanted at 9.5 days and subsequently cultured (Travers *et al*, 1989). The addition of insulin at low physiological concentrations improved growth. However, an increase in both growth and development required higher physiological insulin concentrations (Travers *et al*, 1992).

Pancreatic insulin is detectable from approximately day 15 of gestation in the rat, after which it increases by one hundred fold to approach adult levels by birth (Kakita *et al*, 1983). In addition, the yolk sac demonstrates high levels of a fetal insulin mRNA during late gestation (Rau *et al*, 1989). This insulin represents a considerable amount of the total fetal insulin stores present. Insulin-specific binding sites have been reported to be present in rat conceptuses and their surrounding membranes by 10.4 days of gestation (Unterman *et al*, 1986). In humans, insulin is present in the pancreatic β cells of the human fetus from the 10th week of gestation, although its release is insensitive to glucose until the 28th week.

The mechanisms by which insulin could be acting as a growth factor can be summarised as follows:

i) alteration of cellular nutrition, increasing nutrient uptake and utilisation
ii) direct anabolic action via either the insulin or IGF-I receptor
iii) modulation of IGF (or other growth factor) release from fetal tissues.

The Insulin-like growth factors

Closely related to insulin, both in terms of structure and function, are the insulin-like growth factors (reviewed Humbel, 1990).

Discovery of the insulin-like growth factors

In 1957, Salmon and Daughaday observed that normal rat serum stimulated a 200% increase in the uptake of ^{35}S-sulphate into cartilage *in vitro*, while serum taken from hypophysectomised rats was essentially inactive (Salmon and Daughaday, 1957). It had earlier been established that hypophysectomy in the rat resulted in a cessation of growth, while the subsequent administration of pituitary extracts produced normal or supranormal growth. The simple addition of growth hormone (GH) alone to

the cartilage incubation medium had no effect on ^{35}S-sulphate incorporation, leading Salmon and Daughaday to conclude that some factor or factors must be present in serum which act as intermediaries for pituitary produced growth hormone, and which they termed sulphation factors (reviewed Daughaday, 1989), later renamed somatomedins.

This somatomedin hypothesis, as it became known, received support when it was found that serum exerts insulin-like effects on insulin target tissues, such as muscle and adipose tissue, and importantly, that only a small portion of this activity could be suppressed using antibodies to insulin (Froesch *et al*, 1963). Clearly, factors other than insulin were present in serum which were responsible for this "non-suppressible insulin-like activity" (NSILA). The purification of these factors led to the identification of two peptides present in human plasma which displayed around 50% sequence homology with proinsulin. These peptides were termed IGF-I and IGF-II, with IGF-I being identical to Somatomedin C.

One further observation came in 1972, when Pierson and Temin purified a factor from calf serum with multiplication-stimulating activity (MSA). This factor stimulated DNA synthesis, mitosis and growth in chicken embryo fibroblasts (Pierson and Temin, 1972). It was found to be heat-stable, estimated by gel filtration to have a molecular weight between 4000-5000 Dalton, and was not suppressed by anti-insulin antisera. From this work, Pierson and Temin concluded that this MSA might result from the same molecule responsible for the NSILA seen in serum. Marquardt and coworkers later purified a MSA secreted by a cultured buffalo rat liver cell line (BRL-3A) (Marquardt and Todaro, 1981), identified by them, and others (Rechler *et al*, 1981) to be homologous with human IGF-II.

Thus, three very different lines of work led to the eventual identification and classification of the IGFs.

Originally it was thought that GH stimulated the liver to produce IGF-I, which then mediated its effects on the IGF-I target tissues; ie. IGF-I was acting in an endocrine fashion (Salmon and Daughaday, 1957). While IGFs are continuously synthesised and released from the liver (Schwander *et al*, 1983), IGF-I is now known to be produced by several extra-hepatic sites (D'Ercole *et al*, 1984), and it is now thought more likely that GH stimulates IGF-I production in these various tissues which then act on local cells (reviewed Holly and Wass, 1989).

The Role of IGFs in Development

Midgestation human and rodent tissues express insulin-like growth factors (or IGFs) (reviewed Sara and Hall, 1984; Han *et al*, 1987). For example,

they are released by mouse embryonic explants from the 11th day of gestation, while receptors for both IGF-I and -II are present in mouse embryos from embryonic day 9 (E9) (Smith *et al*, 1987). In the rat, IGF-I is present in term fetal rat serum, (Daughaday *et al*, 1982) with low levels of IGF-I reflecting intrauterine growth retardation (Bernstein *et al*, 1991). Generally, however, IGF-I is the predominant form of somatomedin in the adult, with sites of synthesis being determined precisely in the adult brain, for example (Werther *et al*, 1990). IGF-I receptors have been reported in the adult rat liver (Venkatesan and Davidson, 1990), though it was previously widely thought that the adult liver was devoid of IGF-I binding activity (Massague and Czech, 1982). Venkatesan and Davidson believe that the presence of these receptors is sex and rat strain specific, though this has yet to be confirmed. IGF-II mRNA and protein levels are greater during gestation and decline within a few weeks of postnatal life (Beck *et al*, 1987; Beck *et al*, 1988a; Senior *et al*, 1990). IGF-II is a more effective growth promoter of fetal paw transplants (Liu *et al*, 1989), and IGF-II mRNA has been detected on the 7th day of gestation in the egg cylinder region, (Florance *et al*, 1991) while its receptor is expressed from the two cell stage of mouse development (Harvey and Kaye, 1991). Similarly, IGF-II appears to bind avidly to rat placental tissue, while IGF-I binds only weakly (Daughaday *et al*, 1981). In the rat, therefore, it has been proposed that IGF-II is the principal fetal factor. However, IGF-I mRNA has been detected in several rat fetal tissues, while IGF-II mRNA is present in the adult rat brain (Lund *et al*, 1986), suggesting that the roles of IGFs are more complex.

Work carried out on rat embryos cultured in serum depleted of insulin by affinity chromatography, show that an improvement in growth and development can be achieved by the addition of 10ng/ml IGF-I to the medium (Jefferson *et al*, 1990). This suggests that factors other than insulin may play important roles during development. IGFs have been shown to stimulate growth in all fetal cells so far examined, acting as potent mitogens, stimulating DNA synthesis and cell proliferation. IGF-binding proteins have been detected in amniotic fluid and blastocysts, suggesting a role for IGFs in early development. The IGFs are not present free in the circulation, but are instead bound to specific carrier proteins, and PC13 EC cells have been found to express IGF receptors and secrete these binding proteins (Heath and Shi, 1986), again suggesting a role in development.

Structure of IGFs

The amino acid sequences of IGF-I and IGF-II, elucidated by Rinderknecht and Humbel (1978a; b) reveal peptide hormones with a high degree of homology (approximately 70%) with each other, and to a lesser extent, to proinsulin. Both IGFs are single-chain polypeptides consisting of A and B domains similar to the domains of proinsulin (approximately 50% homology), plus C domains, which are shorter in the IGFs and show no homology to the C domain of proinsulin. In addition, the IGFs possess an additional D domain, which exists as a carboxy-terminal extension of the A domain, and which is not present in proinsulin. Dull *et al* (1984) concluded that IGF-II was initially synthesised as a larger preprohormone, with a molecular weight around 20,000, prior to post translational processing to produce IGF-II. IGF-I consists of 70 amino acid residues (Mr 7649), IGF-II of 67 amino acids (Mr 7471), insulin of 51 amino acids (Mr 5747) and finally, proinsulin of 82 amino acids (Mr 9000). Cysteine residues are present in the same positions in IGF-I and -II as in insulin and proinsulin, suggesting the IGFs are also cross-linked by disulphide bridges (reviewed Roberts and LeRoith, 1988). The gene for IGF-I has been assigned to a location on human chromosome 12, while the IGF-II gene is believed to be located on the short arm of chromosome 11, close to the gene encoding insulin (Tricoli *et al*, 1984).

The fourth member of the insulin family, relaxin, is produced by the corpus luteum and acts to soften the cervix and the sacro-iliac joint and pubic symphysis prior to parturition. This displays only around 35% sequence homology with insulin and the IGFs. Relaxin does not possess any insulin-like activity, which may be because the C-terminal of the B chain is very different from the corresponding sequence in insulin and the IGFs (reviewed Gammeltoft, 1989).

Receptors for Insulin and the IGFs

The actions of insulin, IGF-I and IGF-II are brought about by the hormone binding to specific cell surface receptors, which then initiates one or more intracellular signalling mechanisms (reviewed Czech, 1985).

The isolation and characterisation of the insulin receptor from human placenta, a rich source of the receptor (Posner, 1974), revealed a membrane-associated glycoprotein, which consisted of an α subunit, with an apparent Mr=125,000-135,000. This was initially suggested by visualisation of protein-stained electrophoretic gels containing a preparation of insulin receptors affinity purified from insulin-agarose. (Jacobs *et al*, 1977). Competitive inhibition of this α subunit's affinity for insulin was investigated by increasing the concentration of unlabelled insulin, and such studies revealed a high

affinity for insulin, as would be expected from a specific insulin receptor (Pilch and Czech, 1980). An insulin receptor α subunit of similar characteristics to those described in rodents and humans, has been identified in the hagfish, indicating significant conservation of the properties of this receptor over the last 500 million years of evolution.

In addition to the α subunit, affinity purification studies also revealed the existence of a second subunit type (Jacobs *et al*, 1980). This β subunit had a molecular weight of around 90,000 and was found to be present in the insulin receptor complex in approximately the same stoichiometry as the α subunit.

The general model for the configuration of the insulin receptor was proposed by Jacobs and co-workers (Jacobs *et al*, 1980) using affinity purification and photolabelling techniques. These findings were supported by the work of Massague and workers who used cross-linking techniques (Massague *et al*, 1980). This proposed a receptor complex with an α_2 β_2 stoichiometry in the form of an α-β-β-α heterotetramer connected via disulpide bonds i.e. in an β-S-S-α-S-S-α-S-S-β configuration.

The β subunit was found to undergo autophosphorylation upon the addition of the solubilised insulin receptor in detergent in the presence of [gamma^{32}P]ATP or its analogues (Kasuga *et al*, 1983). This suggested that a tyrosine-specific protein kinase activity is closely associated with the insulin receptor. Kasuga *et al* concluded from their work that this tyrosine kinase activity may be important in the signal transmission required for insulin action. Shortly after this, the human insulin receptor sequence was deduced (Ullrich *et al*, 1985), revealing a ligand-dependent tyrosine-kinase domain with homology to that of the EGF receptor. Both the insulin and IGF-I receptor tyrosine kinases appear to be regulated by the phosphorylation of serine/threonine residues within this intracellular domain (Czech, 1989). Studies using defective tyrosines kinase domains transfected into fibroblast insulin receptors, have demonstrated that autophosphorylation is not impaired, whereas intracellular substrate phosphorylation is reduced (Maegawa *et al*, 1988). This indicates that the autophosphorylation which occurs following ligand binding to the insulin receptor, is not sufficient alone to initiate the biological response.

In addition to the work carried out on the insulin receptor, two distinct types of IGF receptors have also been identified, which differ in their relative affinities for IGF-I and IGF-II (reviewed Rechler and Nissley, 1985). This was first demonstrated by the work of Massague and Czech, who cross-linked [^{125}I]-labelled-IGF-I and [^{125}I]-labelled-IGF-II to both membrane preparations and intact cells using the covalent cross-linker disuccinimidyl suberate (Massague and Czech, 1982).

Subsequent analysis of the solubilised radioligand-receptor complexes using sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) revealed two distinct labelled species of membrane components, consistent with the presence of two types of IGF receptor. The first receptor type was found to be structurally very similar to the insulin receptor and to bind IGF-I with greater affinity than IGF-II, (hence its more usual description as the IGF-I receptor), though also binding insulin at high concentrations. Using artificially produced hybrid insulin/IGF-I receptors, it has been demonstrated that the specificity of the insulin receptor can be drastically altered by substitution into exons 2 and 3 of a sequence from the IGF-I receptor. The hybrid receptor then binds IGF-I with a much stronger affinity than insulin, demonstrating that the ligand binding domain of the insulin receptor must be located within these two exons (Anderson *et al*, 1990). Much work has been carried out as to the precise location of binding sites on the IGF-I receptor, such that using single point mutations, the amino acids responsible have been determined (Bayne *et al*, 1990; Gustafson and Rutter, 1990).

The second receptor type displayed a higher affinity for IGF-II than IGF-I, and showed no significant affinity for insulin. This receptor type (the IGF-II receptor) appeared to consist of one structure, was not disulphide linked to any other membrane component, and appeared similar to a structure previously identified as a cell surface receptor specific for multiplication-stimulating activity (Rechler *et al*, 1980).

The binding characteristics of all three receptor types (that is the insulin and type 1 and 2 IGF receptors) are complicated by the varying levels of cross reactivity which they display. The IGF-I receptor exhibits an affinity for IGF-II that is only 2-3 times lower than that for IGF-I ($Kd=1.5x10^{-9}$), but has a lower affinity for insulin ($Kd=10^{-7}$) (Steele-Perkins *et al*, 1988; Roth *et al*, 1988). The IGF-II receptor binds IGF-II with between 10-50 times higher affinity than it does IGF-I, and displays no affinity for insulin (Massague and Czech, 1982), while the insulin receptor binds insulin with 10-100 times higher affinity than IGF-I or IGF-II (reviewed Gammeltoft, 1989).

Like the insulin receptor, the IGF-I receptor is a membrane glycoprotein of Mr between 300,000 and 350,000, consisting of two α and two β subunits (Mr=135,000 and 90,000 respectively), connected via disulphide bonds to form an α-β-β-α heterotetramer, (Feltz *et al*, 1988) (See figure 1.2). Jacobs *et al* (1983) were able to demonstrate phosphorylation occurring on tyrosine residues of solubilised IGF-I receptors in IM-9 lymphocytes in the presence of ATP, using a monoclonal antibody specific to the IGF-I receptor (the α-IR3 antibody) to immunoprecipitate the receptor. The species precipitated was between 92,000 and 95,000 molecular weight, which is consistent with the phosphorylation

occurring on the β subunit, and phosphorylation was increased when the cells were exposed to IGF-I. The IGF-I receptor also stimulates tyrosine kinase activity in the BRL 3A2 rat liver cell line (Zick *et al*, 1984), suggesting that the IGF-I receptor is a tyrosine-specific protein kinase, capable of autophosphorylation in a manner very similar to the insulin receptor (Yu *et al*, 1986). If the disulphide bonds of the receptor are reduced to produce an α-β dimer, the resulting structure has a much lower affinity for IGF-I, and shows limited autophosphorylation (Tollefsen *et al*, 1991). This demonstrates that interaction between dimers is necessary to propagate the cellular signal.

A further impetus for study in this area came when the complete structure of the human IGF-I receptor was determined using cDNA clones (Ullrich *et al*, 1986). The amino acid sequence described showed a high degree of homology to the insulin receptor. The receptor was initially isolated from solubilised human placental membranes using the specific monoclonal antibody α-IR3, which binds the IGF-I receptor with 100 times greater affinity than it binds the insulin receptor, (Kull *et al*, 1983). The cDNA sequence which was determined predicted a receptor precursor of 1367 amino acids, of which 30 amino acids represents a signal peptide removed during translocation (Ullrich *et al*, 1986). This residue has an Mr of approximately 152,000, which, when it undergoes cleavage at an Arg-Lys-Lys-Arg sequence at position 707 of the precursor, generates α and β subunits of 80,423 and 70,866 respectively, which become 135,000 and 90,000 upon glycosylation. The IGF-I receptor α and β subunits are, therefore, generated from a single polypeptide precursor in the same fashion as the insulin receptor.

The extracellular subunits of both the insulin and IGF-I receptors contain cysteine rich regions. This portion shows only 48% sequence identity in the two receptors, compared with approximately 65% in the surrounding areas, which may be as a consequence of this area being strongly involved in hormone binding. The specific residues involved in IGF-I binding to the receptor are now believed to be exclusively present in this cysteine-rich domain, while the insulin receptor binding sites lie in both the amino- and carboxyl-terminal regions (Schumacker *et al*, 1991). The cysteine spacing observed in the α subunits of the IGF-I and insulin receptors are also seen in the *neu* oncogene-related putative hormone receptor HER2, suggesting a common evolutionary origin.

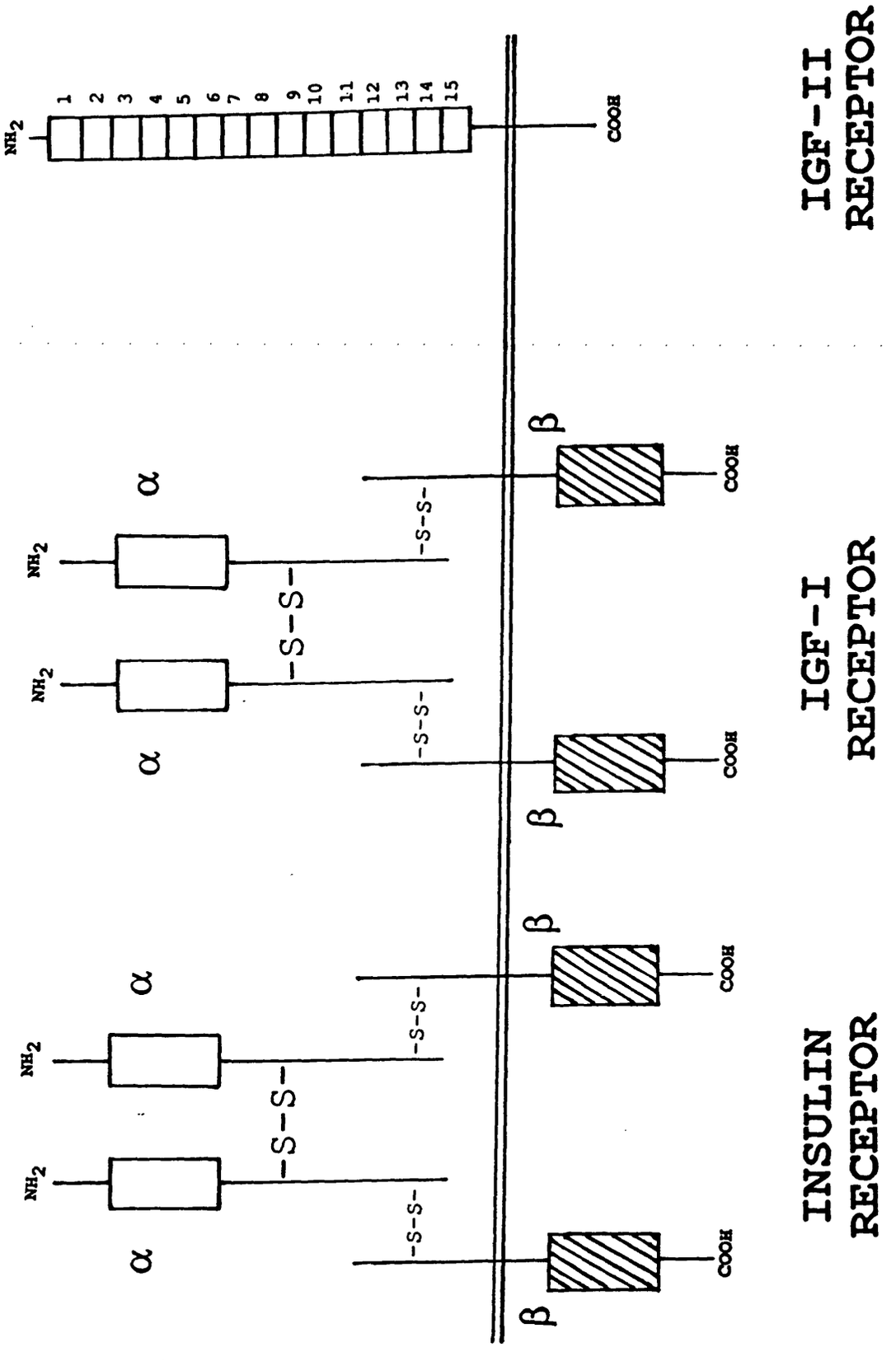

FIGURE 1.2

Schematic comparison of the insulin, IGF-I and IGF-II (mannose-6-phosphate) receptors. (Not to scale.)
In the insulin and IGF-I receptors, the striped boxes represent the tyrosine kinase domains, while the open boxes indicate extracellular cysteine rich regions. In the IGF-II receptor, the boxes labelled 1-15 represent repeat sequences.

The cytoplasmic domain of the IGF-I receptor, which possesses the tyrosine kinase activity, displays the highest degree of homology to the insulin receptor (around 84%), while the C-terminal hydrophilic tails of these receptors show only 44% homology (reviewed Roth, 1988a). One interpretation of this is that the differences in the tail sequences could be responsible for the abilities of the two receptors to mediate different functions. However, work done involving the expression of both insulin and IGF-I receptor cDNAs in Chinese hamster ovary cells indicate that there are no inherent differences in the abilities of the two receptors to mediate rapid and long-term biological responses when expressed in the same cells (Steele-Perkins *et al*, 1988). It may be therefore, that differences in physiological effects may be regulated by mechanisms such as the levels of receptors in various tissues. Intriguingly, stimulation of the insulin receptor has also been shown to produce a significant increase in the phosphorylation of IGF-I receptors (Tartare *et al*, 1991), which may be one mechanism to enhance insulin action in cells where both receptor types are present.

While the IGF-I receptor binds insulin weakly, it will also bind IGF-II with high affinity. The structure of the receptor for IGF-II is quite distinct from that for IGF-I and insulin (see Fig. 1.2), being composed of a single polypeptide of Mr=260,000. It does not possess tyrosine kinase activity or undergo autophosphorylation, and appears to bind neither insulin nor IGF-I. Instead, it appears to be identical to the cation-independent mannose-6-phosphate receptor (Roth, 1988b; Oshima *et al*, 1988; MacDonald *et al*, 1988), which is involved in the targeting of various lysosomal enzymes phosphorylated on mannose residues from the Golgi apparatus and the plasma membrane to the lysosomes (Von Figura and Hasilik, 1986).

The receptor consists of a large extracellular domain, which is 93% of the receptor, a transmembrane spanning domain, and a short (163 residues) intracellular portion. It does not contain the cysteine rich regions seen on the IGF-I and insulin receptors; instead, it is composed of a 150 residue-long sequence repeated 15 times. The extracellular domain also contains a sequence 43% homologous to the type II region of fibronectin, and it may be that this area is involved in binding IGF-II.

It was thought for a long time that the IGF-II receptor did not propagate a signal after it had bound IGF-II. While IGF-II appears to play a role in mammalian development, it has been suggested that it stimulates cell division and metabolism by acting via the IGF-I receptor and initiating the tyrosine kinase pathway (Ballotti *et al*, 1987). This would appear to be the case in *Xenopus laevis* oocytes, as although these express IGF-II receptors, it is the IGF-I receptor which is thought to mediate IGF-II stimulated uptake of hexose

(Janicot *et al*, 1991). The role of the IGF-II receptor is, therefore, unclear, although some work has suggested that the IGF-II receptor can in fact, generate a response via its own receptor. These responses have included the uptake of amino acids and glucose in human muscle cells (Beguinot *et al*, 1985), and glycogen synthesis in hepatoma cells (Hari *et al*, 1987), and were not inhibited by antibodies against the insulin or IGF-I receptors. It is may be that the majority of biological responses to IGF-II are mediated though the IGF-II receptor, and that the IGF-II/mannose-6-phosphate receptor can be thought of as a multifunctional protein involved in the intracellular transport of IGF-II and lysosomal hydrolyses. However, much work remains to be done in this area.

Summary

Insulin, IGF-I and IGF-II appear to play important roles as growth factors in mammalian development. Briefly, insulin and IGF-I show a large amount of homology both in structure, and in their mechanism of action on cell metabolism and division. IGF-I seems to stimulate growth of several tissues before birth and in postnatal and into adult life, and while the primary role of insulin is in glucose homeostasis, its importance as a growth factor is becoming apparent. The actions of insulin and IGF-I are mediated through the insulin and IGF-I receptors, two structurally similar receptors, and there may be a high degree of interaction between these two receptors and these two ligands. The role of IGF-II is not as clear as that of IGF-I, with the receptor for IGF-II being totally dissimilar to those for insulin and IGF-I. The observation that IGF-II binds to the IGF-I receptor with high affinity has lead to the proposal that this is how IGF-II produces its mitogenic effects.

Before the chorio-allantoic placenta is fully formed and functional, the developing rat has been demonstrated to require insulin for growth and development. This necessity for insulin cannot be met solely by embryonic insulin production, suggesting that maternally derived insulin may be an important source at this stage. One possibility is, therefore, that maternal insulin acts on the extraembryonic membranes which surround the embryo, and is transported to the embryo via this route. Other essential growth factors, including the closely related IGFs, may also be derived from the mother and transported to the embryo in a similar fashion.

The rat visceral yolk sac is an extraembryonic membrane which surrounds the embryo during the second half of gestation. This membrane has been implicated in the transfer of the immunoglobulins which confer passive immunity, as well as playing a role in nutrition by digesting and transporting essential molecules from the surrounding maternal fluid. Maternally derived

growth factors may, therefore, be acting on the maternal surface of this membrane to produce a growth effect in the embryo.

This study is an investigation into the processing of insulin and IGF-I by the rat visceral yolk sac, and the distribution of the insulin and IGF-I receptors which may mediate this process.

Aims of the project

Many of the precise effects of this family of growth factors upon development remain unclear as yet, and this project is an attempt to provide further insight into one aspect of their biology; their handling by the yolk sac and subsequent metabolism. The importance of the possible interactions of insulin, IGF-I and IGF-II with the corresponding receptors is also unclear.

In many experiments carried out in this project, the cultured rat anembryonic yolk sac system will be used as the model. This system provides a comparatively simple *in vitro* model for the study of insulin and IGF-I processing and metabolism. Yolk sacs removed from the mother on the equivalent day of gestation will also be investigated to examine whether extensive culture of yolk sacs results in any apparent differences when the processing of these growth factors are concerned.

To summarise, the aims of this project are:

i) an investigation into the handling of insulin and IGF-I by the cultured rat anembryonic yolk sac system using [^{125}I]-radiolabelled insulin and IGF-I.

ii) a parallel investigation into the handling of these factors by yolk sacs explanted on the equivalent day of gestation (day 17).

iii) an investigation into the destination of the insulin and IGF-I following uptake by the yolk sac using fluorescently labelled insulin and IGF-I.

iv) preincubation of cultured anembyronic yolk sacs with a monoclonal antibody which blocks the IGF-I receptor to see if this affects subsequent development.

v) a series of immunocytochemical and/or immunoprecipitation experiments utilizing an antibody specific to the IGF-I receptor to examine receptor location.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

Preparation of Yolk sacs

Mating

Female Wistar rats were placed in cages with male Wistar partners overnight. Mating was confirmed the following morning by the presence of vaginal plugs at the bottom of the cage. For the purpose of timing the gestation, it was assumed that mating occurred at approximately midnight and the animal deemed 0.5 days pregnant at midday.

Explantation of 17.5 day yolk sacs

Wistar females were mated, and pregnancy confirmed as above. The gestation was then allowed to proceed until the 17th day. The animal was then anaesthetised using diethyl ether within a perspex chamber. The animal was removed when unconscious, as judged by the loss of corneal reflex, and anaesthesia maintained by placing the animals head in a nose cone containing ether-soaked gauze. The ventral abdominal wall was then soaked with 70% methanol to prevent fur interfering with the subsequent explantation. A mid-line incision was made and the abdomen opened. The abdominal contents were then displaced to one side with a sterile swab to expose the bifurcation of the abdominal aorta at the common iliac arteries. Exsanguination was then carried out by the insertion of a 1.5mm diameter needle attached to a 10ml Monovette syringe into the aorta. Normally, between 8-10 mls of blood was withdrawn from each animal, and this was immediately centrifuged at 3000 r.p.m. for 10 minutes to be used for serum preparation. The uterus was removed, and then placed in Hank's balanced salt solution (Appendix 1). An incision was next made along the mesometrial border of the uterine musculature, and the embryos plus the surrounding tissue removed. The placental discs were carefully removed using a pair of curved scissors, and the embryo was then separated from the yolk sac taking care not to damage the yolk sac more than was necessary. The amnion was then teased from the yolk sac, which was washed finally in Hank's to remove any blood. Yolk sacs were then placed in the incubation medium, Medium 199 (Appendix 2), before being gassed with $95\%O_2/5\%CO_2$ for 1 minute, and the experiment started.

Fig 2.1 shows the appearance of a 17.5 day yolk sac entirely surrounding the fetus.

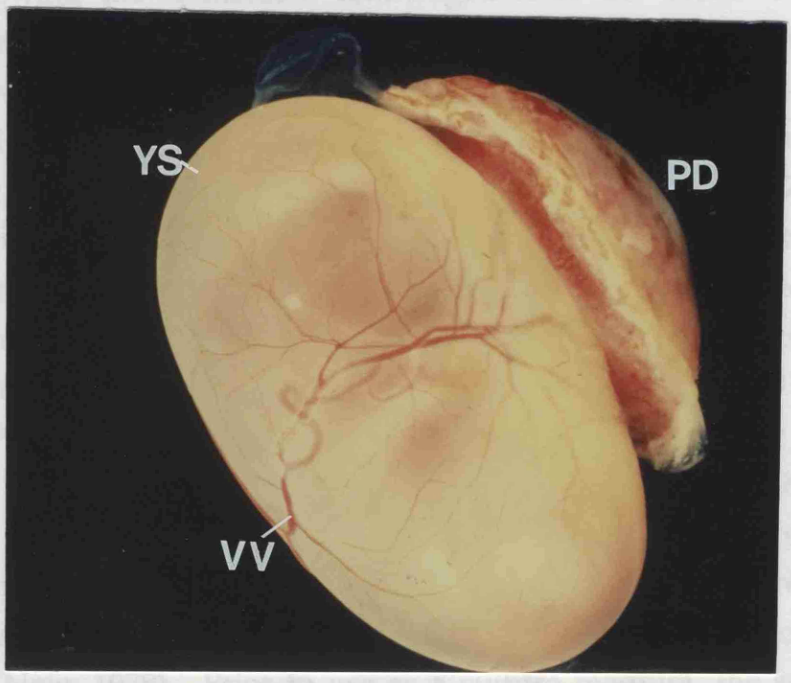

Fig 2.1. 17.5 day rat visceral yolk sac.
The rat conceptus is removed from the mother on the 17th day of gestation, and the uterine tissue removed. This reveals the fetus surrounded by the yolk sac (YS). The placental disc (PD) is then cut away, and the fetus removed from the yolk sac. Note the vitelline vessels (VV), which are well developed. (Magnification: x4)

Explantation of 9.5 day yolk sacs

At 9.5 days of gestation, the pregnant animal was placed in a perspex chamber and anaesthetised and exsanguinated in the same fashion as a 17.5 day pregnant animal.

The uterine horns containing the conceptuses were then dissected out, separated into individual decidual masses, and placed into Hank's balanced salt solution. Using blunt forceps, the uterine tissue was separated from the decidua, which were placed into fresh Hank's solution. Under a dissecting microscope and using two pairs of fine watchmaker's forceps, the decidua was removed from around each conceptus. The Reichert's membrane was then carefully teased open to permit normal development of the embryo and yolk sac (Ellington and New, 1980).

The conceptuses were then transferred to glass bottles containing 2mls of heat-inactivated serum (containing penicillin/streptomycin) and 2mls of Medium 199, before being gassed with 5%CO_2, 90%N_2, and 5%O_2 prior to culture.

Preparation of serum

Following the immediate centrifugation of the blood obtained from exsanguination, the blood was set aside for approximately 30 minutes at room temperature to enable coagulation to occur. The serum must be immediately centrifuged as it has been clearly demonstrated that a delay reduces the growth supporting capacity of the serum (Steele, 1972; Steele and New, 1974). The clear fibrin clot which forms was then squeezed and the tube recentrifuged for a further 10 minutes. After this, the supernatent serum was removed and pooled with serum from several other rats in a 20ml "universal" plastic container. An antibiotic mixture was added to give a final concentration of 100 International Units/ml Penicillin and 100μg/ml Streptomycin. Though it may be suggested that sera from different sources may have differing growth supporting capacities, no differences have been detected, and this pool contains serum from pregnant and non-pregnant females in addition to serum from males. In contrast, heterologous sera (i.e. that from another species) is often harmful (reviewed New, 1978). Prior to use as a culture medium, the serum is heat-inactivated at 56°C for 30 minutes, as this also has been shown to improve its capacity to support embryonic growth, (Steele and New, 1974; New *et al*, 1976a), possibly via the inactivation of serum complement.

Culture of anembryonic yolk sacs

The yolk sacs were cultured following a modification of the rat whole embryo technique originally developed by New (New *et al*, 1973), and subsequently extended to produce anembryonic yolk sacs (Dunton *et al*, 1986). The conceptuses (approximately 10 per bottle) were placed in sealed culture bottles containing 2mls of serum and 2mls of Medium 199, as previously described, and were constantly rotated in a roller incubator at 37°C. After 24 hours, the bottles were gassed with a mixture of 20%O_2, 75%N_2, and 5%CO_2. After 48 hours, the yolk sacs were transferred to fresh medium. The yolk sacs were now separated into 3 yolk sacs per bottle, to which 1.5mls M199 and 1.5mls serum were added. The bottles were then gassed, with 40%O_2, 55%N_2, and 5%CO_2 and returned to the incubator.

Every subsequent 24 hours the bottles were regassed, with the 40%O_2 mixture while the serum/M199 mixture was changed every 48 hours. The length of the culture period was 8 days, i.e. until the yolk sacs were equivalent to 17.5 days of gestation. Figure 2.2 shows a typical anembryonic cultured yolk sac after 8 days.

Fig. 2.2 Anembryonic cultured rat visceral yolk sac.
The conceptus was explanted from the mother at 9.5 days of gestation, and surrounding decidua and Reichert's membrane removed. The yolk sacs were then cultured in a 50:50 mixture of heat-inactivated rat serum and Medium 199 in a roller incubator at 37°C for a further 8 days. (magnification:x5)

Harvesting of anembryonic yolk sacs

Following the addition of the experimental protein, the yolk sacs were harvested. In the case of [^{125}I]-labelled insulin or IGF-I, yolk sacs were harvested between 30 minutes and 4 hours after the addition of the protein. Two 0.5ml samples of the culture medium were removed for estimation of radioactivity. The remaining medium was then removed using a bent needle, and replaced with 1% sodium chloride solution. The bottles were then rolled for approximately 2 minutes to wash the yolk sacs. This washing procedure was repeated twice. The yolk sacs were then carefully floated into a tank containing 1% sodium chloride. A small plastic Petri dish was placed under the yolk sac which was manoeuvred onto it, to enable its removal from the tank. Excess saline was removed from the Petri dish using a syringe. The yolk sac was then burst, releasing the extraembryonic coelomic fluid (EEC) within, which was collected using a 1ml syringe and fine needle. This fluid was made up to 0.5ml by the addition of 1% sodium chloride. The yolk sac tissue was washed in 1% sodium chloride, before being placed in 1ml of 1M sodium hydroxide in a plastic tube.

Counting radioactive samples

As previously stated, two 0.5ml samples of the culture medium, in addition to the extraembryonic coelomic fluid collected, were retained for future radioactive counting. These samples were initially placed in a Pharmacia LKB Minigamma counter, and counted for 30 seconds. After this, the samples underwent acid precipitation. This was performed by the addition of 0.25mls of used serum (that is, serum which has been exhausted by culturing yolk sacs in it), 0.25mls of 40% perchloric acid and 0.25% of trichloroacetic acid. The samples were then spun in an MSE bench centrifuge at 4200 r.p.m. for 30 minutes, before the supernatent was removed and recounted for 30 seconds on the Minigamma counter.

The yolk sac tissue, which had been placed in 1ml of 1M NaOH, was also counted on the Minigamma counter for 30 seconds. These samples then underwent an assay to determine their protein content.

Estimating the protein content

The amount of protein in the yolk sac was estimated using a modification of the Lowry method (Lowry *et al*, 1951).

Stock solutions prepared prior to assay were:

1)1M sodium hydroxide (40g/litre)

2)3M hydrochloric acid (3.67mls of conc. HCl made up to 11ml with distilled water.)

3)Sodium carbonate (2g anhydrous Na_2CO_3 per 100ml distilled water.)

4)Copper sulphate (2g $CuSO_4.5H_2O$ per 100ml distilled water.)

5) Sodium tartrate (2g $(CH(OH).COONa)_2.2H_2O$ per 100ml distilled water.

6)Bovine Serum Albumin (5mg/ml B.S.A./ml 1M NaOH)

7)Folin Ciocalteu's reagent (BDH)

Folin A solution.

This must be prepared fresh on the day of use, by the addition of 1ml of (4) and (5) to 100mls of (3)

Folin B solution.

This is prepared immediately before use by diluting Folin Ciocalteu's reagent (7 above) 50/50 with distilled water.

Method

The yolk sac, which had been placed in 1ml of 1M NaOH in a sealed tube, prior to counting on the LKB Minigamma monitor, was whirlimixed and then incubated at 37°C for at least 2 hours. After this time, the sample was removed and thoroughly whirlimixed to ensure the yolk sac had dissolved completely.

0.145ml of 3M HCl (2) were then added to each tube and mixed. Two 0.5ml aliquots were then pipetted from each tube into two clean 9ml plastic tubes. To these, 2.5mls of Folin A solution were added, and they were then left at room temperature for 20 minutes.

0.25mls of Folin B were then added to each tube, which was immediately mixed, before leaving at room temperature for 45 minutes.

The absorbance of each tube was read at 750nm in a spectrophotometer (LKB Biochrom Ultraspec 4050).

In addition to the experimental samples, a series of standard solutions of B.S.A. were prepared from the stock solution (6) as follows: 0, 20, 40, 60, 80, and 100µl were taken and made up to 1ml with 1M NaOH. 0.145ml of 3M HCl were added to these standards, after which they were treated in the same manner as the samples.

A graph was then plotted of the protein concentration of the standards against their absorbance values, which should give a straight line, the gradient of which is used to calculate the protein concentration of the dissolved yolk sac.

Photography

All photographs of embryos (plus Figs. 2.1 and 2.2) were taken using a Tessovar Photo microscope loaded with Kodak EPY-64 Tungsten film and using 3 point incidental light from a fibre optic light source. All film processing was carried out by Leicester University's Central Reprographics Unit.

CHAPTER THREE

PROCESSING OF INSULIN BY THE RAT VISCERAL YOLK SAC

Introduction

In the human fetus, profound growth retardation is observed in cases of pancreatic agenesis, while the infants of diabetic mothers are frequently larger than average, (Johnson, 1985) thus reflecting the importance of insulin for human fetal growth. High levels of genes encoding for insulin have been detected in placental tissue from diabetic mothers, which may come about as a response to high glucose levels in the circulation. The overall result may be an increase in glucose utilization leading to the increase in birth weight and abnormalities seen.

Similar evidence exists which implicates insulin as a growth factor during the development of the rat. Culture of rat embryos from the early head-fold stage (approximately 9.5 days gestation) in insulin-depleted serum, results in growth retardation, plus an associated increase in morphological abnormalities (Travers *et al*, 1989). Embryos cultured in glucose-deficient media develop significant abnormalities (Ellington, 1987), while pathological changes have been reported in the visceral yolk sac endoderm of diabetic rat embryos cultured *in vitro* (Zusman *et al*, 1987). These observations stress the importance of glucose control in the developing embryo.

As there is no evidence that insulin is able to cross the placenta from mother to developing embryo (Underwood and D'Ercole, 1984), it can be assumed that any insulin detected in the fetus probably results from fetal production.

In the rat, pancreatic insulin is detectable from the 15th day of gestation, after which there is a marked increase in levels of approximately 100 fold up to birth (Kakita *et al*, 1983). However, since the early 1980's, evidence has accumulated which suggests that the insulin present during rat development is not limited to the fetal pancreas (Muglia and Locker, 1984). The yolk sac, the extraembryonic membrane which surrounds the embryo, has been shown to contain insulin mRNA, immunoreactive insulin, plus a pro-insulin species (Rau *et al*, 1989) which are detectable from embryonic day 16 (E16) and increase until birth. Further, Giddings and Carnaghi, (1989) have demonstrated synthesis and release of insulin by the yolk sac while fetal liver has also been shown to express insulin mRNA, this time from E13 (Giddings and Carnaghi, 1990).

Insulin receptors are present in early somite rat embryos (day 9.5) and membranes (Unterman *et al*, 1986). However, the relative numbers of receptors are low in comparison to IGF-I receptors, and one possibility proposed is that insulin's role may be to stimulate the secretion of IGF-I by embryonic tissues (Gluckman, 1986).

The internalisation and subsequent degradation of insulin has been studied in a variety of cell types, and it appears that insulin degradation is a

complex process. It has been demonstrated that insulin binds to its receptor on human lymphocyte cell surface microvilli (IM-9 line), (Carpentier, 1989) and that the resultant hormone-ligand complex migrates to non-villous areas where internalization occurs by pinching off the plasma membrane and forming coated vesicles (Carpentier *et al*, 1992). Similar internalisation of a ligand-receptor complex had earlier been reported for insulin in rat hepatocytes (Gorden *et al*, 1982). Following this receptor-mediated endocytosis, [^{125}I]-insulin has been detected within endosomes and lysosomes in IM-9 cells (Carpentier, 1989), within lysosomes in dog kidney, while colloidal gold labelled insulin has been traced to endosomes and a population of dense bodies within mouse blastocysts (Heyner *et al*, 1990). Such dense bodies could be lysosomes, though no enzymatic characterisation was performed.

The internalisation of the insulin receptor which occurs when insulin binds is a rapid process, with $t_{1/2}$ approximately 10 minutes (Knutson *et al*, 1983), and involves amino acid residues, including a tyrosine, present in the juxtamembrane region of the receptor (Backer *et al*, 1990). After internalisation, the receptor may theoretically either undergo degradation or be recycled to the cell membrane. Such recycling has in fact been demonstrated in cultured fibroblasts (Knutson, 1992) and IM-9 lymphocytes (Carpentier, 1989). Such internalized receptors are phosphorylated and have been shown to persist in this state after the dissociation of insulin, but to dephosphorylate prior to their recycling to the plasma membrane (Backer *et al*, 1989). Studies utilising mutated insulin receptors which could not undergo phosphorylation inserted into Chinese hamster ovary cells, have shown that the autophosphorylation of the insulin receptor which occurs following hormone binding, is necessary for internalization to occur (Carpentier *et al*, 1992). Some work has suggested that the actual endocytosis of the insulin receptor is not necessary for the biological response to insulin to be seen, (McClain, 1990), rather that the role of endocytosis is to direct the availability of the hormone, rather than control the insulin signalling response.

It would appear, therefore, that following endocytosis, a proportion of internalised insulin is channelled to the lysosomes for degradation in some cell types at least.

However, work carried out to investigate the degradation of insulin in human fibroblasts concluded that in this system at least, breakdown of the protein was occurring via a non-lysosomal pathway (Kooistra and Lloyd, 1985). These conclusions were based upon the observation that the addition of the lysosomotropic agents chloroquine and ammonium chloride, which concentrate within lysosomes and inhibit their function by raising pH, did not affect the rate of insulin digestion. Conversely, agents known to inhibit pinocytosis such

as the metabolic inhibitors 2,4-dinitrophenol and sodium fluoride (Duncan and Lloyd, 1978) appeared to have little effect on insulin degradation in human fibroblasts, suggesting that internalisation may not be important for insulin processing. Instead, cell-surface proteases were concluded to be responsible for insulin breakdown. This work supported the earlier findings of Caro *et al* (1982) who reported that around half of the insulin degradation preformed by rat hepatocytes occurred at the plasma membrane.

The degradation of insulin is further complicated by the existence of an intracellular protease, termed insulin-degrading enzyme, whose site of action has not yet been precisely determined and remains controversial. The activity of this enzyme has been investigated in simian kidney cells (Kuo *et al*, 1991), where the addition of lysosomotropic agents did not affect insulin degradation, indicating a non-lysosomal pathway. The activity of this enzyme was also concluded to be mainly intracellular, although how a cytosolic enzyme would gain access to internalised insulin present within endosomes is unclear. One possibility is that IDE acts within endosomes, perhaps existing initially on the cell surface to be internalised along with the insulin-receptor complex when it is somehow activated.

The uptake of insulin has previously been quantified in rat visceral yolk sacs taken from the mother at 17.5 days gestation, referred to throughout this thesis as 17.5 day explanted yolk sacs (Livesey, PhD thesis, 1979). The culture of such yolk sacs, first described by Williams *et al* (1975a,b), has provided an enormous amount of data concerning the uptake of various macromolecules by pinocytosis. This yolk sac system has been used to further investigate the processing and subsequent metabolism of insulin. In addition, the handling of insulin by the cultured anembryonic yolk sac system developed by Dunton *et al* (1986) has also been examined, to investigate whether the culture of yolk sac cells results in any appreciable differences concerning the processing of this important molecule.

Radiolabelling of insulin

Introduction

Although a variety of radioisotopes can be used to label proteins, in practice the vast majority of work is carried out using [^{125}]Iodine. This is partly due to the ease of counting samples directly with a gamma monitor without the need for liquid scintillant, but is mainly due to the much higher detectable count rate produced in comparison with ^{14}C and ^{3}H, for example.

The most commonly used method for [^{125}I]-labelling proteins has been the chloramine T method, in which the [^{125}I] is oxidised to form a species which readily reacts with tyrosine residues in the protein (reviewed Bailey, 1989; Bolton, 1985). Chloramine T is the sodium salt of the N-monochloro derivative of p-toluene sulphonamide, which in aqueous solution, breaks down to produce hypochlorous acid. This acid then oxidises the [^{125}]Iodide to cationic iodine I^+, which at pH 7.5 becomes incorporated into the tyrosine residues of the protein to form monoiodotyrosine principally (see fig.3.1).

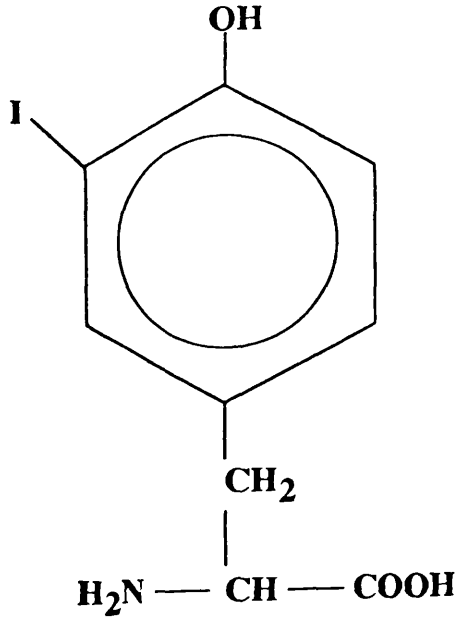

Fig. 3.1 Monoiodotyrosine, the principal iodination product produced by the chloramine T method.

In the case of insulin, it is believed that the tyrosine present at residue 14 of the A-chain is preferentially iodinated by the chloramine T method (Freelender and Cathou, 1971).

When free chloramine T is used, the reaction must be terminated by the addition of a reducing agent, such as sodium metabisulphite, when it has been judged that sufficient incorporation has occurred. The iodination products and

the iodinating reagents must then be separated, most commonly by dialysis or column chromatography.

The use of free chloramine T has now been superseded by the production of an insoluble derivative of a closely related reagent (the sodium salt of N-chloro-benzene sulphonamide) covalently linked to non-porous polystyrene beads (Markwell, 1982) (fig.3.2).

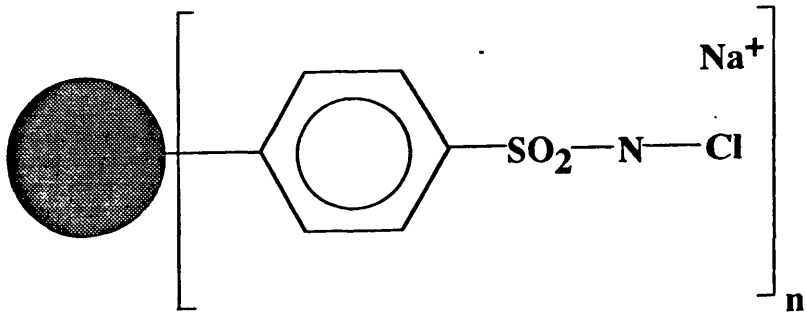

Fig.3.2 Structure of Iodobead.
These iodobeads, as they are termed, efficiently incorporate [^{125}]I into protein over a wide pH and temperature range, removing the harsh chloramine T component, while the reaction is easily terminated by removing the bead from the reaction mixture, so eliminating the need for reducing agents and the separation of reaction products and reagents.

Materials and methods

1mg of porcine insulin (Sigma) was dissolved in 50µl of 0.01M hydrochloric acid, before being made up to 200µl with 50mM phosphate buffer. Four Iodobeads (Pierce) were then added to the insulin, the mixture shaken, and left at room temperature for 5 minutes. 1mCi (10µl) of [^{125}]Iodine (Amersham) was then added, and the reaction mixture left for a further 10 minutes. The Iodobeads were then removed.

A 2ml disposable desalting column (Pierce), prepacked with cross-linked, beaded cellulose (Excellulose GF-5), had previously been prepared by running BSA (bovine serum albumin) (5mg/ml in distilled water) down it to presaturate non-specific binding sites. The column was then washed with 50mM phosphate buffer for approximately two hours, before the 200µl insulin

sample was loaded and washed through with further phosphate buffer. Between 15-20 fractions of approximately 1ml were collected from the column in 3.5ml Sarstedt tubes.

Evaluation of labelling

A 1µl sample from each of the fractions collected was made up to 1ml with phosphate buffer, and then the amount of radioactivity in each determined on an LKB gamma counter. Each sample was counted for 30 seconds, and the number of counts was then plotted against the fraction number.

Fig.3.3 shows a typical graph obtained when the number of counts are plotted against fraction number for insulin.

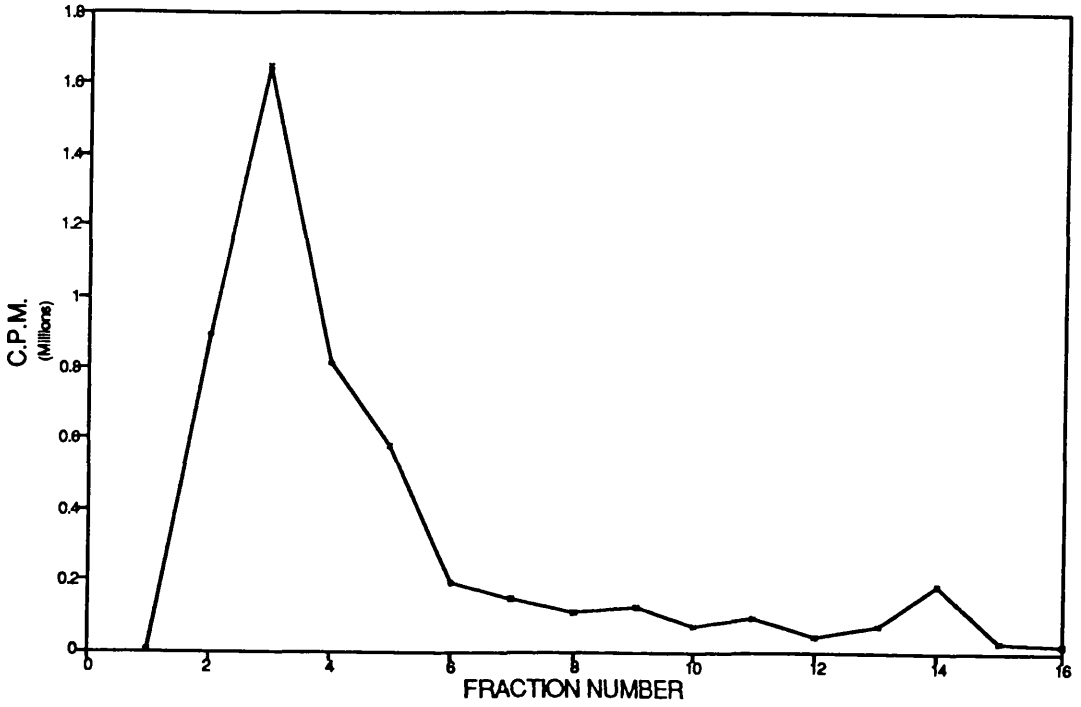

Fig. 3.3. Labelling of insulin with [^{125}]Iodine.
1mg of insulin was made up in 50mM phosphate buffer and 4 Iodobeads added. After 5 minutes, 1mCi of [^{125}]Iodine was added. When the reaction was complete, the labelled insulin sample was run down a pre-packed cellulose column, and eluted with 50mM phosphate buffer. Fractions were collected, and a 1µl sample from each made up to 1ml and counted on a gamma counter to determine which fraction contained the [^{125}I]-insulin.

From the graph, all the labelled insulin (ie. a total of 1mg) is estimated to have come off in fractions 2 to 5. The total counts in these fractions are then added together, and the proportion of [^{125}I]-insulin in each fraction calculated. For example, the total counts in fractions 2 to 5 = 2.3918×10^9 counts per minute

(c.p.m.). Each fraction collected was also weighed to determine the exact volume collected. If we assume that the total 1mg of insulin is divided between these fractions, then the relative amount of insulin present in each fraction can be calculated from:

$$\frac{\text{number of counts in that fraction}}{\text{total number of counts}} \times 100$$

e.g. for fraction 3,

$$\frac{(1{,}648{,}738 \times 458)}{2.3918 \times 10^9} \times 100 = 31.57$$

or, fraction 3 contains 31.57% of the insulin labelled. Assuming that 1mg, or 1000µg, of insulin has been labelled, this equals 315.7µg of insulin.

For fraction 3, the fraction weighed 0.4584g, which we will assume is equal to 0.4584ml. So, 315.7µg of insulin is present in 0.4584ml,

$$\frac{315.7\mu g}{0.4584ml} = 688.7\mu g/ml$$

Efficiency of Labelling

The total counts assumed to be bound to protein are contained within fractions 2 to 5. A 1µl sample of each of these fractions was counted to calculate the amount of protein in that sample (see above). Samples were also weighed to give an estimate of their volume.

Fraction 2 contained 951,616 c.p.m. in 1µl taken from 595µl, fraction 3 contained 1,648,738 c.p.m. in 1µl taken from 458µl, fraction 4 contained 806,366 c.p.m. in 1µl taken from 756µl, and fraction 5 contained 580,692 c.p.m. in 1µl taken from 827µl.

The total reaction volume was 200µl from which a 1µl sample diluted to give a 1:10 dilution contained 1,305,646 c.p.m.

So the labelling efficiency can be calculated from:

$$\frac{(951616 \times 595)+(1648738 \times 458)+(806366 \times 756)+(580692 \times 827)}{(652823 \times 10) \times 200}$$

$$= \frac{2.3918 \times 10^9}{2.61 \times 10^9}$$

$$= 91.61\%$$

This high value appears to confirm that Iodobeads do result in efficient labelling of protein with [^{125}I]-Iodine.

Acid precipitation

A series of acid precipitations was set up to determine which concentration or combination of acid produced the maximum protein precipitation. A 1ml sample of [^{125}I]-insulin was divided into 10x100µl aliquots, to which 0.25mls of exhausted serum was added. The addition of this excess of protein assists the precipitation of the much smaller quantity of [^{125}I]-labelled protein. To each of these samples, the following combination of perchloric acid (P.A.) and trichloroacetic acid (T.C.A.) was added:

Sample number
1: 0.25mls 20% P.A. + 0.25mls 30% T.C.A.
2: " "
3: 0.25mls 30% P.A. + 0.25mls 40% T.C.A.
4: " "
5: 0.25mls 30% P.A. + 0.25mls 50% T.C.A.
6: " "
7: 0.25mls 40% P.A. + 0.25mls 50% T.C.A.
8: " "
9: 0.5mls 50mM phosphate buffer
10: "

The samples were then spun for 30 minutes at 4100 rpm in a bench top centrifuge, before the supernatants were removed and counted using a gamma counter.

Results

Sample number	Counts per minute
1	2780
2	3688
3	3046
4	2234
5	2904
6	2540
7	2276
8	2258
9	34136
10	32248

It was impossible to produce 100% precipitation, so because 40% perchloric acid plus 50% T.C.A. reduced the number of counts in the supernatant by the greatest amount, this combination of acids was used for all subsequent acid precipitations.

Stability of [^{125}I]-labelled insulin

Once the insulin had been radiolabelled the fractions were stored frozen at -20°C. If the percentage of soluble products at 37°C is plotted against time (fig. 3.4) it can be seen that [^{125}I]-labelled insulin remains relatively stable over the time course.

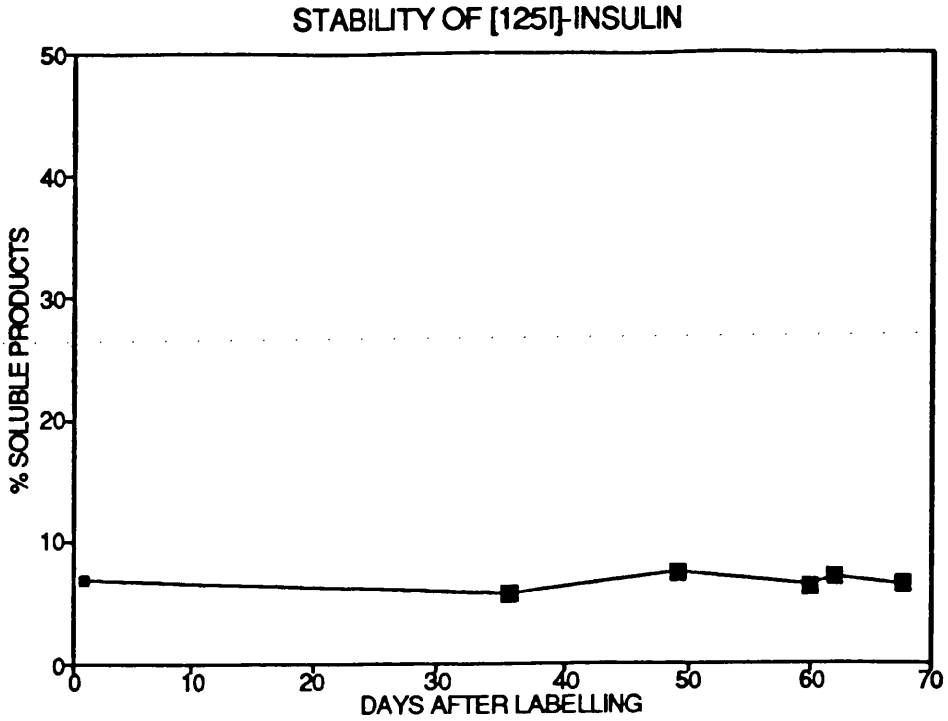

Fig 3.4 Stability of Radiolabelled insulin with time.
Samples of [^{125}I]-insulin were incubated at 37°C without tissue present. After 4 hours, the sample was subjected to acid precipitation, and the percentage of acid soluble [^{125}I] calculated. As any acid soluble [^{125}I] could not be associated with tissue digestion products, this was taken to represent radio-label no longer attached to the protein. This value is plotted here against time to demonstrate the stability of the [^{125}I] and insulin complex.

Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs

Rat conceptuses were explanted at 17.5 days gestation as described in Chapter 2. The fetuses were removed from the yolk sacs with as little damage to the yolk sacs as possible, by cutting off the placental disc and carefully removing the fetus. The amnion was then teased away from the inner surface of the yolk sac using jewellers forceps.

The yolk sacs were then placed in 3mls M199 in glass bottles, either 2 or 3 yolk sacs per bottle, and gassed with 95%O_2, 5% CO_2 for 1 minute before the glass bottles were stoppered with a sterile rubber bung, and placed in a roller incubator at 37°C. The yolk sacs were then allowed to equilibrate with their surrounding environment for between 15 to 30 minutes, before their M199 was removed using a bent needle attached to a 5ml syringe, and replaced with 3mls M199 which contained [^{125}I]-insulin at a concentration of 7μg/ml. The yolk sacs were then regassed with 95%O_2 and returned to the incubator.

Samples were taken after 30, 60, 120, 180, and 240 minutes. Two 0.5ml aliquots of culture medium were removed, and the yolk sacs then washed thoroughly in cold 1% saline three times, before being blotted dry on tissue

paper and placed in 2.5mls 1M NaOH prior to counting on a gamma counter and the subsequent assay for protein.

Results

Table 3.1 shows the results of 4 separate experiments using explanted yolk sacs, where *n*, the number of yolk sacs used, is between 8 and 10.

The values for percentage digestion are derived by taking the average from the number of acid soluble counts/ ml medium (minus the amount of solubles produced/ml when no tissue is present) in a sample taken from each bottle of yolk sacs, divided by the total number of counts in the medium. This value was then divided by the total amount of yolk sac tissue present in each bottle, to normalise these results for any variation in yolk sac size, or in the number of yolk sacs present in a bottle. Both these figures are shown in Table 3.1 below. In addition, the range of digestion products from which these averages are calculated is also given as it is not statistically acceptable to calculate standard errors for transformed data.

TIME (mins)	AVERAGE % DIGESTION (% of added substrate which becomes acid soluble) in total medium	per mg protein
30	34.14 (Range= 25.16...47.7)	6.00 (Range= 4.5..6.8)
60	53.08 (Range= 44.0..69.5)	11.5 (Range = 9.09..17.02)
120	71.80 (Range= 61.2..76.1)	14.3 (Range =11.2..20.1)
180	75.83 (Range= 73.22..77.8)	15.15 (Range = 12.1..18.4)
240	77.19 (Range= 75.8..78.3)	16.89 (Range = 11.7..23.5)

Table 3.1. Percentage of [^{125}I]-insulin digested by 17.5 day explanted yolk sacs.

When the percentage [^{125}I]-insulin digestion is plotted (fig 3.5a), it is apparent from this graph that over the first hour of incubation [^{125}I]-insulin is rapidly degraded to produce acid soluble material. After this time, however, the level of digestion appears to level off, so that after 2 hours of incubation, around 75% of the total protein present has been digested.

When the percentage [^{125}I]-insulin digestion per mg of yolk sac protein is now plotted (fig 3.5b), then a similar pattern is seen. From this graph it becomes apparent that after 4 hours of the incubation, approximately 16% of the insulin present has been digested per mg of yolk sac protein.

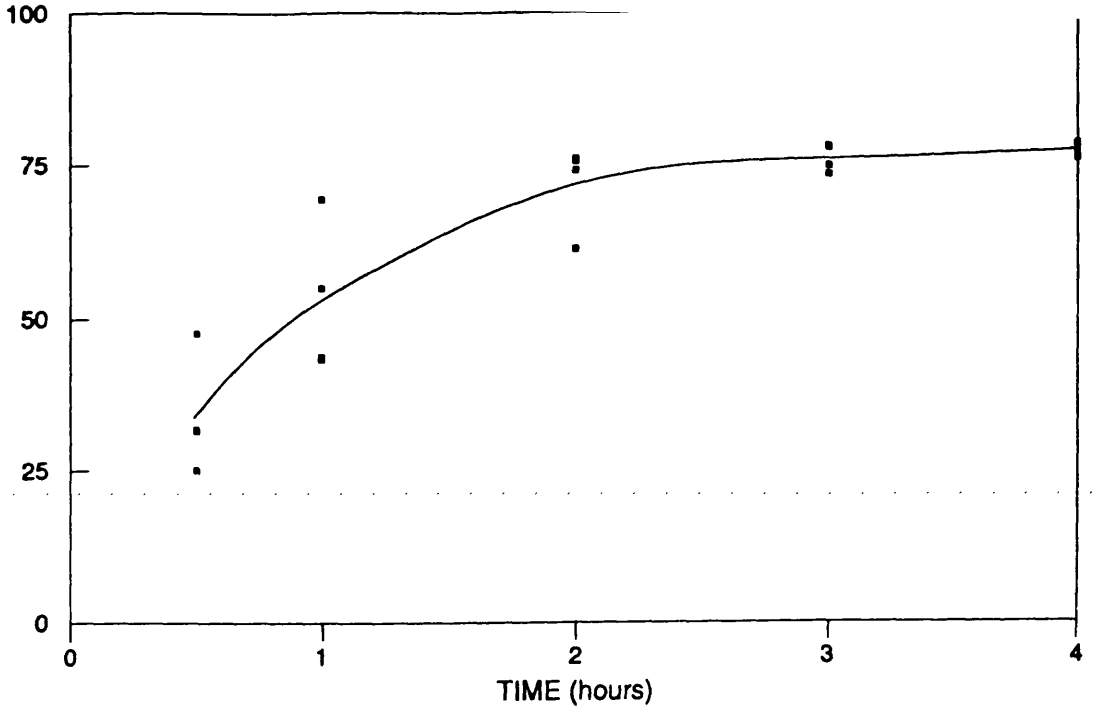

Fig. 3.5a Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs.

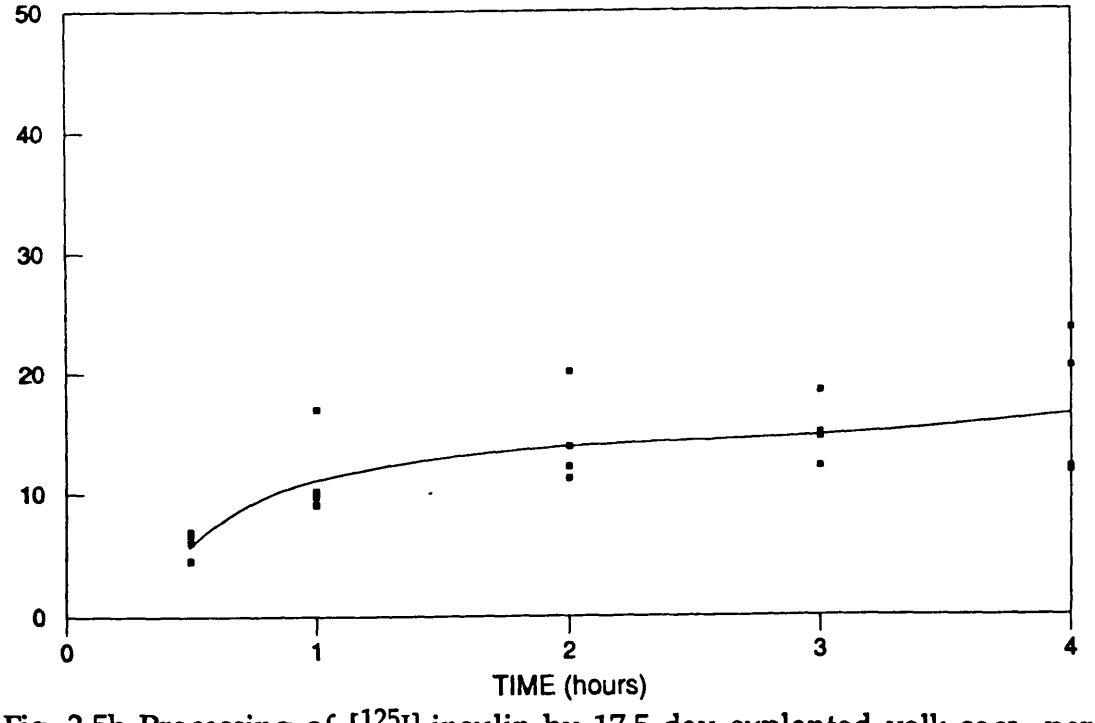

Fig. 3.5b Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs per mg yolk sac protein.

Yolk sacs were removed from the mother on the 17th day of gestation, and washed in M199. They were then placed in glass bottles containing M199 plus [^{125}I]-insulin at 7μg/ml. Yolk sacs were then gassed with 95%O_2 and rotated at 37° C for between 30 minutes and 4 hours. At each time point examined, two 0.5 ml samples of the culture medium were removed, and counted for radioactivity. These samples then acid precipitated by the addition of 0.25mls exhausted serum, 0.25mls 40% perchloric acid, and 0.25ml T.C.A., followed by centrifugation for 30 minutes at 4100rpm. The supernatants were then recounted for radioactivity, as this represents the amount of acid soluble digestion products in the medium samples. The values calculated are plotted here for 4 experiments, while the solid line represents the average calculated from each individual measurement. Fig 3.5b shows the results in Fig 3.5a normalised for the amount of protein present.

It is also possible to examine the levels of radioactivity associated with the yolk sac tissue rather than that within the medium, by dividing the c.p.m. measured in the system by the total number of counts present in the medium. This should provide information as to whether any appreciable level of tissue accumulation is occurring. Since a large proportion of the counts present in the incubation medium are in the form of soluble digestion products, this figure is actually an underestimate of the amount of intact protein which becomes tissue-associated. However, the kinetics involved make a corrected calculation extremely complex, and this procedure has not been performed. The values calculated are those normalised for the amount of yolk sac tissue present.

This level of tissue-associated radioactivity is shown in Fig. 3.6, from which it can be seen that the level of tissue associated radioactivity falls from the initial value of 5.7% per mg protein after 30 minutes to a steady low level of between 2 to 3% after 2 hours incubation. This pattern suggests that there is little progressive accumulation of radioactivity within the yolk sac tissue.

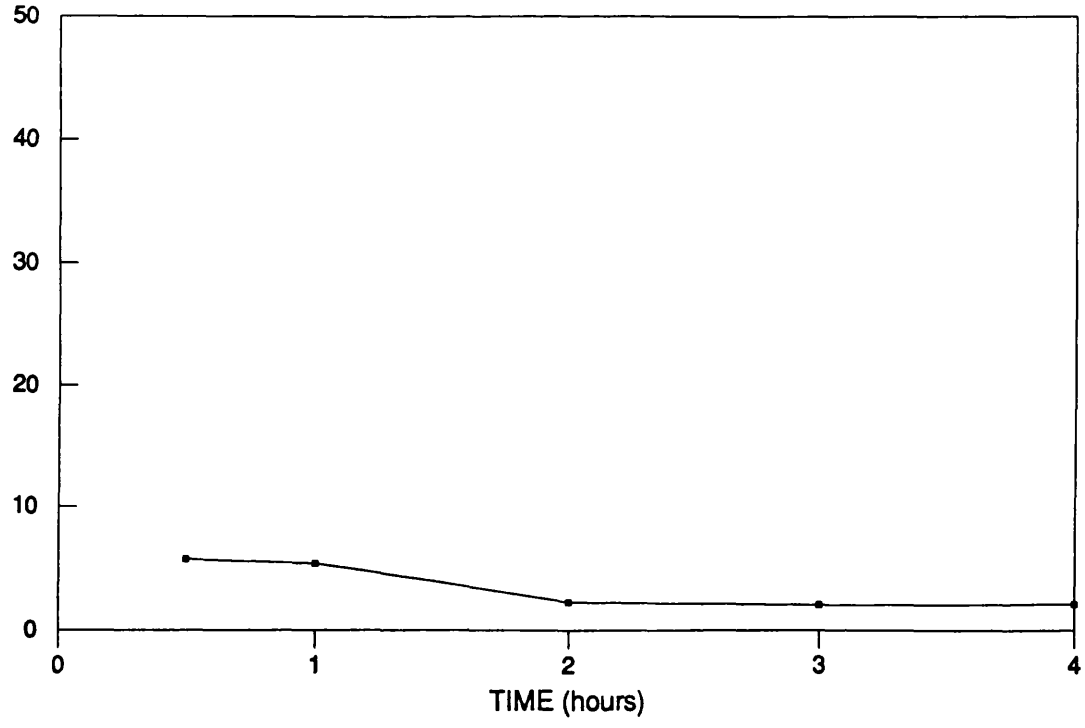

Fig. 3.6 Percentage of the total radioactivity associated with yolk sac tissue per mg yolk sac protein.
The level of radioactivity associated with yolk sac tissue can be estimated by taking the average number of counts measured in each yolk sac, and diving this value by the total number of counts in the incubation medium. Each point is the average of between 8 and 10 yolk sacs.

Attempts to Elucidate the Mechanism of Insulin Digestion

The above experiments demonstrate that exposure of 17.5 day explanted yolk sacs to 1μg/ml [^{125}I]-insulin results in the production of a large amount of low molecular weight digestion products, such that after 2 hours the amount of intact substrate is much depleted. However, these experiments do not provide information as to the mechanism of this breakdown, ie. is the majority of insulin being broken down via internalisation and subsequent transport to the lysosomal system, or is some other process occurring?

It was decided to investigate this question by using a variety of compounds known to inhibit pinocytosis. The compounds used were the glycolysis inhibitors sodium fluoride and monoiodoacetate, both at 10μg/ml; the electron transport uncoupler 2,4-dinitrophenol at 50μg/ml; and the calcium chelating agent EGTA (ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid) at 5mM. The yolk sacs were preincubated with these inhibitors for approximately 30 minutes prior to the addition of [^{125}I]-insulin to allow some equilibration to occur. In addition, yolk sacs were also incubated in the presence of [^{125}I]-insulin at low temperature (4° C).

Figs 3.7.a,b,c,d and e show the various effects on the production of digestion products seen in the presence of each of these compounds, and at low temperature.

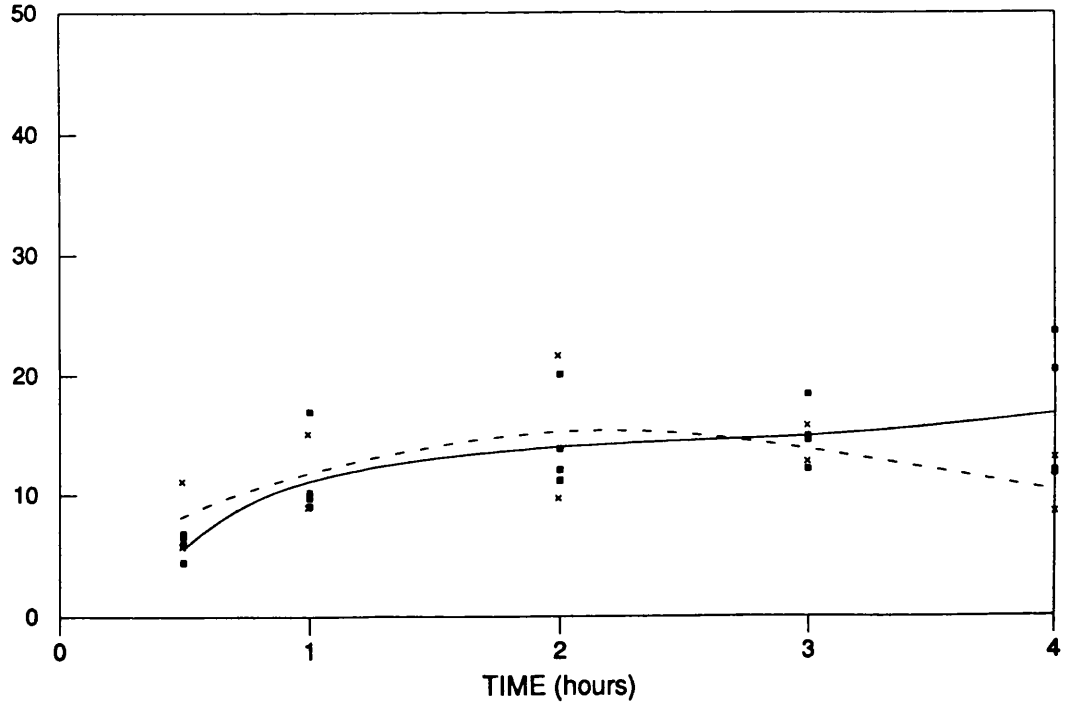

Fig. 3.7a. Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs in the presence of sodium fluoride.

Yolk sacs were explanted on the 17th day of gestation, and washed in M199. The yolk sacs were then placed in M199 which contained 10μg/ml of the glycolysis inhibitor sodium fluoride, and rotated at 37° C for 30 minutes before 7μg/ml [^{125}I]-insulin was added. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that in the presence of sodium fluoride.

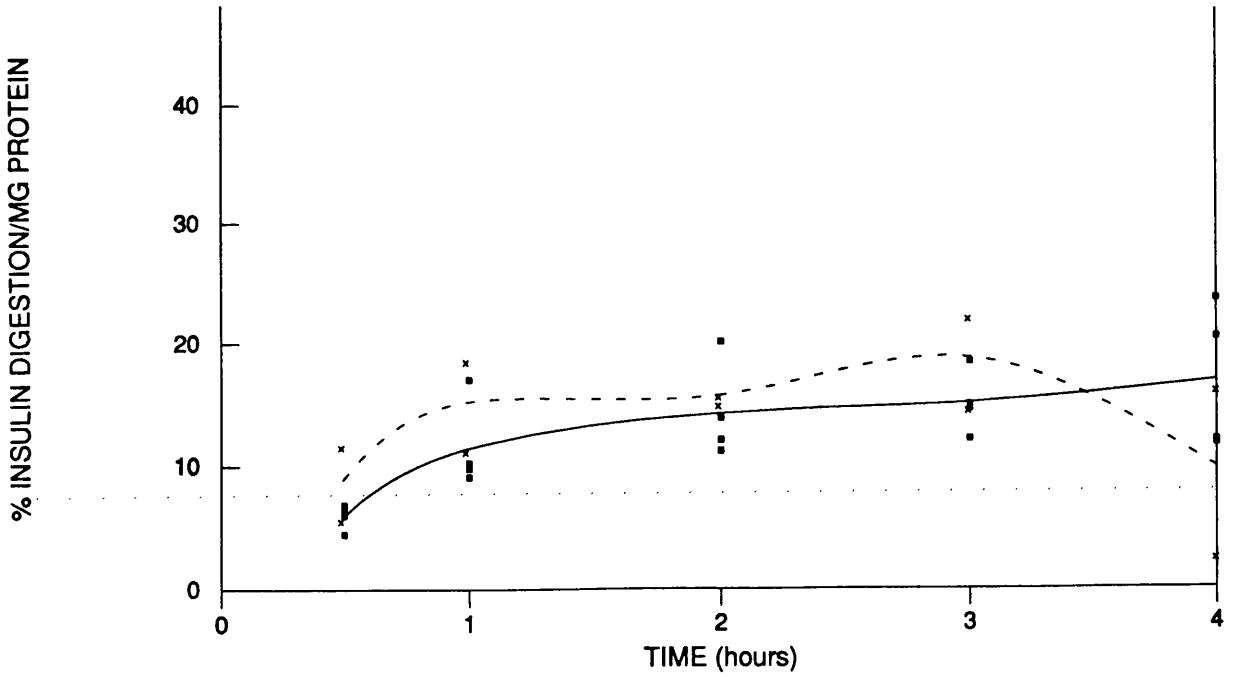

Fig. 3.7b. Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs in the presence of monoiodoacetate.

Yolk sacs were explanted on the 17th day of gestation, and washed in M199. They were then placed in fresh M199 containing 10μg/ml of the glycolysis inhibitor monoiodoacetate, and rotated at 37° C for 30 minutes. After this, the [^{125}I]-insulin (7μg/ml) was added. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that in the presence of monoiodoacetate.

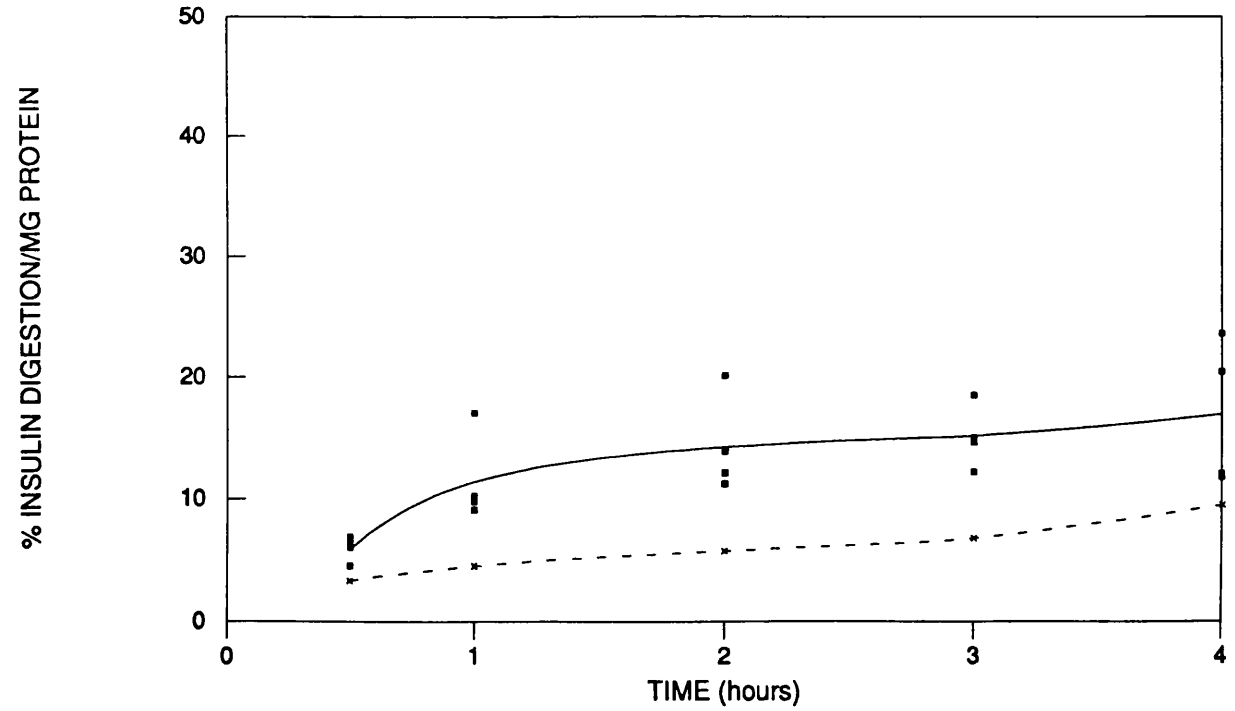

Fig. 3.7c. Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs in the presence of 2,4-dinitrophenol.

Yolk sacs were explanted on the 17th day of gestation, and washed in M199. Tissue was then placed in M199 containing 50μg/ml 2,4,dinitrophenol, an uncoupler of oxidative phosphorylation. After 30 minutes 7μg/ml [^{125}I]-insulin was added to the incubation medium. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg protein in a series of control yolk sacs, while the dashed line shows that in the presence of dinitrophenol.

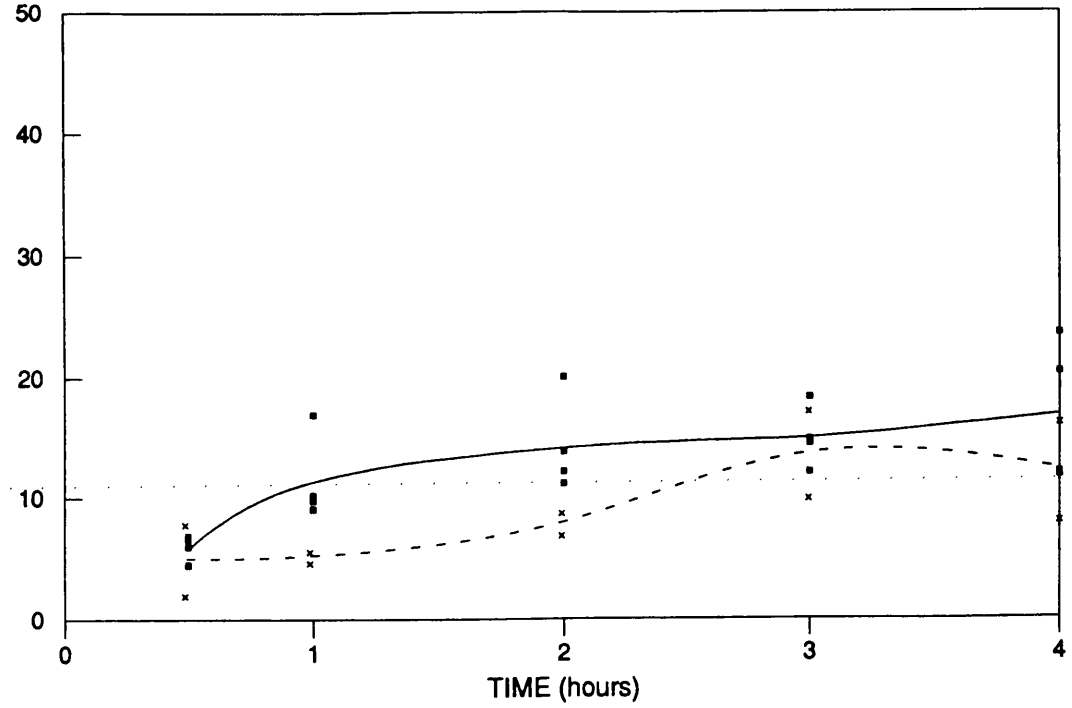

Fig. 3.7d. Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs in the presence of the calcium ion chelator, EGTA.

Yolk sacs were explanted from the mother on the 17th day of gestation, and washed in M199. They were then placed in M199, which contained the Ca^{2+} chelator EGTA at 5mM for 30 mins. After this, [^{125}I]-insulin (7μg/ml) was added and time points taken 30 mins to 4 hours later. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg protein in a series of control yolk sacs, while the dashed line shows that in the presence of EGTA.

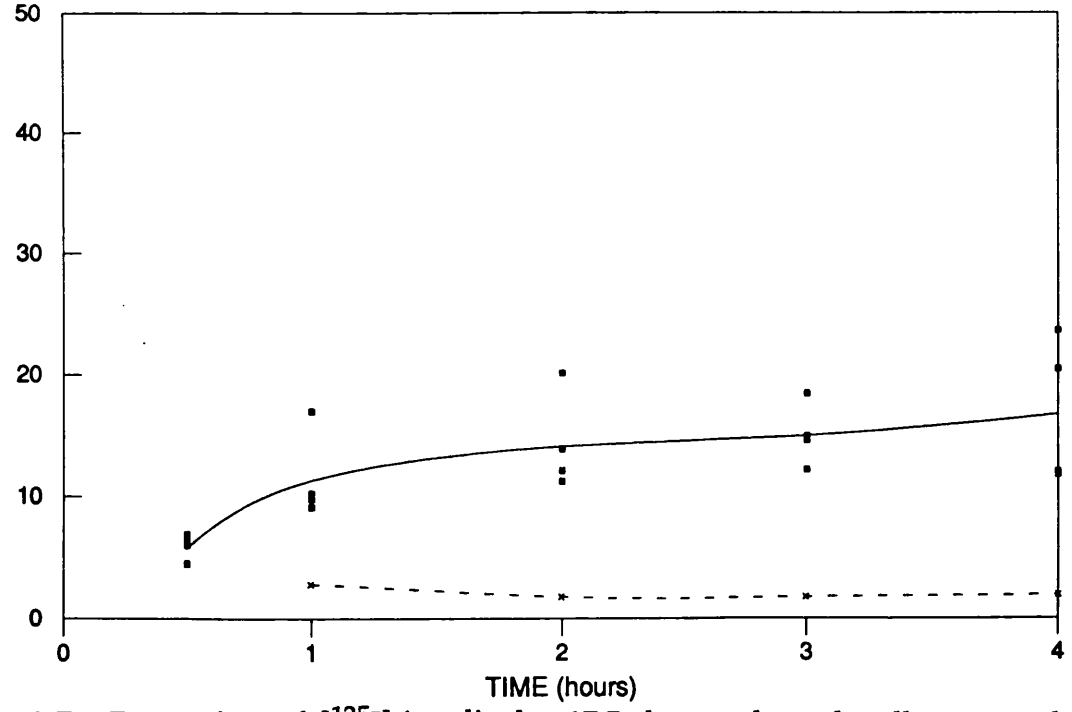

Fig. 3.7e. Processing of [^{125}I]-insulin by 17.5 day explanted yolk sacs at low temperature.

Yolk sacs were explanted from the mother on the 17th day of gestation, and washed in M199. They were then placed in M199, at 4°C for 30 mins. After this, [^{125}I]-insulin (7μg/ml) was added and time points taken 30 mins to 4 hours later. 0.5ml samples of the incubation medium were taken at each time point, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that at low temperature.

10μg/ml of the glycolysis inhibitor sodium fluoride appears to produce only a very small effect on the level of [^{125}I]-insulin digested over the first three hours of incubation. Only the four hour time point shows any reduction in the amount of degradation seen, with sodium fluoride appearing to reduce this to 64% of that seen in the control yolk sacs, although this is probably not significant.

The other glycolysis inhibitor tested, monoiodoacetate, rather than reduce the amount of protein digestion seen, actually appears to increase it over the first 3 hours of the experiment. Acid soluble materials are produced over the first 30 minutes of incubation, followed by a levelling off after this. Overall, this substance has the least effect on uptake of [^{125}I]-labelled insulin by 17.5 day explanted yolk sacs over the time course tested in this experiment.

The effect of 50μg/ml of the inhibitor of oxidative phosphorylation, 2,4-dinitrophenol, appears to produce a more dramatic effect on the level of [^{125}I]-insulin digested than the addition of the glycolysis inhibitors described above. In the presence of this compound, digestion appears to be reduced to less than 60% of that seen in the controls over the course of the experiment, although the production of acid soluble material does appear to increase linearly over the 4 hours of incubation.

A marked reduction in digestion is also apparent when the effect of the calcium chelator EGTA is examined, with the only discrepancy being the slight increase seen at the 3 hour time point, when the level of digestion rises to only slightly less (93.3%) than that seen in the control yolk sacs series. Overall, however, a significant reduction in the level of protein digested is seen in the presence of this compound.

By far the most marked reduction in [^{125}I]-insulin digestion is seen when yolk sacs are incubated at low temperature (4°C). In this case, the level of digestion never rises above 25% of that seen in the controls.

The level of tissue associated radioactivity in the presence of each of these compounds, and also at 4°C, can be calculated as previously described for the control yolk sacs. These values are shown in Table 3.2.

% TISSUE ASSOCIATED/MG PROTEIN

TIME (hrs)	CONTROL	+EGTA	+NaF	+MIA	+DNP	Low Temp
0.5	5.75	1.47	10.30	8.08	5.50	-
1	5.40	1.58	6.64	5.77	4.60	1.33
2	2.28	1.63	3.97	2.62	4.31	1.04
3	2.06	1.69	1.69	3.15	4.11	0.95
4	2.12	1.84	1.99	1.42	4.10	0.96

Table 3.2. Table showing the percentage of [^{125}I]-insulin which was associated with the tissue per mg of yolk sac tissue when 17.5 day explanted yolk sacs were exposed to the radiolabelled ligand for 4 hours. The equivalent values are also shown for yolk sacs in the presence of a number of metabolic inhibitors and at low temperature. Each value has been expressed as a percentage by dividing the counts per minute/ml associated with that department by the total counts present in the experimental system.

From these results, it would appear that the glycolysis inhibitors sodium fluoride and monoiodoacetate initially increase the amount of tissue associated radioactivity, before falling to the same low levels seen in the control series. The presence of 2,4-dinitrophenol results in a steady level of tissue associated material, which does not seem to fall with time, while the presence of EGTA results in a lowering of the relative level of tissue associated material seen with time. At 4°C, the amount of radioactivity associated with the yolk sac is also significantly reduced at all the time points examined.

Processing of [^{125}]I-labelled insulin by cultured anembryonic rat visceral yolk sacs

Rat conceptuses were explanted at 9.5 days gestation and subsequently cultured for a further 8 days with 2 or 3 yolk sacs per bottle as described in Chapter 2.

In the course of culturing the anembryonic yolk sacs, it was observed that occasionally the fluid within them appeared slightly pink by the 8th day of culture. One possibility was that these yolk sacs had developed leaky epithelia, and were no longer capable of selectively taking up substances from their surrounding medium. Consequently, they would be highly permeable to the incubation medium. This medium contains phenol red in the M199, which might account for the pink colouration seen.

Yolk sacs which appeared normal to the naked eye, plus those which appeared slightly pink, were fixed in 10% formal buffered saline for 3 hours. After fixation, the specimens were transferred to 70% propan-2-ol overnight, and subsequently dehydrated in 90% ethanol (for 2 hours), followed by 2 changes of 100% ethanol for 2 hours each. The yolk sacs were then cleared in toluene, and transferred to paraffin wax at 56°C for a total of 4 hours, which included one change of wax.

Sections were cut at 5μm using a Leitz microtome, and stained with either Haematoxylin and Eosin or Masson's trichrome. (For protocol, see Sheehan and Hrapchak, 1980).

The results are shown in Fig 3.8 a,b,c and d.

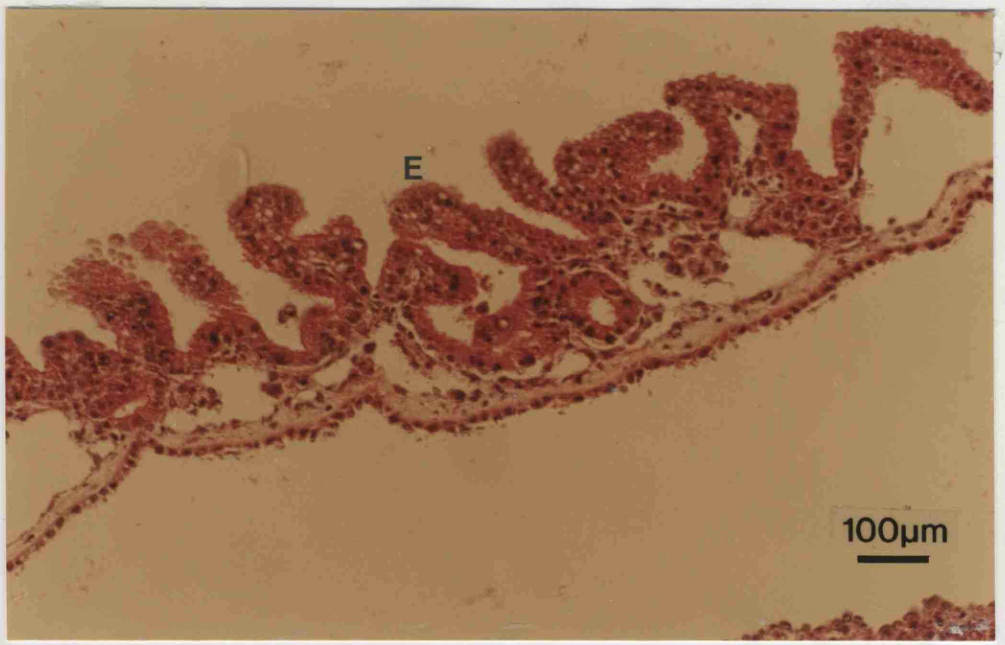

Fig 3.8a Histology of cultured, anembryonic yolk sac which appeared normal, stained with Haematoxylin and Eosin.

Rat conceptuses were removed from the mother on the 9th day of gestation, and subsequently cultured for 8 days at 37°C in a roller incubator in 50:50 rat serum/M199. After this time, the yolk sacs were examined by eye.

This yolk sac was fixed in 10% formalin phosphate-buffered saline (pH7.4) . The tissue was then dehydrated through alcohols and cleared in toluene before being embedded in parrafin wax. 5µm sections were then cut and the sections mounted and stained with haematoxylin and eosin.

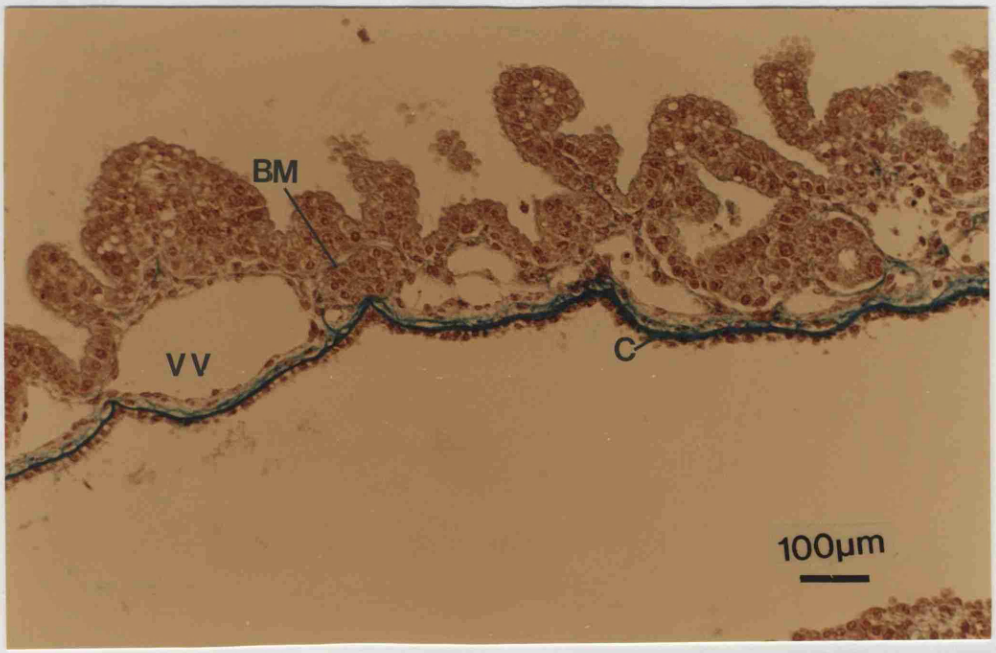

Fig 3.8b. Histology of cultured, anembryonic yolk sac which appeared normal, stained with trichrome.

Rat conceptuses were explanted on the 9th day of gestation and cultured for the next 8 days in 50:50 rat serum/M199. After this time, yolk sacs were examined to see whether they appeared normal.

This yolk sac was fixed in 10% formalin phosphate-buffered saline (pH7.4). The yolk sac was then dehydrated through alcohols, cleared in toluene and embedded in parrafin wax. 5µm sections were cut, mounted and stained with Masson's trichrome, which stains nuclei black and collagen blue.

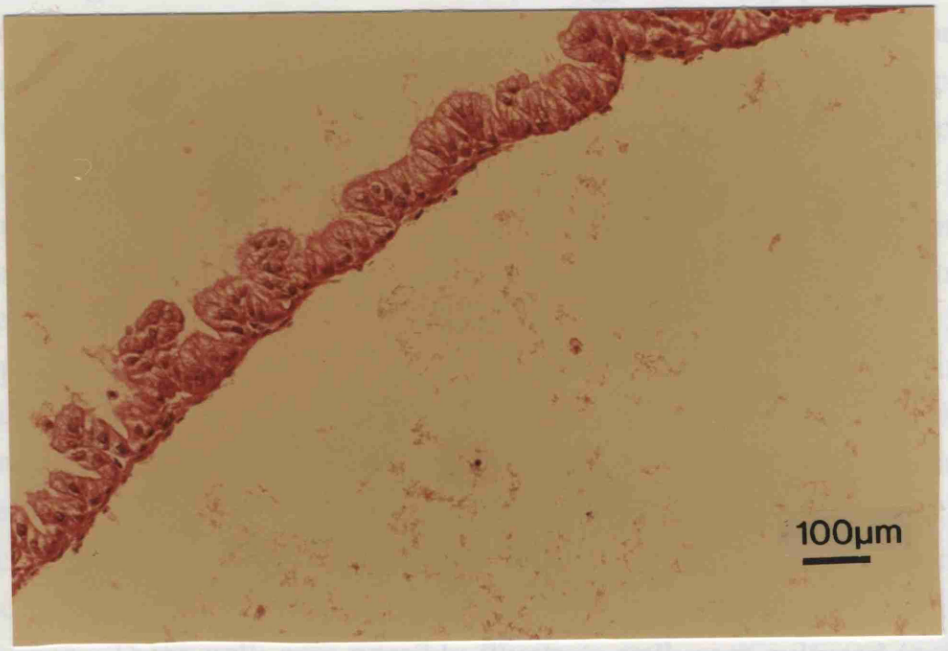

Fig 3.8c. Histology of cultured, anembryonic yolk sac which appeared pink to the naked eye by the 8th day of culture.

Rat conceptuses were explanted on the 9th day of gestation and cultured for the next 8 days in a 50:50 mix of rat serum and M199. After this time, yolk sacs were examined by eye to see whether they appeared normal, or had developed a pinkish tinge to the fluid within them.
This yolk sac appeared pink. It was fixed in 10% formalin phosphate-buffered saline The yolk sac was then dehydrated through alcohols, cleared in toluene and wax embedded. 5µm sections were then cut, mounted and stained with haematoxylin and eosin.

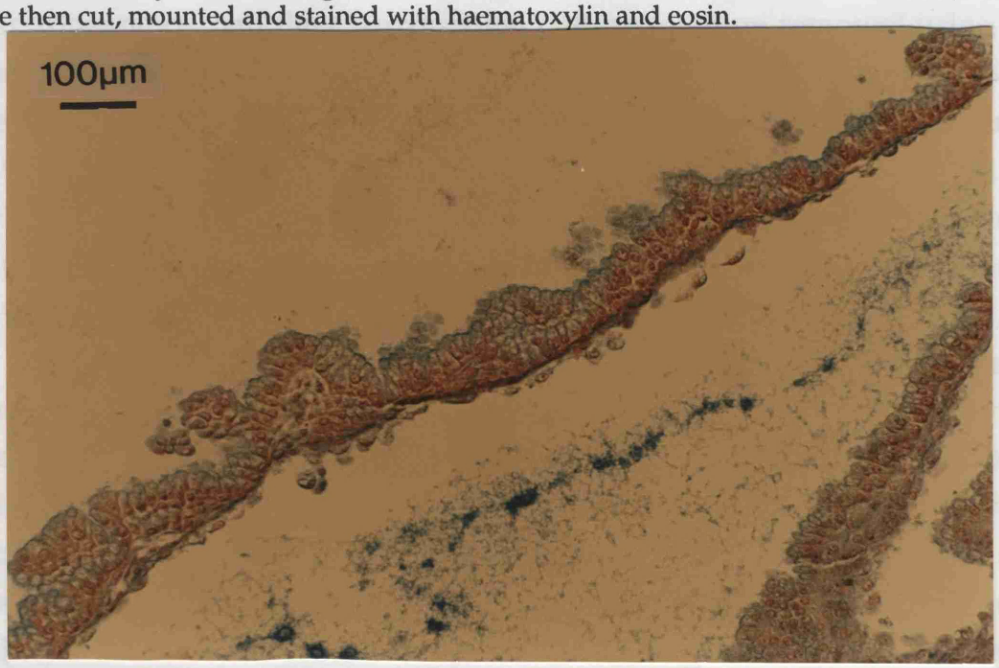

Fig 3.8d. Histology of cultured, anembryonic yolk sac which appeared pink to the naked eye by the 8th day of culture.

Rat conceptuses were explanted on the 9th day of gestation and cultured for the next 8 days in 50:50 rat serum/M199. After this time, yolk sacs were examined by eye to see if they appeared normal, or if the fluid they contained had developed a pink colouration.
This yolk sac was one in which this pink colouration had appeared. It was fixed in 10% formalin phosphate-buffered saline, before being dehydrated through alcohol, cleared in toluene and wax embedded. 5µm sections were then cut and stained with Masson's trichrome, which stains nuclei black and collagen blue.

Figs 3.8 a and b show the morphology of healthy yolk sac tissue. The endodermal cells (E) appear as distinct, well-rounded bodies, supported by the basement membranes (BM). Fig 3.8 b shows a good example of a space within which a vitelline blood vessel would form (VV). Beneath this, the trichrome has strongly stained a continous line of collagen fibres blue (C). Such a network would support the tissue and add strength. In contrast, Figs 3.8. c and d show the morphology of a yolk sac which had taken on this pinkish appearance. The pictures are at the same magnification, but those yolk sac cells in Fig 3.8 c and d appear smaller, more compact and less clearly defined than those in Fig 3.8 a and b. These sections do not contain the large spaces within which blood vessels would form, apparent in the normal tissue. Most striking, however, is the lack of staining for collagen in Fig 3.8d. This would suggest that these yolk sacs do not possess the supportive framework seen in the normal looking ones, and that perhaps fluid is able to leak across this membrane in a non-specific manner. These yolk sacs possibly illustrate yolk sacs cultured in suboptimal conditions for some reason, resulting in subsequent tissue death.

Any yolk sacs demonstrating this pinkish appearance (around 10% of the total yolk sacs cultured), were not subsequently used for experiments, as it was considered that an epithelium which may be leaky could give spurious results.

Those yolk sacs which had been examined and judged normal based on their appearance, had their surrounding culture medium removed using a bent needle attached to a 5ml syringe, and replaced with M199 prewarmed to 37° C. The yolk sacs were then replaced in the incubator and allowed to wash in fresh M199 for approximately one minute. This M199 was removed and again replaced with more M199. This washing procedure was carried out to ensure that no trace of serum remained within the bottle. After the second wash, the M199 was replaced with 3mls M199 which contained [^{125}I]-insulin at 7μg/ml.

The yolk sacs were regassed with 95%O_2/5%CO_2 for approximately one minute before being returned to the roller incubator. Yolk sacs were harvested after 30, 60, 120, 180 and 240 minutes. Two 0.5ml samples of the incubation fluid were taken for analysis, before the yolk sac was carefully transferred out into a large excess of fluid by submerging the bottle containing the yolk sacs in a large container of 1% saline. The yolk sacs were then floated on to Petri dishes, and the saline around them removed using a fine needle and syringe. Yolk sacs were then burst, and the fluid within them (referred to an extraembryonic fluid although these yolk sacs are anembryonic) was collected. Tissue was placed in 1ml 1M NaOH for counting and subsequent analysis for protein.

Results

Fig. 3.9 shows the results of 4 separate [^{125}I]-labelled insulin experiments, where *n* lies between 7 and 13. The only difference between the two systems is that when cultured anembryonic yolk sacs are used the counts present in the extraembryonic coelomic fluid must also be taken into consideration separately.

TIME (mins)	AVERAGE % DIGESTION (% of added substrate which becomes acid soluble) in total medium	per mg protein
30	9.5 (Range = 6.4...14.2)	6.95 (Range = 5.3..9.0)
60	15.32 (Range = 11.0...21.8)	11.19 (Range = 7.9..17.3)
120	21.38 (Range = 18.2...26.3)	10.71 (Range = 3.6..16.4)
180	25.72 (Range =23.9...27.5)	17.33 (Range = 16.7..17.9)
240	40.2 (Range = 40.2...44.9)	31.46 (Range = 13.9..43.02)

Table 3.3. Percentage digestion of [^{125}I]-insulin by cultured, anembryonic yolk sacs.

The values for percentage digestion are derived as described earlier for the 17.5 day explanted yolk sacs. In this case, the production of acid soluble digestion products from [^{125}]I-labelled insulin appears to increase gradually over the 4 hours incubation. When these figures are plotted (Fig 3.9a), the gradual production of acid soluble material is apparent.

Once again, this value was then divided by the total amount of yolk sac tissue present in each bottle, to normalise these results for any variation in yolk sac size, or in the number of yolk sacs present in a bottle. Fig 3.9b shows the percentage [^{125}I]-insulin digestion per mg of yolk sac protein. In this case, degradation appears to increase dramatically over the final 2 hours of the experiment. From this graph it becomes apparent that after 4 hours of the incubation, approximately 32% of the insulin present has been digested per mg of yolk sac protein. This is approximately twice as much as the level of insulin degradation per mg yolk sac protein calculated for the 17.5 day explanted yolk sacs. However, the total amount of insulin degradation occurring in the explanted yolk sacs was higher because the total amount of protein present was greater.

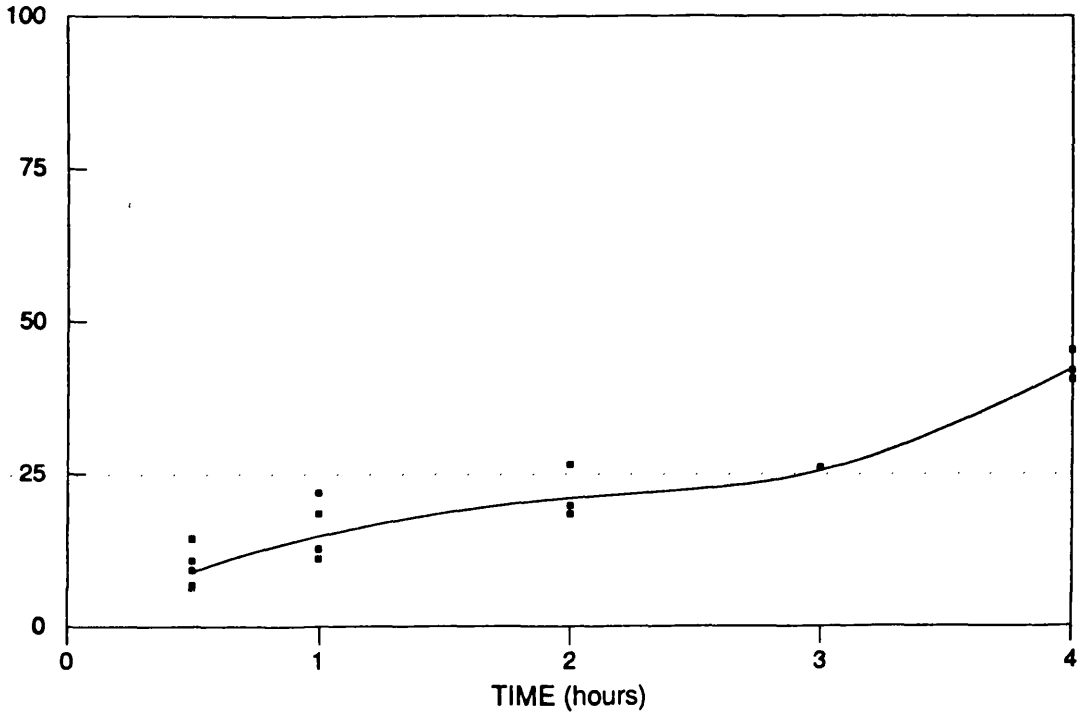

Fig 3.9a. Processing of [^{125}I]-insulin by cultured anembryonic yolk sacs.

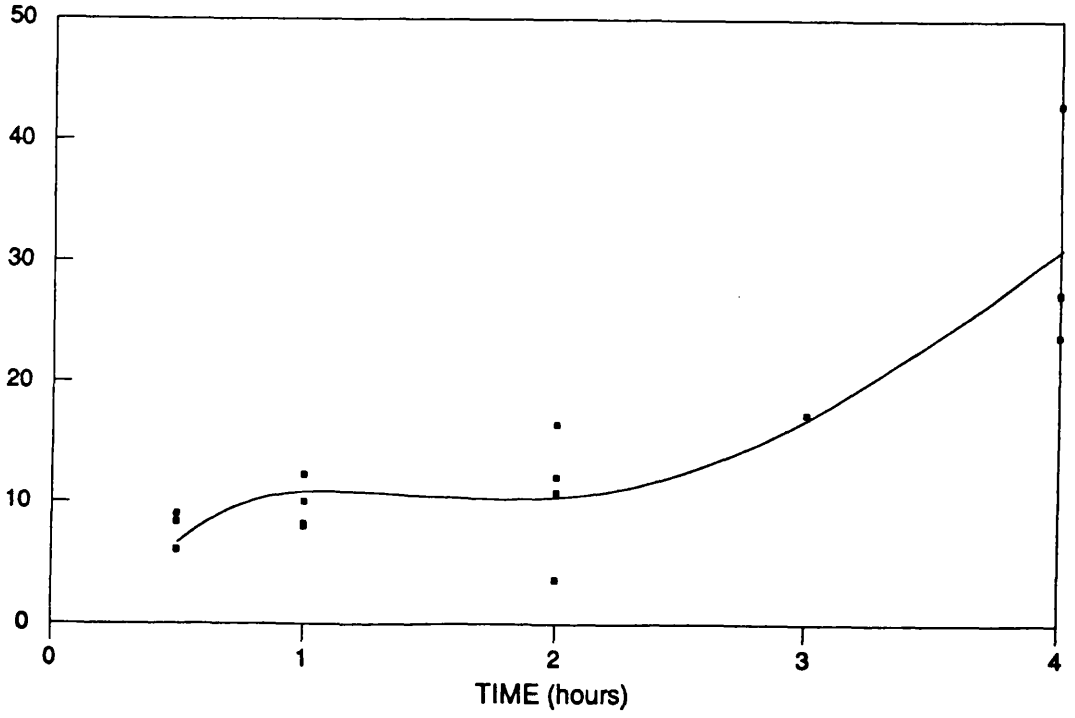

Fig 3.9b. Processing of [^{125}I]-insulin by cultured, anembryonic yolk sacs per mg yolk sac protein.

Yolk sacs were explanted from the mother on the 9th day of gestation, and cultured for the subsequent 8 days in a 50:50 mixture of M199 and rat serum. After this, the incubation medium was replaced with fresh M199 alone to wash the yolk sacs. This was removed and M199 containing [^{125}I]-insulin at 7μg/ml added. The yolk sacs were then incubated for between 30 minutes and 4 hours. At the end of the incubation period, medium surrounding the yolk sacs was removed, counted for radioactivity, subjected to acid precipitation, and then recounted. The values calculated are plotted here for 4 experiments, while the solid line represents the average calculated from each individual measurement. Fig 3.9b shows the results in Fig 3.9a normalised for the amount of protein present.

It is also possible to examine the levels of radioactivity associated with the yolk sac tissue rather than that within the medium, by dividing the c.p.m. measured in the tissue by the total number of counts present in the system. This is shown in Fig. 3.10, from which it can be seen that the level of tissue associated radioactivity rises from the initial value of 8.4% after 30 minutes to a maximum of 15.2% after 2 hours, only to fall over the final 2 hours of the experiment.

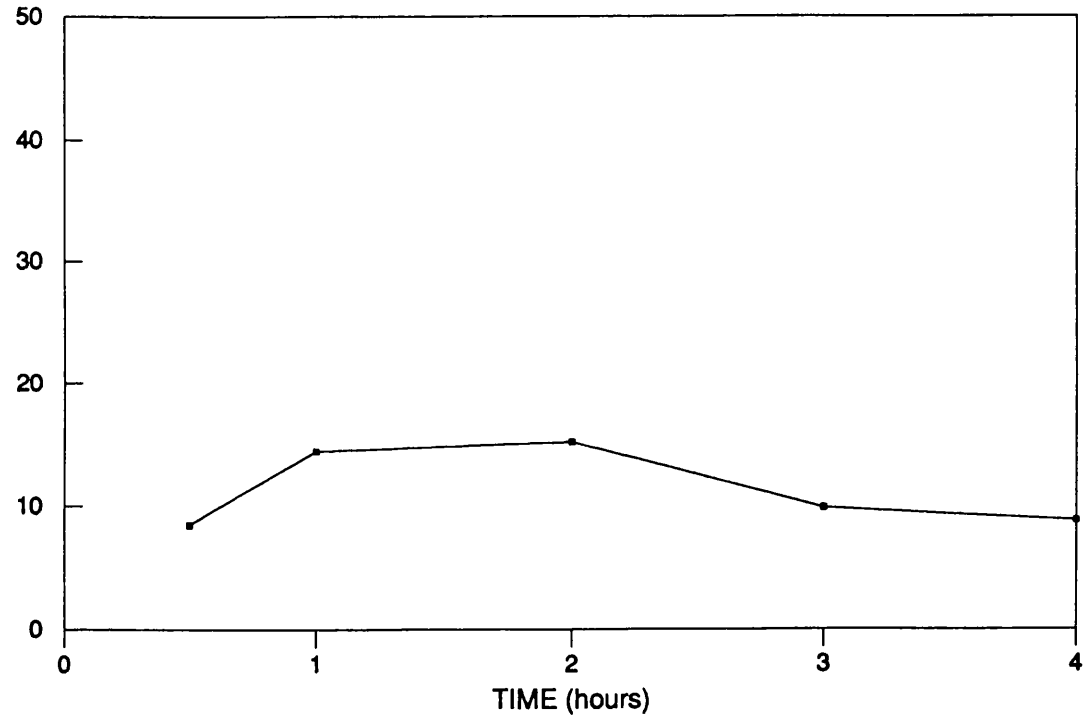

Fig 3.10 Percentage of the total radioactivity which is tissue-associated when 17.5 day cultured yolk sacs are exposed to [^{125}I]-insulin.
The level of radioactivity associated with yolk sac tissue can be estimated by averaging the counts measured in each yolk sac. Each point is the average of between 7 to 13 yolk sacs).

Culture of the anembryonic yolk sac as a closed vesicle allows an examination of the fluid within the yolk sac, which can be further analysed. The proportion of radioactivity associated with this component is calculated from the total c.p.m. measured in the fluid collected when the yolk sacs are burst, divided by the total number of counts in the system. This can subdivided further by counting the proportion of these counts which are acid soluble by subjecting the fluid collected to acid precipitation. This is shown in Fig 3.11, in

which the solid line shows the percentage radioactivity which is associated with this EEC fluid, while the dashed line below illustrates the proportion of these counts which are acid soluble.

The number of counts associated with the fluid rises steadily over the 4 hours of the experiment. At its final point maximum, 39.2% of the total radioactive counts present are associated with this fluid. However, when the acid soluble component is examined, it becomes apparent that the majority of the counts present within the EEC fluid are associated with acid soluble material. These results clearly demonstrate that the majority of counts detectable within the yolk sac are associated with the soluble products of digestion.

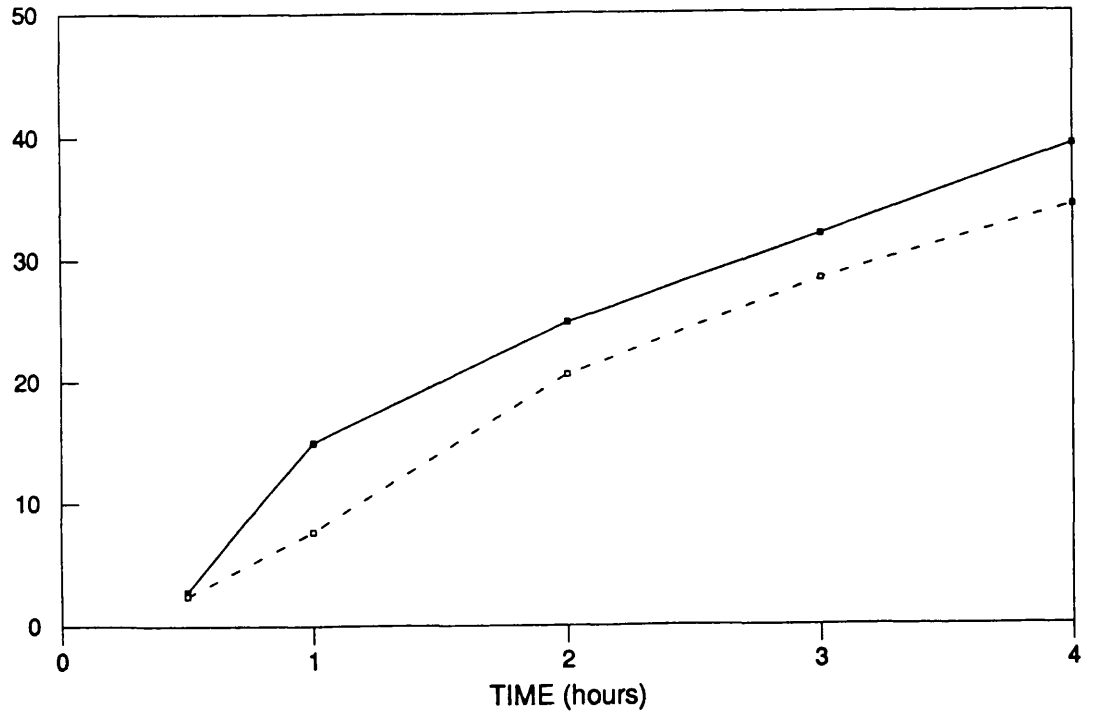

Fig 3.11 Percentage of the total radioactivity which is associated with the EEC fluid when 17.5 day cultured yolk sacs are exposed to [^{125}I]-insulin.
Yolk sacs were removed from the mother on the 9th day of gestation, and subsequently cultured for 8 days in 50:50 rat serum/M199. After this, the incubation medium was removed and replaced with M199 containing [^{125}I]-insulin at 7μg/ml. Yolk sacs were harvested between 30mins and 4 hours later, and the EEC fluid within them carefully collected using a fine needle and syringe. The level of radioactivity associated with yolk sac EEC fluid can be estimated by averaging the counts measured in each yolk sac. Each point is the average of between 7 to 13 yolk sacs. This fluid was also acid precipated to determine what proportion was associated with the products of digestion, and this is shown in the dashed line.

Because the cultured yolk sac develops as a closed vesicle, the number of counts which are present in the EEC fluid can also be used to examine the mechanism by which radioactivity accumulates within this vesicle. The fluid within these yolk sacs was collected, and then weighed to give an estimate of its volume. The total counts in the medium at the beginning of the experiment was taken to equal 7μg/ml of intact insulin and using this figure, a concentration within the EEC was estimated from the number of counts and its volume. It was then acid precipitated as previously described, to give the proportion of the counts which were associated with intact protein to those associated with breakdown products.

The difference between the total counts, and the counts which were associated with acid soluble digestion products in this fluid was taken as an estimate of the amount of intact [^{125}I]-insulin within that fluid. This estimated amount of intact protein is plotted against time in fig 3.12, in which each point represents the amount of intact protein estimated within individual yolk sacs. The line on this graph has been fitted using linear regression analysis.

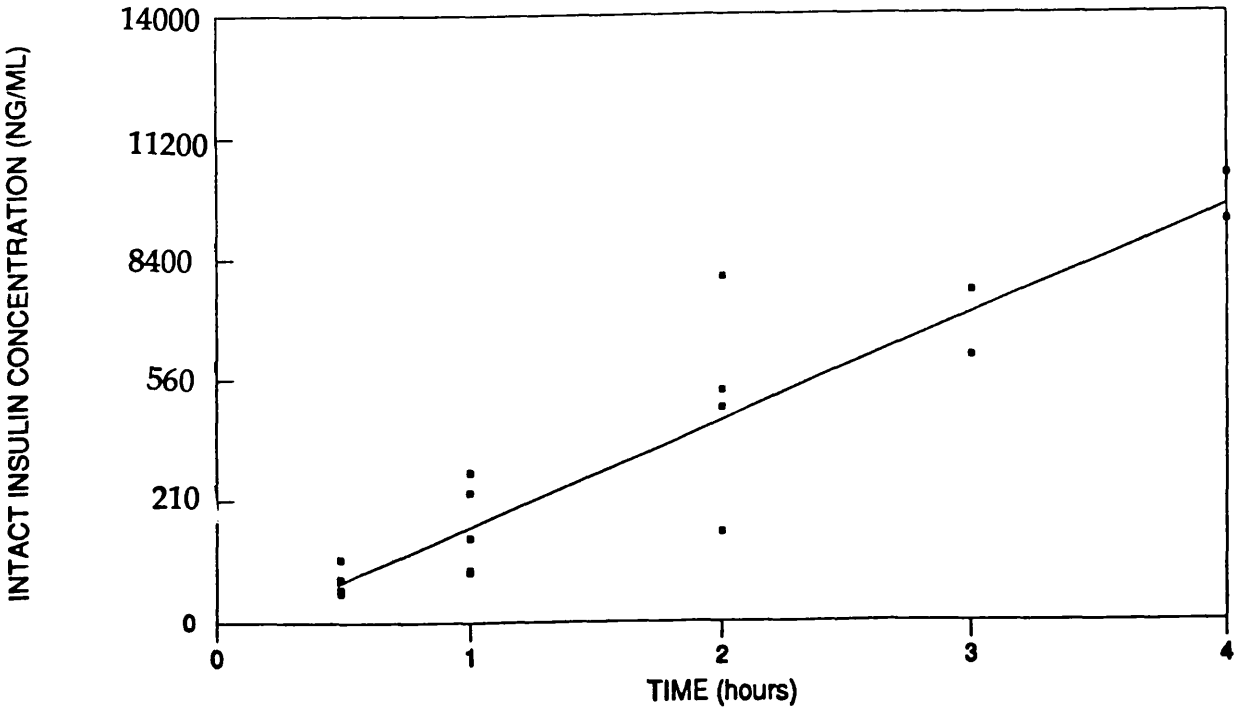

Fig 3.12 Accumulation of Intact [^{125}I]-insulin within cultured, anembryonic yolk sacs.

Yolk sacs were removed from the mother on the 9th day of gestation, and subsequently cultured for 8 days in 50:50 rat serum/M199. After this, the incubation medium was removed and replaced with M199 containing [^{125}I]-insulin at 7μg/ml. Yolk sacs were harvested between 30mins and 4 hours later, and the EEC fluid within them carefully collected using a fine needle and syringe. This fluid was then weighed to give an estimate of its volume. EEC fluid was then subjected to acid precipitation, and the estimated amount of intact [^{125}I]-insulin plotted to see how this varied with time.

In addition to the amount of intact protein which was accumulating within the EEC fluid, the amount of acid soluble material within this fluid has also been plotted against time (fig 3.13).

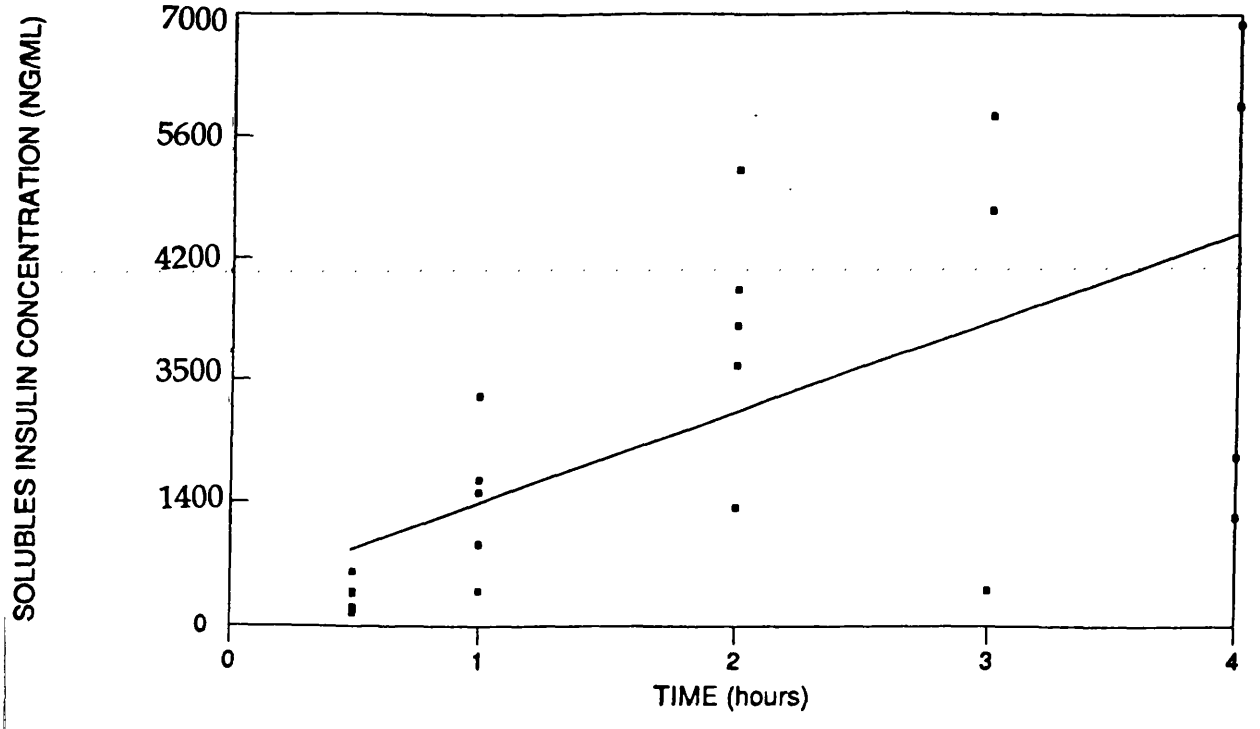

Fig 3.13 Accumulation of acid soluble material within cultured, anembryonic yolk sacs.
Yolk sacs were removed from the mother on the 9th day of gestation, and subsequently cultured for 8 days in 50:50 rat serum/M199. After this, the incubation medium was removed and replaced with M199 containing [^{125}I]-insulin at 7μg/ml. Yolk sacs were harvested between 30mins and 4 hours later, and the EEC fluid within them carefully collected using a fine needle and syringe. This fluid was then weighed to give an estimate of its volume. EEC fluid was then subjected to acid precipitation, and the acid soluble concentration (ie. ng of insulin digested per ml) was plotted to see how this varied with time.

It can be seen from these graphs that there is a linear accumulation of intact [^{125}I]-insulin within the EEC fluid over the 4 hours of the experiment. After 4 hours incubation, there is approximately 840ng/ml of intact protein within the EEC fluid. Similarly there is a gradual accumulation of acid soluble digestion products, and it appears that there are approximately five times as many radioactive counts associated with these soluble digestion products than with the intact protein. The observation that intact protein appears to accumulate within the yolk sac at a steady rate with time, despite the fact that the concentration of intact protein is the incubation medium is known to be falling dramatically, may suggest that some transport mechanism does exist in this system.

Attempts to Elucidate the Mechanism of Insulin Digestion

As in the case of the 17.5 day explanted yolk sacs, the effects of various metabolic inhibitors upon [^{125}I]-insulin processing were tested using the anembryonic system in an attempt to elucidate the mechanism by which this degradation was occurring. Again, experiments were carried out using the glycolysis inhibitors sodium fluoride and monoiodoacetate (both at 10μg/ml), the electron transport inhibitor dinitrophenol (50μg/ml), the calcium chelator EGTA (10μg/ml), and also at 4°C. All bottles of yolk sacs were preincubated at 37°C with these metabolic inhibitors, or at 4°C, to allow some equilibration to occur, before the addition of [^{125}I]-insulin at 7μg/ml.

Figures 3.14 a,b,c,d,and e show the results of these experiments.

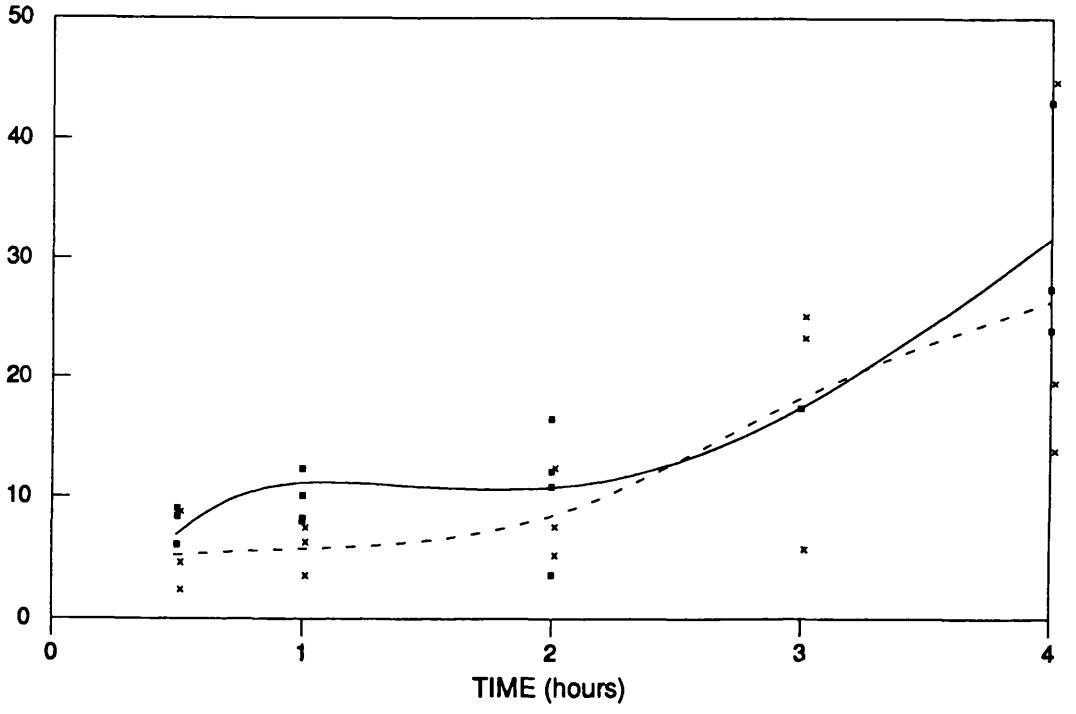

Fig 3.14a Processing of [^{125}I]-insulin by 17.5 cultured anembryonic yolk sacs in the presence of sodium fluoride.

Rat conceptuses were explanted from the mother on the 9th day of gestation and cultured for the next 8 days. Yolk sacs were then washed with fresh, warmed M199 before the introduction of M199 containing 10μg/ml of the glycolysis inhibitor sodium fluoride, and rotated at 37°C for 30 minutes before 7μg/ml [^{125}I]-insulin was added. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that in the presence of sodium fluoride.

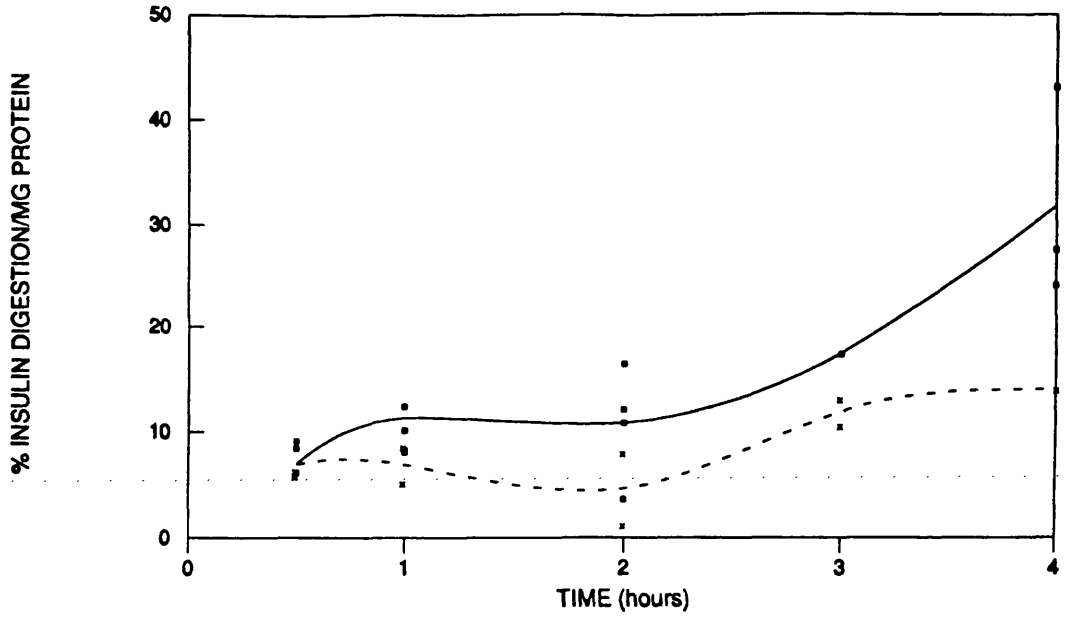

Fig 3.14b Processing of [^{125}I]-insulin by 17.5 cultured anembryonic yolk sacs in the presence of monoiodoacetate.

Rat conceptuses were explanted from the mother on the 9th day of gestation and cultured for the next 8 days. Yolk sacs were then washed with fresh, warmed M199 before the introduction of M199 containing 10μg/ml of the glycolysis inhibitor monoiodoacetate and rotated at 37°C for 30 minutes before 7μg/ml [^{125}I]-insulin was added. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that in the presence of monoiodoacetate.

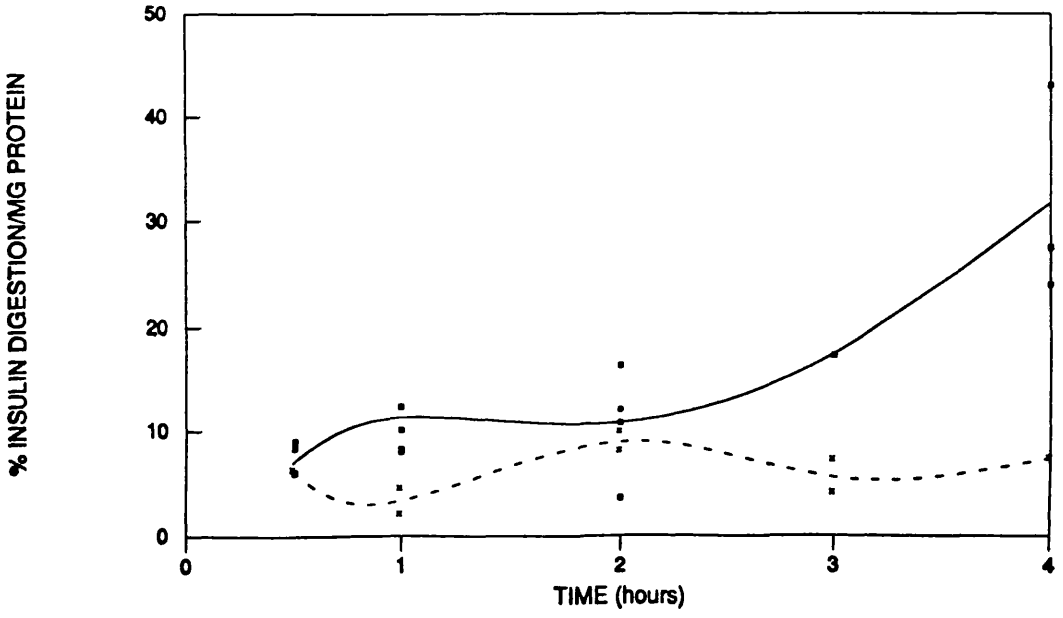

Fig 3.14c Processing of [^{125}I]-insulin by 17.5 cultured anembryonic yolk sacs in the presence of 2,4-dinitrophenol.

Rat conceptuses were explanted from the mother on the 9th day of gestation and cultured for the next 8 days. Yolk sacs were then washed with fresh, warmed M199 before the introduction of M199 containing 50μg/ml of the electron transport inhibitor dinitrophenol and rotated at 37°C for 30 minutes before 7μg/ml [^{125}I]-insulin was added. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that in the presence of dinitrophenol.

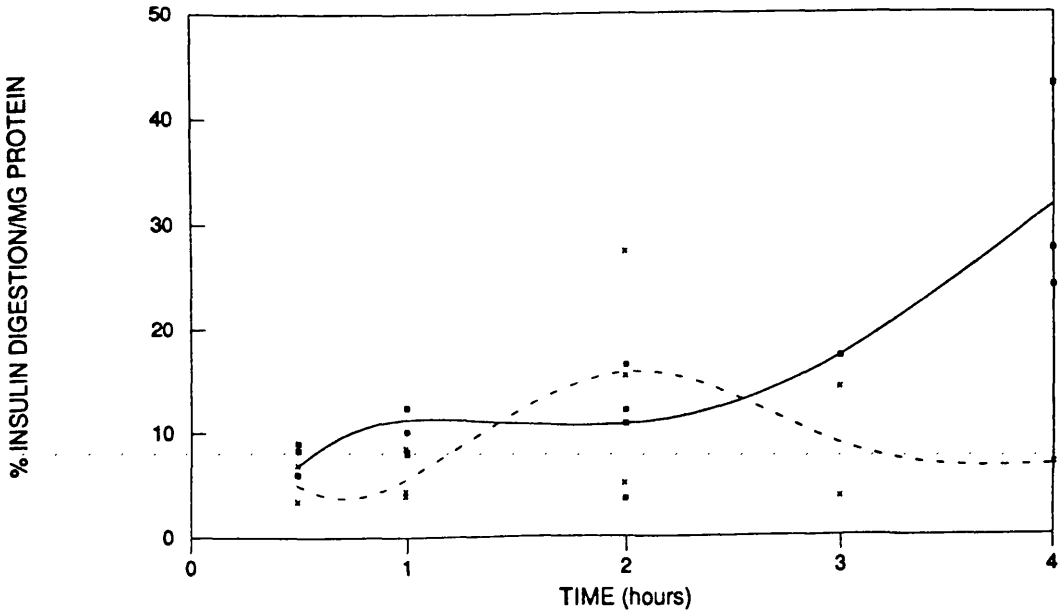

Fig 3.14d Processing of [^{125}I]-insulin by 17.5 cultured anembryonic yolk sacs in the presence of EGTA.

Rat conceptuses were explanted from the mother on the 9th day of gestation and cultured for the next 8 days. Yolk sacs were then washed with fresh, warmed M199 before the introduction of M199 containing 5mM of the Ca^{++} chelator EGTA. Yolk sacs were then rotated at 37° C for 30 minutes before 7μg/ml [^{125}I]-insulin was added. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control volk sacs, while the dashed line shows that in the presence of EGTA.

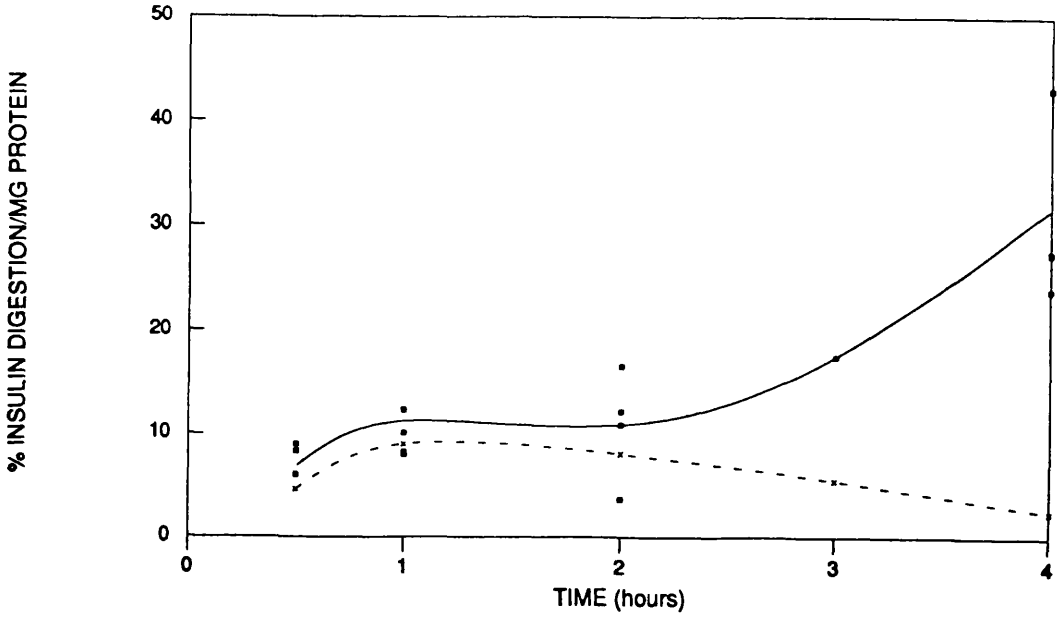

Fig 3.14e Processing of [^{125}I]-insulin by 17.5 cultured anembryonic yolk sacs at low temperature.

Rat conceptuses were explanted from the mother on the 9th day of gestation and cultured for the next 8 days. After this, yolk sacs were washed in M199 before being placed in a roller incubator at 4° C. 30 minutes later, [^{125}I]-insulin (7μg/ml) was added to the incubation medium. Tissue was harvested between 30 minutes and 4 hours later. Samples of the incubation medium were collected for radioactive counting, acid precipitated and then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that at low temperature.

The addition of 10μg/ml of the glycolysis inhibitor sodium fluoride to the incubation medium results in a small reduction in the level of acid soluble digestion products seen over the first 2 hours of incubation. At the 3 hour time point, however, the level of digestion is approximately equal to that seen in the control series, while at the 4 hour time point degradation is 85% that seen in the controls. These small fluctuations are probably not significant. However, the addition of the other glycolysis inhibitor tested, monoiodoacetate, does result in a significant inhibition of protein degradation over the course of the experiment.

In the case of 2,4,-dinitrophenol, this compound also appears to significantly reduce the amount of acid soluble digestion products formed after 1 hour of incubation. After the 4 hours of incubation, the percentage of [^{125}I]-insulin is only 24% of the control value seen.

When EGTA is present in the incubation medium, the pattern seen is very similar to the effects of 2,4-dinitrophenol, except that at the 3 hour time point, the amount of insulin degraded is actually greater than that seen in the controls. After 4 hours, the levels is again significantly less than that seen in the control yolk sac, at around 24% of the control value.

As was the case with the 17.5 day explanted yolk sac, the most obvious reduction in digestion of [^{125}I]-insulin is seen when the yolk sacs are incubated at low temperature. Here the level of protein digestion gradually falls with time to only 8% seen in the control yolk sacs after 4 hours.

The level of tissue associated radioactivity in the presence of each of these compounds, and also at 4°C, can be calculated as previously described for the control yolk sacs. In addition, the level of radioactivity associated with the EEC fluid can also be examined in the case of each of these inhibitors. These values are shown in Table 3.4, which shows the percentage of tissue associated radioactivity in comparison with the controls, as well as the percentage associated with the EEC fluid collected from within the yolk sac vesicle. In the first column of the EEC values, the values in brackets represent the proportion of radioactive counts which were associated with acid soluble material.

% TISSUE ASSOCIATED/MG PROTEIN

TIME (hrs)	CONTROL	+EGTA	+NaF	+MIA	+DNP	Low Temp
0.5	8.44	0.87	12.44	7.90	0.90	4.93
1	14.48	2.72	7.28	9.25	2.71	3.59
2	15.21	1.01	5.97	4.97	4.31	2.38
3	9.81	1.48	10.05	8.83	5.91	5.33
4	8.76	4.85	8.11	5.11	2.30	3.20

% EEC ASSOCIATED/MG PROTEIN

TIME (hrs)	CONTROL	+EGTA	+NaF	+MIA	+DNP	Low Temp
0.5	2.80 (2.59)	0.17	2.34	5.38	3.37	0.06
1	14.92 (7.59)	2.03	5.69	10.39	1.04	0.04
2	24.85 (20.49)	1.29	9.44	8.79	11.29	0.36
3	31.96 (28.33)	1.18	31.78	21.18	5.06	0.47
4	39.19 (34.19)	2.34	35.95	7.93	2.23	0.15

Table 3.4 Tables showing the percentage of [^{125}I]-insulin which was associated with the tissue and with the extraembryonic fluid per mg yolk sac protein when 17.5 day cultured anembryonic yolk sacs were exposed to the radiolabelled ligand for 4 hours. The equivalent values are also shown for yolk sacs in the presence of a number of metabolic inhibitors and at low temperature. Each value has been expressed as a percentage by dividing the counts per minute/ml associated with that department by the total counts present in the experimental system.

From these results, it would appear that the glycolysis inhibitors sodium fluoride and monoiodoacetate have little effect upon the amount of tissue associated radioactivity in comparison with the control yolk sacs. The presence of 2,4-dinitrophenol results in a steady low level of tissue associated material, which does not seem to fall with time, while the presence of EGTA results in a lowering of the relative level of tissue associated material seen with time. At 4°C, the amount of radioactivity associated with the yolk sac is also significantly reduced at all the time points examined.

Discussion

These experiments indicate that the digestion of [^{125}I]-insulin occurs at an extremely rapid rate in both the cultured and in the corresponding explanted yolk sac systems. In the case of the 17.5 day explanted yolk sacs, digestion is very rapid over the first hour of incubation and is almost complete after 4 hours. In cultured, anembryonic yolk sacs, digestion of this protein occurs over the entire 4 hours of incubation, with a steady increase in the level of acid soluble digestion products appearing in the incubation medium with time.

The mechanism which has previously been proposed for insulin uptake in the explanted model, (Livesey, 1979) is via receptor-mediated pinocytosis. In this, the ligand binds to specific cell surface receptors resulting in the subsequent invagination of the plasma membrane and the introduction of endocytic vesicles (or pinosomes) into the cell. This process acts, therefore, as a selective concentration mechanism, whereby the intracellular concentration of certain molecules can be raised, without the concomitant increase in fluid which would result from fluid-phase pinocytosis.

In the case of the yolk sacs explanted directly from the mother on the 17th day of gestation, the production of low molecular weight, acid soluble material occurs very quickly. At the end of the second hour of incubation around 75% of the total substrate added (here [^{125}I]-insulin) has been depleted. However, the remaining 25% of protein does not appear to be broken down over the final 2 hours of incubation. This may indicate that a large proportion of the receptors binding insulin have been down-regulated from the cell surface, and are not available to bind the remaining substrate. When the level of degradation is normalised for the amount of yolk sac tissue present, after 4 hours, approximately 20% of the [^{125}I]-insulin originally present has been broken down per milligram of protein. Most of the work carried out using 17.5 day yolk sacs placed them in a much greater volume of fluid per yolk sac than is used in these experiments. Here, the volume has been reduced to bring it in line with that used for anembryonic yolk sacs, with the result that substrate depletion has resulted.

The level of radioactive counts which is associated with the yolk sac tissue remains comparatively low at all stages of the incubation, falling to around 2% per mg protein of the total counts after 4 hours. This indicates that little accumulation of radiolabelled ligand is occurring within the tissue. The binding of ligand to the yolk sac tissue must be, therefore, the rate limiting step in the production of acid soluble material, because if the converse were true (and digestion were the rate limiting step) the level of radiolabelled ligand associated with yolk sac should increase with time.

In an attempt to establish the mechanism by which insulin digestion was occurring a variety of compounds known to inhibit pinocytosis were investigated. Work carried out by Livesey and Williams (1979) demonstrated that [^{125}I]-BSA digestion occurred solely in the 17.5 day yolk sac as a result of intracellular lysosomal action, but it is not possible to exclude the activity of such extracellular enzymes on [^{125}I]-insulin degradation without carrying out the appropriate experiments. In the experiments reported here, both sodium fluoride and monoiodoacetate have been tested as endocytosis inhibitors. Sodium fluoride (or more specifically F^-ions) is an inhibitor of glycolysis, the process by which glucose is converted to pyruvate with the production of adenosine triphosphate (ATP). Under aerobic conditions, the pyruvate formed in this process is used to fuel the citric acid cycle and electron transport chain, which in turn produce a vast excess of energy in the form of ATP. In the case of sodium fluoride, the compound is acting as a metal complexing agent, either inhibiting the function of a metalloenzyme, or removing the metal ion from such an enzyme (see Fig 3.15). All kinases require Mg^{2+} (or some other divalent metal ion) for activity, and two essential steps in glycolysis are catalysed by such kinases: the initial conversion step from glucose to glucose-6-phosphate, and the phosphorylation of fructose-6-phosphate to fructose 1,6 diphosphate. Fluoride ions could, therefore, be acting in either of these reactions. Monoiodoacetate also produces its inhibitory effect during glycolysis, although at a later stage in the metabolic pathway than sodium fluoride. Monoiodoacetate acts specifically to inhibit the enzyme glyceraldehyde-3-phosphate dehydrogenase, which is responsible for the conversion of glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate, a by-product of which reaction is the production of NADH. In the case of explanted yolk sacs neither of these compounds significantly affected the amount of [^{125}I]-insulin which was broken down. The most obvious explanation of these results are that the high rate of protein digestion seen is not occurring via an energy consuming process, which would seem to exclude receptor-mediated pinocytosis and suggest that some cell mediated event which did not involve internalisation was occurring. These results contrast with the findings of Duncan and Lloyd (1978) who reported that monoiodoacetate abolished the pinocytic uptake of [^{125}I]-PVP, [^{125}I]-BSA, and colloidal [^{198}] Au by 17.5 day yolk sacs. However, all these compounds are taken up non-specifically. Perhaps of more relevance to the processing of insulin are the findings of Kooistra and Lloyd (1985), who found that monoiodoacetate had no effect on the processing of insulin in human fibroblasts.

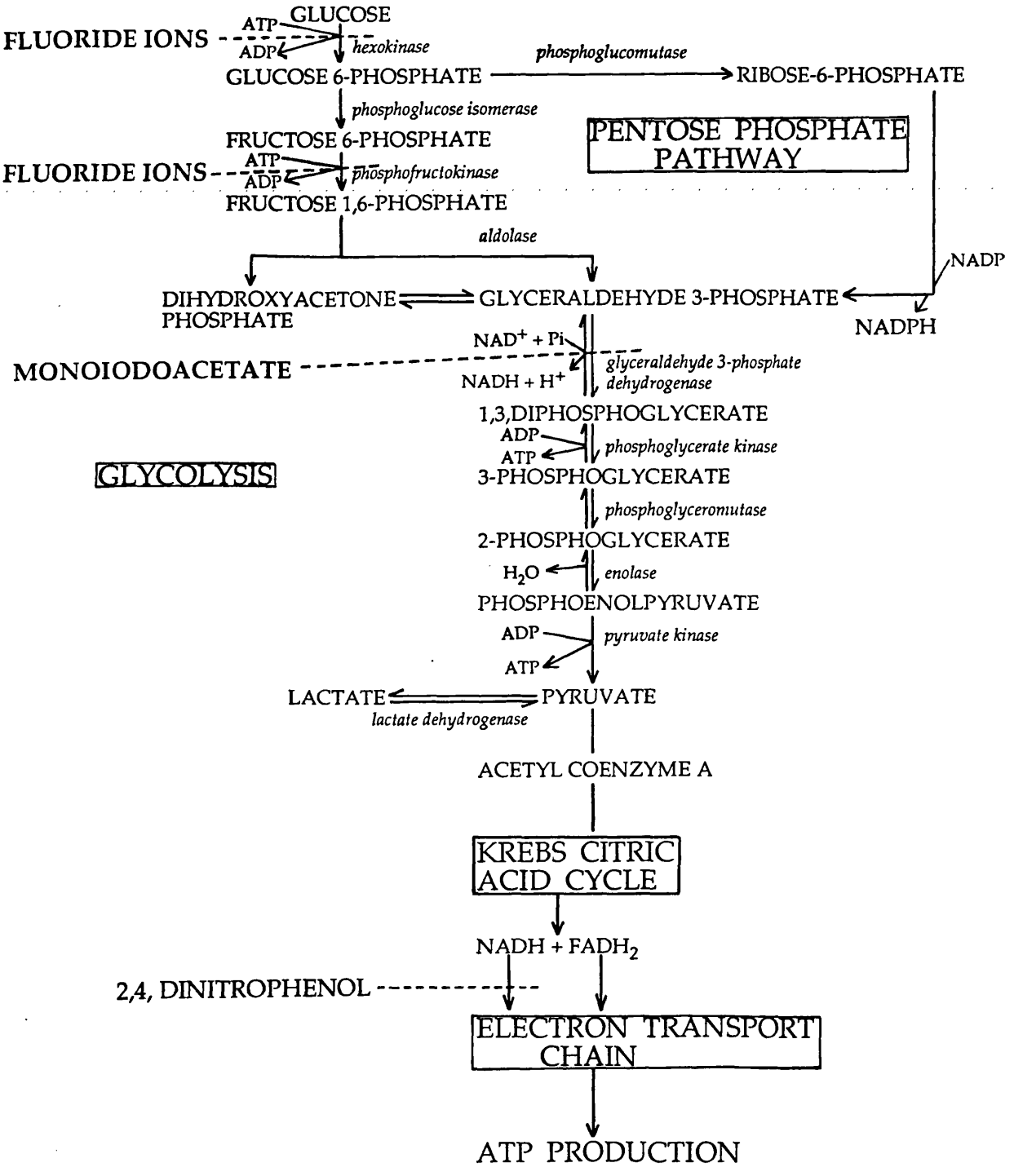

Fig. 3.15 Sites of Action of Metabolic Inhibitors

The finding that at low temperature there is a significant reduction in the amount of protein digestion seen may also support the hypothesis that a cell surface enzyme is responsible for insulin degradation in this system. A reduction in temperature will result in the inhibition of a variety of enzymes, which may explain this result. However, low temperature is also a known inhibitor of pinocytosis. This result may also be interpreted, therefore, as echoing the findings of Duncan and Lloyd (1978), who found a similar reduction in the processing of exogenous ligands in the 17.5 day explanted yolk sac by reducing temperature. Low temperature has two principal effects: firstly, the supply of metabolic energy (in the form of ATP) will be reduced. But secondly, and it is believed, more importantly, the mobility and fluidity of the membrane components is drastically reduced. In the case of endocytosis, recent work has established that endosome-lysosome fusion is not inhibited at low temperature, (Haylett and Thilo, 1991). Rather it appears that pinocytosis is affected earlier, prior to this fusion step, with a reduction in membrane invagination, decreasing pinosome function. Fluidity is critical for many membrane-localised phenomena, including membrane fusion. It appears that there is a virtual cessation of pinocytosis when temperatures fall below 20° C, as this is close to the transition temperatures of membrane lipids, below which their movement virtually ceases. This result cannot, therefore, support a pinocytic rather than an ectoenzyme theory of insulin degradation.

A very similar situation is found in the case of EGTA inhibition. Once again a significant reduction in the level of insulin degradation is seen in the presence of this Ca^{2+} chelator. It is known that endocytosis requires the presence of free Ca^{2+} ions in the medium, as uptake is inhibited when the Ca^{2+} chelator is added (Duncan and Lloyd, 1978). That this is due to the removal of Ca^{2+}, rather than to some pharmacological effect of EGTA, is demonstrated by the rapid reversal of this effect when Ca^{2+} ions are added back to the medium.

Precisely why Ca^{2+} ions are so important within this system remains unclear. The role of Ca^{2+} within the cell has been extensively examined, in particular its role in secretion. Like receptor-mediated endocytosis, this involves an interaction between a ligand and a membrane bound receptor. Although many ligands act in the same fashion, a common pathway leading to secretion is initiated following binding of the ligand to the receptor, and it is known that this pathway involves Ca^{2+}. The general cellular mechanisms seen in secretion may be closely related, if not the same, as those seen in endocytosis. For example, one theory is that Ca^{2+} interacts with cytoskeletal components to release secretory vesicles stored within the cell, by allowing them to fuse with the plasma membrane. This is supported by the observation that Ca^{2+} is involved in the movement of spindle microtubules during meiosis and mitosis.

These same cytoskeletal components may be equally important in endocytosis as in exocytosis. Indeed, there is evidence that cytochalasin B and colchicine, known inhibitors of cytoskeletal components, do inhibit pinocytosis (Duncan and Lloyd, 1978). The actions of Ca^{2+} may be more general however, regulating enzymes and ion channels, which again may be similar in the two processes of secretion and uptake.

However, although EGTA is a known inhibitor of pinocytosis, its effects on reducing [^{125}I]-insulin degradation is not enough to confirm that pinocytosis is in fact occurring. Another explanation is that the enzyme responsible for insulin degradation in this system is a Ca^{2+} dependant enzyme.

The final inhibitor tested, 2,4-dinitrophenol, also produced a significant reduction in the level of insulin degraded. This result is much more difficult to explain in terms of an ectoenzyme being responsible for insulin degradation. Dinitrophenol acts as an uncoupler of oxidative phosphorylation; this is the process by which ATP is produced as electrons are transferred from NADH (formed in glycolysis) to O_2, via a series of electron carriers present on the inner surface of the mitochondrial cell wall. In addition, it also stimulates mitochondrial ATPase, resulting in an increase in intracellular P_i, which favours rapid glycogenolysis. In contrast, dinitrophenol is not reported to act as an enzymatic inhibitor. This is the only evidence that the degradation of insulin is occurring via an energy dependant pathway in this system.

The results seen in these inhibition experiments mean that two entirely different mechanisms can be proposed for the degradation of insulin in the 17.5 day explanted yolk sac. Firstly, the lack of effect seen in the presence of sodium fluoride and monoiodoacetate may indicate that an energy requiring internalisation step is not required; rather that digestion may occur via an enzyme at the cell surface. In this system, the result seen in the presence of dinitrophenol can only be interpreted as an anomaly. Alternatively, the reduction in degradation seen in the presence of EGTA and dinitrophenol may indicate that an energy consuming process is required, and that the 17.5 day yolk sac is somehow able to by-pass glycolysis and produce energy via some other intracellular pathway.

The results seen when the cultured, anembryonic yolk sacs are considered, strongly reflect those seen in the system described above. The only significant difference appears to be that the total rate of protein degradation is less in this type of yolk sac, so that the level of percentage digestion products does not level off; rather, it gradually increases over the 4 hours incubation period. When this results are examined in terms of the total protein content, these yolk sacs actually appeared to degrade more [^{125}I]-insulin per milligram of protein than the 17.5 day explanted equivalent. However, these yolk sacs are

considerably smaller and do not deplete the incubation medium of substrate as rapidly.

The cultured yolk sacs also exhibit low levels of radioactivity associated with tissue over the 4 hours of these experiments. In this system, however, the yolk sac develops as a closed vesicle making it possible to examine the accumulation of radioactive counts within this fluid. When this fluid was investigated, it was discovered that the vast majority of the counts were associated with acid soluble material rather than with intact protein. What this may mean is that a proportion of the insulin degraded by the yolk sac could be made available to the developing embryo. If an ectoenzyme were responsible for insulin degradation, this would mean that low molecular weight radiolabelled breakdown products were crossing this epithelium.

The experiments utilising known inhibitors of pinocytosis in this culture system, give very similar results to those seen in the explanted yolk sacs. The only difference is that the glycolysis inhibitors sodium fluoride and moniodoacetate do result in a slight reduction in the production of acid soluble material. EGTA, 2,4-dinitrophenol and low temperature reduce degradation of [^{125}I]-insulin to an extent similar to that seen in the explanted yolk sacs. This again means that it is impossible to conclude definitely whether extracellular degradation or intracellular degradation following internalisation, is occurring. However, the results seen with the glycolysis inhibitors in the cultured system may mean that an energy dependant internalisation process is required for digestion of insulin, in this system at least. One possible explanation which may account for the anomaly seen in the presence of the glycolysis inhibitors is that yolk sacs recently explanted have been demonstrated to possess large glycogen stores which are not present in those which have been cultured (Decatris, 1988). Glycogen can readily be converted to glucose-1-phosphate, which is in turn converted to glucose-6-phosphate by the action of phosphoglucomutase, so providing a substrate for glycolysis in these yolk sacs. In the case of yolk sacs which have undergone culture for eight days, they might be expected to be somewhat metabolically depleted. Additionally, there could also be intracellular pyruvate stores again exhausted in the cultured yolk sacs, providing another possible substrate for energy product.

The results of these experiments to not enable the precise mechanism of [^{125}I]-insulin degradation to be determined in the yolk sac. Two further methods of investigation should now be employed in an attempt to finally establish whether degradation is extracellular or intracellular. Firstly, the time course of the release of digestion products should be examined. If digestion is occurring principally via an extracellular mechanism, then the addition of radiolabelled protein should result in the production of acid soluble digestion

material in the incubation medium almost instantaneously. Alternatively, uptake of insulin into the cell followed by transfer to the lysosomes and subsequent release of digestion products across the lysosomal membrane, should results in a time lag for the production of acid soluble products. The other approach would be to utilise agents known to inhibit lysosomal activity as this would result in an inhibition of [^{125}I]-insulin degradation if uptake were occurring via this route. Chloroquine and ammonium chloride are both weak bases known to be concentrated within the lysosomes were they result in an increase in pH resulting in a decrease in the activity of lysosomal enzymes. Leupeptin is also known to inhibit cysteine-proteinases, present in the lysosomes, and the addition of these compounds should add further information.

Another possibility, not investigated in these experiments, is that degradation may be occurring intracellularly, but via a non-lysosomal pathway. This could be investigated partly by studying the localisation of fluorescently labelled insulin to investigate whether such labelled insulin could be detected within lysosomes.

CHAPTER FOUR

PROCESSING OF INSULIN-LIKE GROWTH FACTOR I (IGF-I) BY THE RAT VISCERAL YOLK SAC.

Introduction.

Closely related to insulin, both in terms of structure and function, are the insulin-like growth factors-I and -II (IGF-I and -II) (Zapf and Froesch, 1986). These molecules are also believed to play a major role in development (Sara and Hall, 1980).

The IGFs have been demonstrated to produce insulin-like effects on all the insulin target tissues so far studied. For example, in adipose tissue, the effects observed include an increase in glucose metabolism (Froesch *et al*, 1966), the stimulation of glucose transport (Zapf *et al*, 1978), and inhibition of lipolysis (Froesch *et al*, 1985). In these cases, however, IGFs prove to be only one fifth to one hundredth as potent as insulin in producing these responses. In the adult, IGF-I is most abundant in the liver, though lower levels are detectable in many other tissues, while IGF-II is only expressed significantly in the brain (Murphy *et al*, 1987).

IGFs have also been shown to stimulate cell proliferation in a wide variety of cells grown in culture: both IGF-I and -II stimulate DNA synthesis and cell proliferation in cultures of human fibroblasts as well as transformed fibroblast cell lines (Froesch *et al*, 1985). In particular, IGFs have been demonstrated to act as potent mitogens in fetal cells cultured from several species, including chick embryo fibroblasts (Zapf *et al*, 1978) and fetal rat brain cells from embryonic day 15 and 16 (E15 and 16)(Lenoir and Honegger, 1983), prompting the suggestion that they play an important role during development (reviewed Underwood and D'Ercole, 1984; Sara and Hall, 1984). This theory is further strengthened by reports that several fetal tissues or cells secrete either IGF-I or -II when grown in culture (D'Ercole *et al*, 1980; reviewed Nissley and Rechler, 1984), while specific IGF receptors are present in many fetal tissues examined; for example, specific IGF-I receptors have been reported in the human fetal liver (Chernausek *et al*, 1987). Like insulin, there is no evidence that IGF-I crosses the placenta, at least in the case of dogs, sheep and rats (D'Ercole and Underwood, 1980), and so any IGF-I detectable in the fetus must be of fetal origin. Indeed, several fetal tissues have been demonstrated to produce IGFs, including 11 day mouse embryonic limb bud micromass cultures, and 21 day fetal myoblasts (Milner and Hill, 1984).

In man, IGFs circulate bound to specific carrier proteins (Ooi and Herington, 1988). Six classes of binding protein (or IGFBPs) have so far been identified in human serum (Shimasaki *et al*, 1991; Sheikh *et al*, 1992). The majority of IGFs in the circulation bind to IGFBP-3, which has a molecular weight of 150kDa, and is growth hormone dependent (Baxter, 1988). This is present only during the later stages of gestation, when it binds most of the circulating IGF-I and -II (Gargosky *et al*, 1991). Another, smaller molecular

weight protein (25kDa) is IGFBP-1, which has been found to be identical to placental protein 12 and pregnancy associated endometrial α1-globulin (Koistinen *et al*, 1986). This is found in higher concentrations in cord blood and amniotic fluid (Drop *et al*, 1984), and has been demonstrated by immunocytochemical techniques to be present in human fetal tissue between 14-16 weeks of gestation (Hill *et al*, 1989). This particular binding protein appears to be GH-independent (Povoa *et al*, 1984) but insulin-dependent, (Cotterill *et al*, 1988) and has also been demonstrated to act as an inhibitor of DNA synthesis (Liu *et al*, 1991). The remaining well characterised BP, IGFBP-2, has been isolated from the rat liver cell line BRL 3A (Mottola *et al*, 1986; Binkert *et al*, 1989) and has a high affinity for IGF-II. The functions of the remaining binding proteins have yet to be elucidated. Although the potency of IGFs to produce insulin-like effects on target tissue is only 6% that of insulin (Guler *et al*, 1987), their plasma concentration (approximately 800ng/ml in adults) is 1000 times greater than the insulin concentration (Baxter, 1986). Thus, one important role of these binding proteins is to prevent them binding to IGF receptors and producing this enormous potential insulin-like activity. Another important role they play is to dramatically lengthen the plasma half life of the IGFs, since by increasing the molecular weight they protect the proteins from kidney filtration. They therefore play a role in the modulation of IGF effects, as they deliver IGFs to their target tissues, while having no action upon insulin (Adamo *et al*, 1992). It has also been proposed that IGFBP-1 may play a role in the regulation of fetal growth in the human, (Wang and Chard, 1992) as intrauterine growth retardation resulting from a reduction in uteroplacental circulation is also associated with decreased IGF-I levels and a corresponding increase in IGFBP-1, suggesting the two may be connected. The mechanism of the interaction between binding protein, IGF-I, and fetal growth remains to be elucidated, however.

Sara *et al*, (1981) were able to detect IGF-I in the human fetus at 22 weeks of gestation, while a high affinity IGF-I receptor appeared around the 17th week (Sara *et al*, 1983). After this, levels of both hormone and receptor rise with gestational age. Levels of IGF-I in the umbilical cord at delivery show a positive correlation with birth weight, though are still at a lower level than is seen in the adult. In contrast, IGF-II is present during late gestation and shows no correlation with birthweight, gestational age, or with IGF-I levels (Gluckman *et al*, 1983; Bennett *et al*, 1983).

In the mouse, IGF-I appears to be produced by the developing liver from E11, with production levels increasing with liver growth until E16. Hepatic synthesis decreases in this case after birth (D'Ercole *et al*, 1980). This work also demonstrated secretion of immunoreactive IGF-I from E11 limb bud

mesenchymal micromass cultures, and fetal heart, brain, intestine, kidney and lung at E17. IGF-I receptors have been reported in fetal mouse liver cells and implicated in the proliferation and differentiation of erythroid progenitor cells (Akahane *et al*, 1987). IGF-I is also believed to play a role in the normal development of preimplantation mouse embryos, with addition of IGF-I to the culture medium of two-cell embryos stimulating the number of cells in the resulting blastocysts entirely by increasing the number of cells in the inner cell mass (Harvey and Kaye, 1992).

Similarly, work carried out on the developing rat has revealed low levels of IGF-I in the fetus, which increase to adult levels after birth, at around the time growth becomes GH-dependent (Hall and Sara, 1983). In marked contrast, IGF-II is the predominant IGF present in developing rat sera, with high constant levels detectable between days 11 to 14 (Rotwein *et al*, 1987) which fall dramatically after birth (Moses *et al*, 1980; Beck *et al*, 1988a). There is an abrupt decrease in the expression of hepatic IGF-II between 18 to 20 days after birth, which corresponds with a surge in plasma glucocorticoid levels. This observation has lead to the proposal that circulating glucocorticoids may play a role in the control of IGF-II production postnatally (Beck *et al*, 1988b). There appears, therefore to be a developmental switch in the rodent from fetal to adult forms of IGF which occurs around the time of weaning.

IGFs produce their effects by binding to cell surface receptors, which span the cell membrane and produce intracellular changes in the concentrations of second messengers, though precisely which systems are effected remains unclear. It has been reported that IGF receptors appear prior to those for insulin during chick development, with specific IGF-I binding detectable in day 2 chick embryos, followed by insulin binding on day 3 (Bassas *et al*, 1985). These stages of chick development correspond approximately to E11 and E13 in the rat. However, Mattson *et al* (1988), have reported specific IGF-I binding to peri-implantation mouse embryos, suggesting a role for IGF-I in early rodent development.

Werner and coworkers (1989) carried out a comprehensive investigation into the developmental regulation of the rat IGF-I receptor gene around the time of birth, using a specific cDNA probe to hybridise with mRNA encoding for part of the receptor's α subunit. Expression of the IGF-I receptor gene decreased gradually from the maximal levels seen (on E20 in liver and brain, and between E20 and P8 in stomach) throughout the postnatal period up to day 50. In contrast, expression of the IGF-I gene in these tissues increased over the same developmental period. The expression of the IGF-I receptor mRNA suggests that this receptor may be involved in the growth and differentiation of

certain organs in the rat. However, when IGF-I receptor gene expression is at its highest, IGF-I gene expression is low. One possible explanation for this apparent anomaly is that some other ligand is acting at the IGF-I receptor to produce its mitogenic effects. Insulin, with its structural similarity to IGF-I is one obvious candidate for this role, as is IGF-II, whose gene expression is maximal during rat fetal development (Brown *et al*, 1986). Both these ligands are capable of interaction with the IGF-I receptor.

Another possibility is suggested by the report of a fetal insulin-like growth factor I receptor (Alexandrides and Smith, 1989), present in fetal rat skeletal muscle and which has a larger molecular weight β subunit than the adult receptor. This receptor does not appear to bind insulin, but it undergoes phosphorylation in the presence of low concentrations of both IGF-I and insulin. This may be due to insulin producing a phosphorylation of fetal IGF-I receptors by some cross-reaction.

The precise interaction which occurs during rat development between insulin and IGF-I and their corresponding receptors is, therefore, far from clear. In comparison with insulin, the processing of IGF-I has not been investigated in the rat visceral yolk sac. IGF-I is thought to be taken up via receptor-mediated endocytosis in the cultured rat liver cell line BRL-3A2 (Geary *et al*, 1981) and primary fetal rat neurons (Nielsen *et al*, 1991). Receptor-mediated endocytosis and degradation of both IGF-I and -II has been confirmed in cultured neonatal rat astrocytes (Auletta *et al*, 1992), in which both ligands were found to be rapidly internalised after binding to their respective receptors, and to be subsequently degraded to amino acids. However, experiments with the lysosomal inhibitor, chloroquine, resulted in a reduction in the amount of degradation of both IGFs seen, and also produced retention of IGF-I within cells, while IGF-II was recycled to the cell surface. This suggests, that in astrocytes at least, there are some differences between the intracellular processing of these two ligands.

In the present study, it was decided to investigate processing of IGF-I in both the cultured anembryonic yolk sac system, and the corresponding 17.5 day explanted system to see how this compared to that of insulin. As IGF-I and insulin are such structurally similar molecules it was decided to investigate whether IGF-I was processed by a similar mechanism to that envisaged for insulin, or whether any differences were apparent between these systems. This information may provide an insight into the role of insulin and IGF-I during rat development.

Radiolabelling of IGF-I

The procedure followed for radiolabelling of IGF-I with [^{125}]Iodine was essentially that used for the labelling of insulin, the only difference being that the amount of IGF-I labelled had to be reduced because of the much higher cost of this protein in comparison with insulin. 2.25mg of IGF-I (CIBA-Geigy) had previously been dissolved in 225µl of 0.1M acetic acid, and split into 25µl aliquots. 475µl of 50mM phosphate buffer was added to one of these aliquots, before four Iodobeads (Pierce) were added to the protein for 5 minutes. After this, 1mCi of [^{125}]Iodine (Sigma) was added and the reaction mixture left for a further 10 minutes, before being run down a 2ml disposable cellulose column (Pierce). The column had again been prepared by the addition of B.S.A. at 5mg/ml, and the protein was washed through using 50mM phosphate buffer. Fifteen fractions were collected and 1µl samples from each were made up to 1ml with 50mM phosphate buffer to enable counting on the gamma counter.

Evaluation of labelling

Fig. 4.1 shows the graph obtained when the number of counts in each fraction is plotted against the fraction number.

In this case, all the labelled IGF-I (here a total of 250µg) was estimated to have been eluted in the second and third fractions. If we assume all 250µg of IGF-I is present in these fractions then the proportion in each can be calculated as before from

$$\frac{\text{number of counts in that fraction}}{\text{total number of counts}} \times 100$$

ie. for fraction 2,

$$\frac{(1747866 \times 925)}{2.02 \times 10^9} \times 100 = 80.03\%$$

and for fraction 3,

$$\frac{(590902 \times 685)}{2.02 \times 10^9} \times 100 = 20.07\%$$

These proportions mean that fraction 2 contains 200.07µg, while fraction 3 contains 50.17µg, of the IGF-I. When the fractions are weighed therefore, it is possible to calculate the concentration of [^{125}I]-IGF-I.

Fraction 2 contains 200.075µg IGF-I in 0.9252ml, which equals 216.3µg/ml or 216.3ng/µl.

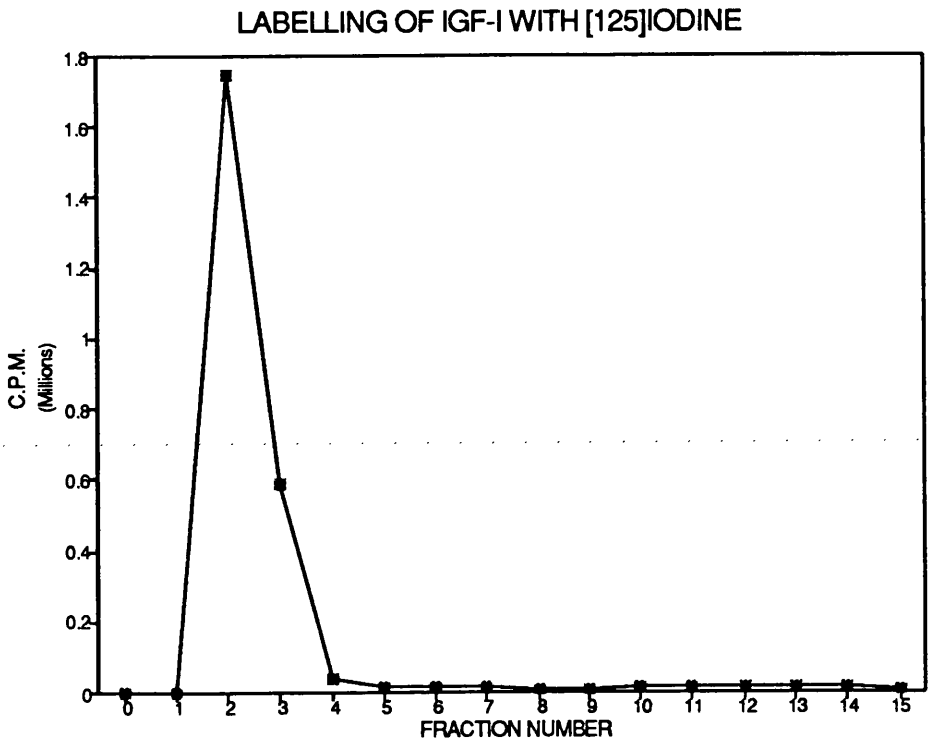

Fig. 4.1. Labelling of IGF-I with [^{125}]Iodine.
250µg of IGF-I in 25µl 0.1M acetic acid was added to 475µl of 50mM phosphate buffer, and 4 Iodobeads (Pierce) added for 5 minutes. 1mCi of ^{125}Iodine was then added, and the reaction mixture left for a further 10 minutes at room temperature to allow iodination to occur. The mixture was then run down a 2ml disposable column, and eluted with 50mM phosphate buffer. 15 fractions were collected in total, and counted using an LKB gamma counter to determine which contained the [^{125}I]-IGF-I.

Efficiency of Labelling

The total counts assumed to be associated with protein are contained within fractions 2 and 3.

1µl samples were taken from both of these fractions and counted for radioactivity. The fractions were then weighed to estimate their volumes.

Fraction 2 contained 1747866 c.p.m. in 1µl taken from a total of 925µl,

and fraction 3 contained 590902 c.p.m. in 1µl from a 685µl total.

The total reaction volume was 500µl, from which a 1µl sample contained 3015570 c.p.m.

so the efficiency of labelling can be calculated from:

$$\frac{(1747866 \times 925)+(590902 \times 685)}{(3015570 \times 500)} \times 100$$

$$= 83.27\%$$

Stability of [^{125}]I-IGF-I

Following the iodination of IGF-I, the [^{125}I]-labelled IGF-I (hereafter referred to as [^{125}I]-IGF-)I was stored at -20°C. In this case, if the percentage of soluble products is plotted against time (fig. 4.2) it can be seen that the stability of the radioligand decreases dramatically with time. Iodinated IGF-I, therefore, appears to be considerably less stable than [^{125}I]-insulin .

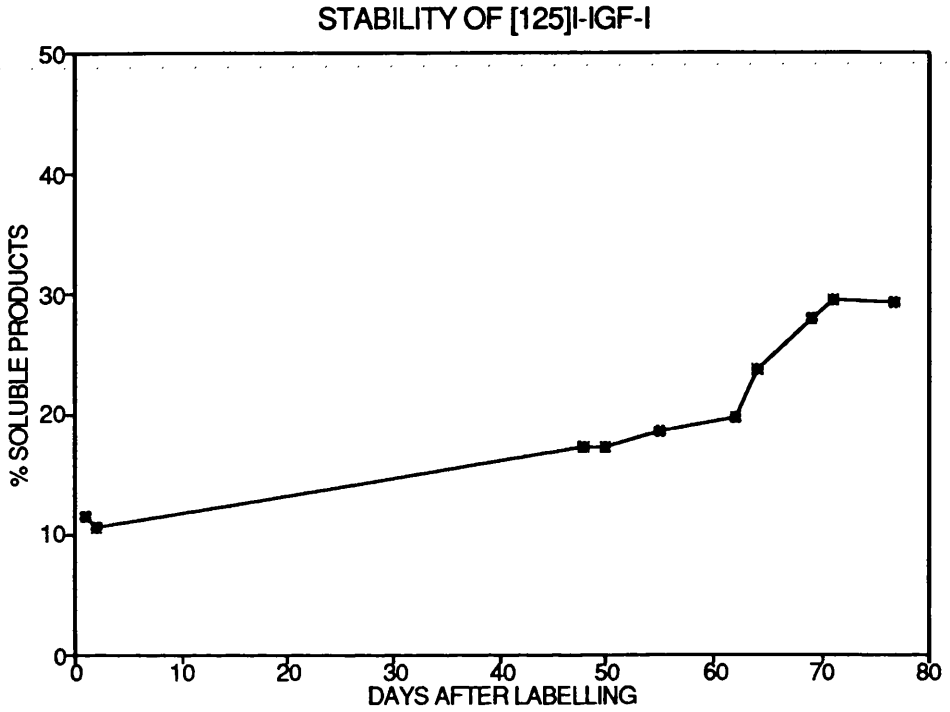

Fig. 4.2 Stability of [^{125}I]-IGF-I with time.
The percentage soluble products produced in a sample of medium which has not been exposed to yolk sac tissue, is here taken as an estimate of the stability of the [^{125}]I-IGF-I complex. If this is plotted against time, it can be seen that approximately 60 days after the initial labelling procedure there is a significant increase in the percentage soluble products, suggesting that [^{125}I] and IGF-I are dissociating.

Processing of [^{125}]I-labelled IGF-I by 17.5 day explanted yolk sacs

Rat conceptuses were explanted at 17.5 days of gestation and the yolk sacs prepared as previously described for [^{125}I]-labelled insulin experiments. Yolk sacs were placed in bottles which contained 3mls M199 with [^{125}I]-IGF-I at 1μg/ml, and placed in roller incubators at 37°C. Harvesting of yolk sacs took place after 30, 60, 120, 180 or 240 minutes of incubation, with samples of incubation medium being taken as before, and the yolk sacs themselves being placed in 1M NaOH.

Results

Table 4.1 shows the results of [^{125}I]-IGF-I digestion by 17.5 day explanted yolk sacs, where *n*, the number of yolk sacs used, is between 4 and 6.

TIME (mins)	AVERAGE % DIGESTION (% of added substrate which becomes acid soluble) in total medium	per mg protein
30	38.04 (Range = 35.46...40.57)	4.56 (Range = 3.6..5.6)
60	57.07 (Range = 56.88...57.25)	7.54 (Range = 5.86..9.22)
120	65.75 (Range = 62.13...69.37)	8.23 (Range = 6.03..10.7)
180	72.38 (Range = 69.49...75.28)	7.24 (Range = 7.24)
240	67.81 (Range = 66.04...69.57)	9.13 (Range = 9.13)

Table 4.1 Percentage of [^{125}I]-IGF-I digested by 17.5 day explanted yolk sacs.

The values for percentage digestion are derived by taking the average from the number of acid soluble counts/ml (minus the amount of solubles produced/ml when no tissue is present) for each vessel which contained [^{125}I]-IGF-I, and dividing by the total number of counts in the medium. The range of values from which this average has been calculated is also given as it is not statistically valid to calculate standard errors for transformed data. These values are plotted in fig 4.3a, where the actual values for percentage digestion in each experiment have been plotted, while the line shows the average between these points shows the average. This value was then divided by the total amount of yolk sac tissue present in each bottle, to normalise these results for any variation in yolk sac size, or in the number of yolk sacs present in a bottle (fig 4.3b).

As was the case when [^{125}I]-insulin was investigated using this system, a large proportion of the radiolabelled protein present in the medium has been degraded over the first hour of incubation. The amount of acid soluble digestion products formed then increases slowly over the next 2 hours, before appearing to fall. Once again, most of the protein present in this system is degraded over the time course of the experiment.

When the percentage [^{125}I]-IGF-I digestion per mg of yolk sac protein is plotted (fig 4.3b), the amount rate of digestion is seen to rise over the first hour of the experiment, and then remain fairly constant.

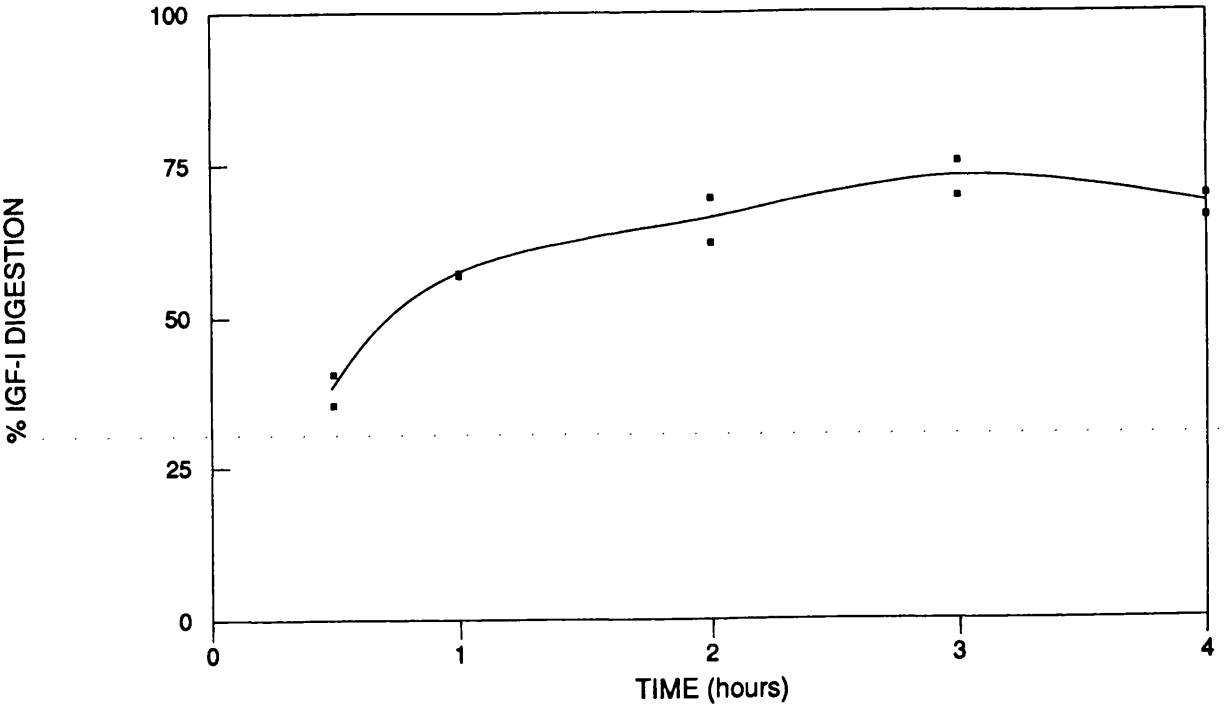

Fig 4.3a Processing of [^{125}I]-IGF-I by 17.5 day explanted yolk sacs.

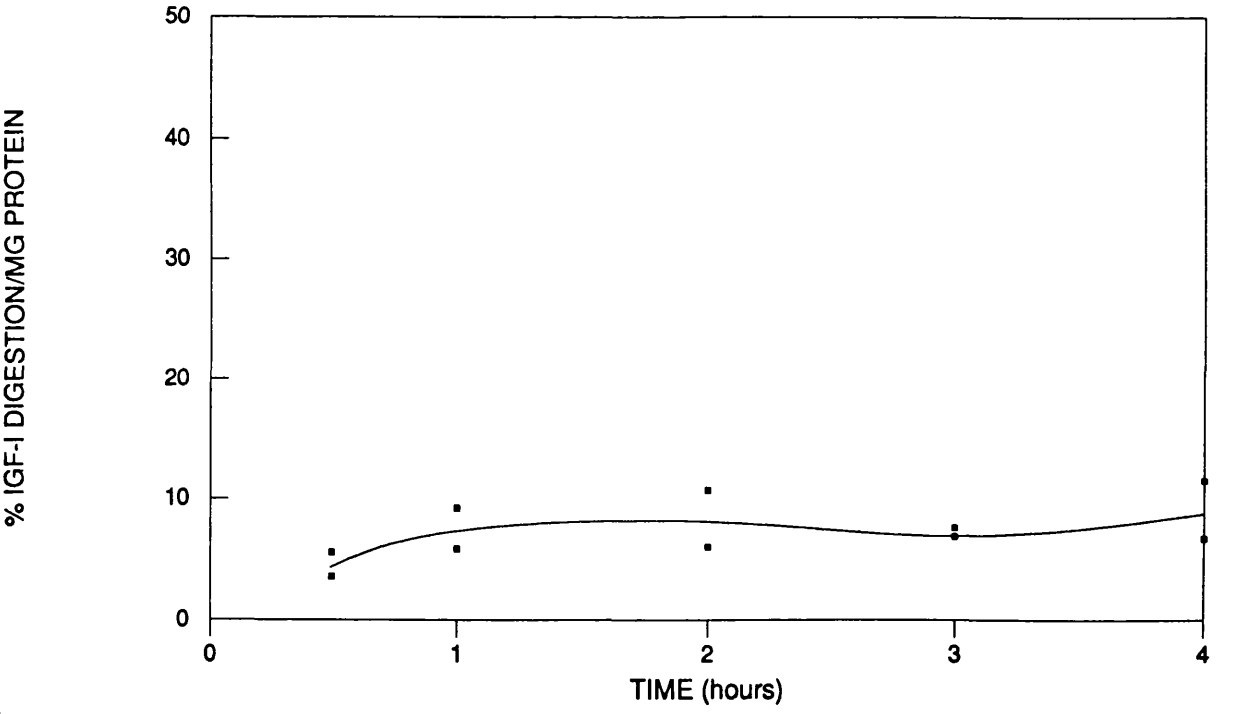

Fig. 4.3b Processing of [^{125}I]-IGF-I by 17.5 day explanted yolk sacs per mg yolk sac protein.

Yolk sacs were removed from the mother on the 17th day of gestation, and washed in M199. They were then placed in glass bottles containing M199 plus [^{125}I]-IGF-I at 1µg/ml. Yolk sacs were then gassed with 95%O_2 and rotated at 37° C for between 30 minutes and 4 hours. At each time point examined, two 0.5 ml samples of the culture medium were removed, and counted for radioactivity. These samples then acid precipitated by the addition of 0.25mls exhausted serum, 0.25mls 40% perchloric acid, and 0.25ml T.C.A., followed by centrifugation for 30 minutes at 4100rpm. The supernatents were then recounted for radioactivity, as this represents the amount of acid soluble digestion products in the medium samples. The values calculated are plotted here for 2 separate experiments, while the solid line represents the average calculated from each individual measurement. Fig 4.3b shows the results in Fig 4.3a normalised for the amount of protein present.

With this protein, it is once again possible to examine the levels of radioactivity which are associated with tissue as opposed to the the incubation medium. This is achieved by dividing the number of counts in the yolk sac tissue by the total number of counts present within the system. As was also the case with [^{125}I]-Insulin, this figure will be an underestimate of the amount of intact protein which becomes tissue-associated, due to the high proportion of degraded protein in the incubation medium. The values plotted are again those which have been normalised for the amount of yolk sac tissue present.

The level of tissue-associated radioactivity is shown in Fig. 4.4, from which it can be seen that that the level of tissue associated radioactivity per milligram protein is initially around 10% of the total counts present. However, the percentage soon falls, eventually reaching a low level of around 3% of the total number of counts present after 4 hours. This pattern suggests that there is little accumulation of radioactivity within the yolk sac tissue, rather that an initial rapid binding event is followed by the release of radioactivity into the medium. It is not possible to determine what proportion of these counts may represent intact protein initially bound and then degraded from protein bound and then possibly released intact.

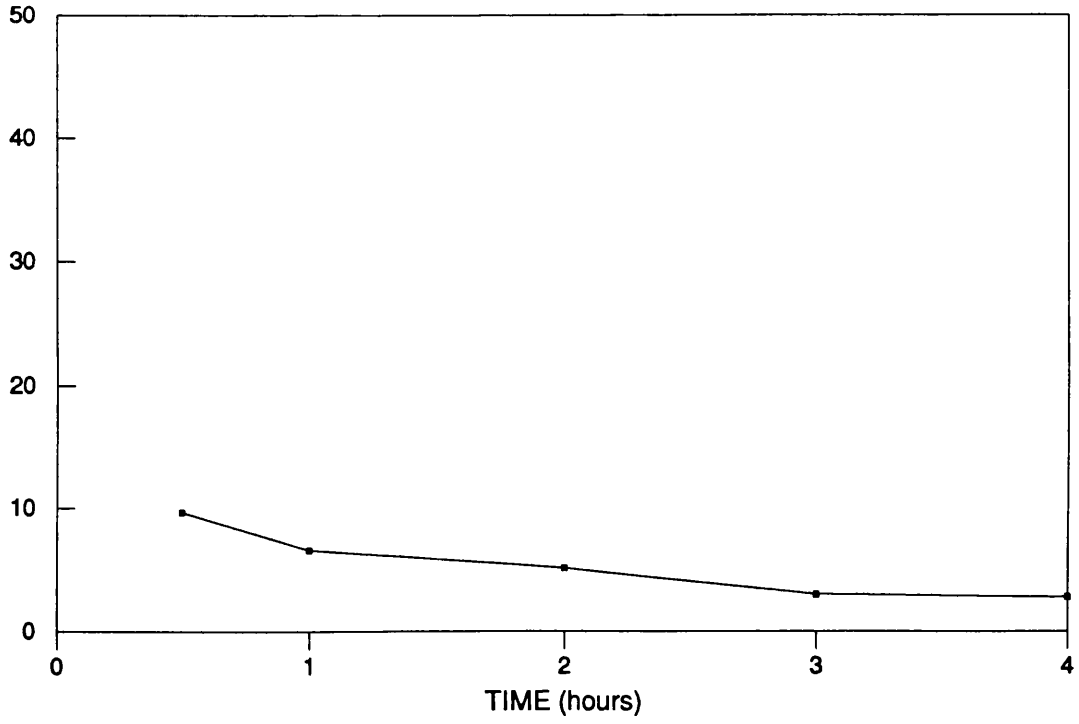

Fig. 4.4 Percentage of the total radioactivity associated with yolk sac tissue.
The level of radioactivity associated with yolk sac tissue can be estimated by taking the average number of counts measured in each yolk sac, and diving this value by the total number of counts in the incubation medium. Each point is the average of between 4 and 6 yolk sacs.

Attempts to Elucidate the Mechanism IGF-I Digestion.

The above experiments demonstrate that exposing 17.5 day explanted yolk sacs to 1μg/ml [^{125}I]-IGF-I results in the production of a large amount of low molecular weight digestion products. The degradation of [^{125}I]-IGF-I was next examined in two situations which may help to elucidate the mechanism of degradation. As with insulin, the same questions must be proposed: namely, is the majority of degradation of this protein occurring via an intracellular or extracellular route.

Yolk sacs were preincubated in the presence of the glycolysis inhibitor sodium fluoride at 10μg/ml for 30 minutes before the addition of [^{125}I]-labelled IGF-I. The presence of this compound should reduce the rate of digestion products formed if an internalisation step is required, as this is known to require energy. Additionally, yolk sacs were also incubated at low temperature (4°C) prior to the addition of labelled IGF-I. The yolk sacs were again harvested after 30, 60, 120, 180, and 240 minutes, and the results plotted in Fig.4.5 a and b.

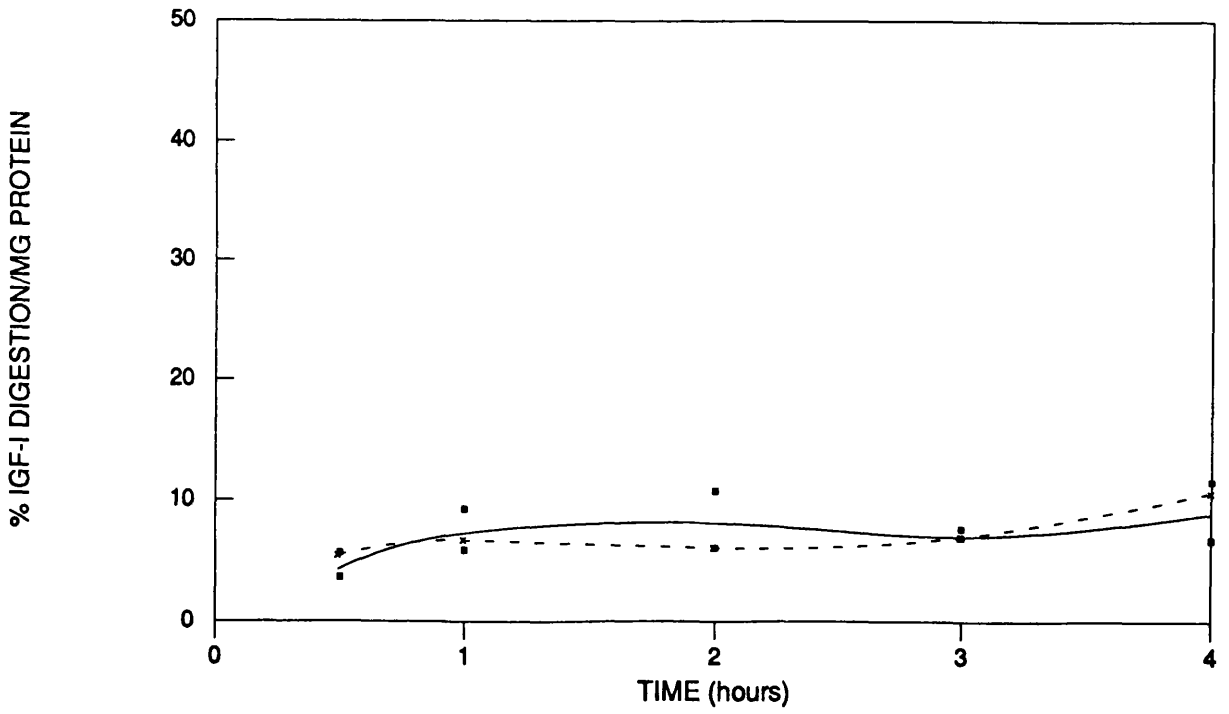

Fig. 4.5a. Processing of [^{125}I]-IGF-I by 17.5 day explanted yolk sacs in the presence of sodium fluoride.

Yolk sacs were explanted on the 17th day of gestation, and washed in M199. The yolk sacs were then placed in M199 which contained 10μg/ml of the glycolysis inhibitor sodium fluoride, and rotated at 37°C for 30 minutes before 1μg/ml [^{125}I]-insulin was added. 0.5ml samples of the incubation medium were taken between 30 minutes and 4 hours later, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that in the presence of sodium fluoride.

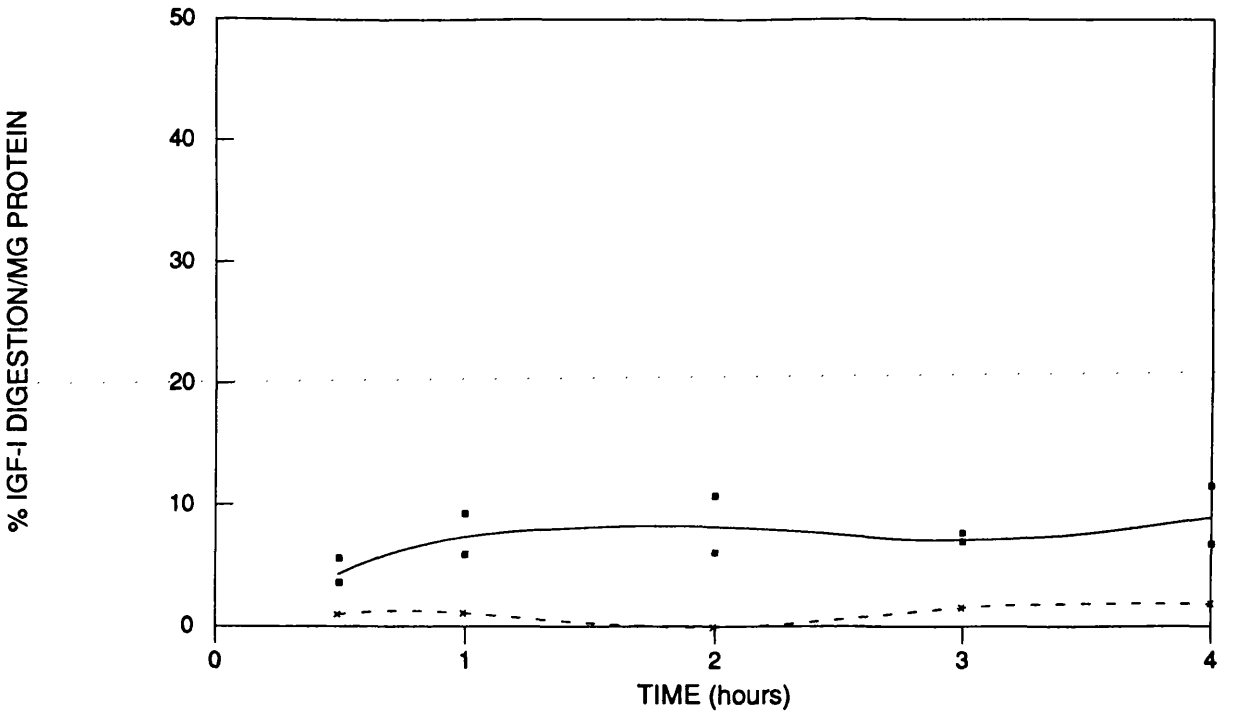

Fig. 4.5b. Processing of [^{125}I]-IGF-I by 17.5 day explanted yolk sacs at low temperature.

Yolk sacs were explanted from the mother on the 17th day of gestation, and washed in M199. They were then placed in M199, at 4°C for 30 mins. After this, [^{125}I]-insulin (1μg/ml) was added and time points taken 30 mins to 4 hours later. 0.5ml samples of the incubation medium were taken at each time point, counted for radioactivity, acid precipitated, then recounted. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that at low temperature.

The addition of 10μg/ml sodium fluoride does not appear to effect the rate at which acid soluble digestion products are released into the incubation medium. In contrast, incubation of 17.5 day explanted yolk sacs to 4°C dramatically reduces the rate of protein degradation seen, with over 70% inhibition seen at all time points tested.

The level of tissue associated radioactivity in the presence of sodium fluoride, and also at 4°C, can be calculated as previously described for the control yolk sacs. These values are shown in Table 4.2.

In each situation, the highest value recorded for percentage tissue-associated radioactivity is seen after 30 minutes incubation, after which the levels fall to a steady low level.

TIME (hrs)	% TISSUE ASSOCIATED/MG PROTEIN CONTROL	+NaF	Low Temp
0.5	9.66	9.35	6.16
1	6.57	7.60	4.49
2	5.10	3.96	4.14
3	2.99	4.12	2.59
4	2.78	2.55	3.15

Table 4.2. Table showing the percentage of [^{125}I]-IGF-I which was associated with the tissue per mg of protein when 17.5 day explanted yolk sacs were exposed to the radiolabelled ligand for 4 hours. The equivalent values are also shown for yolk sacs in the presence of a metabolic inhibitor and at low temperature. Each value has been expressed as a percentage by dividing the counts per minute/ml associated with that department by the total counts present in the experimental system.

Processing of [^{125}]I-IGF-I by cultured anembryonic rat visceral yolk sacs

Rat conceptuses were explanted at 9.5 days gestation and subsequently cultured for a further 8 days with 2 or 3 yolk sacs per bottle as described in Chapter 2. Once again, cultured yolk sacs were carefully washed with warm M199 to remove all traces of serum present in their culture medium, before the addition of 3mls of M199 containing [^{125}I]-IGF-I at 1μg/ml. Yolks sacs were then regassed with 95%O_2/5%CO_2, prior to their return to the roller incubator for the duration of the experiment.

In most cases, harvesting was carried out after 60, 120, 180 and 240 minutes, with the EEC fluid and samples of the incubation medium being collected in addition to the yolk sac tissue.

Results

Results for the digestion of [^{125}I]-IGF-I are shown in Table 4.3. The figure quoted are for the average percent degradation of [^{125}I]-IGF-I seen at the various time points as determined by the proportion of acid soluble to insoluble material present in the incubation medium, snd this value normalised for the amount of protein present in each vessel. Once again, the range of value for digestion from which this figure is the calculated average is shown. In these experiments, the number of yolk sacs investigated lies between 4 and 6, although in one case (the 30 minute time point) only one value for digestion was obtained.

TIME (mins)	AVERAGE % DIGESTION (% of added substrate which becomes acid soluble) in total medium	per mg protein
30	10.3	3.84
60	16.35 (Range = 10.63..22.07)	5.84 (Range = 2.39..9.29)
120	25.68 (Range = 24.49..26.87)	6.54 (Range = 4.88..8.21)
180	48.48 (Range = 32.17..56.82)	13.25 (Range = 11.04..17.39)
240	56.15 (Range = 54.23..58.07)	10.45 (Range = 9.76..11.15)

Table 4.3 Percentage digestion of [^{125}I]-IGF-I by cultured, anembryonic yolk sacs.

The amount of acid soluble digestion products formed from the degradation of [^{125}I]-IGF-I increases gradually over the 4 hours of the experiment, so that at the end of the time course approximately 50% of the protein has been degraded. These figures are plotted in fig 4.6a.

This value is then divided by the total amount of yolk sac protein present (fig 4.6b). Here, approximately 13% of the [^{125}I]-IGF-I present at the start of the experiment has been degraded per mg yolk sac protein after 4 hours of incubation.

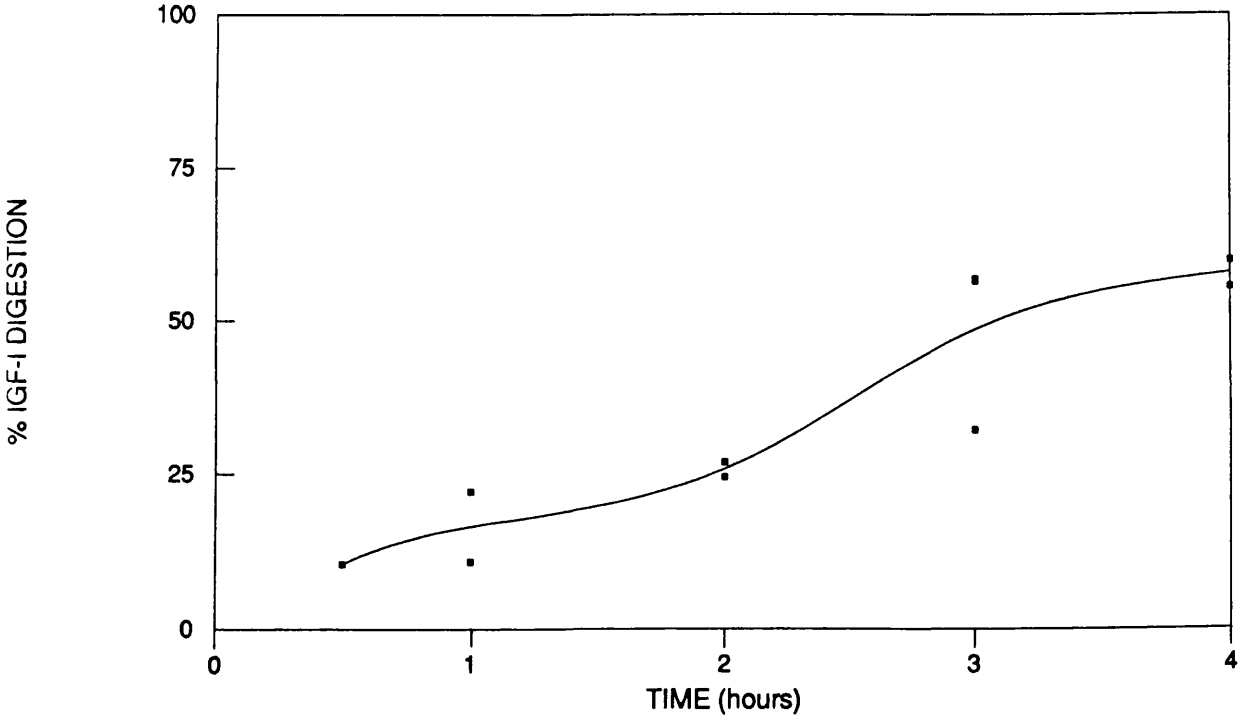

Fig 4.6a Processing of [^{125}I]-IGF-I by cultured anembryonic yolk sacs.

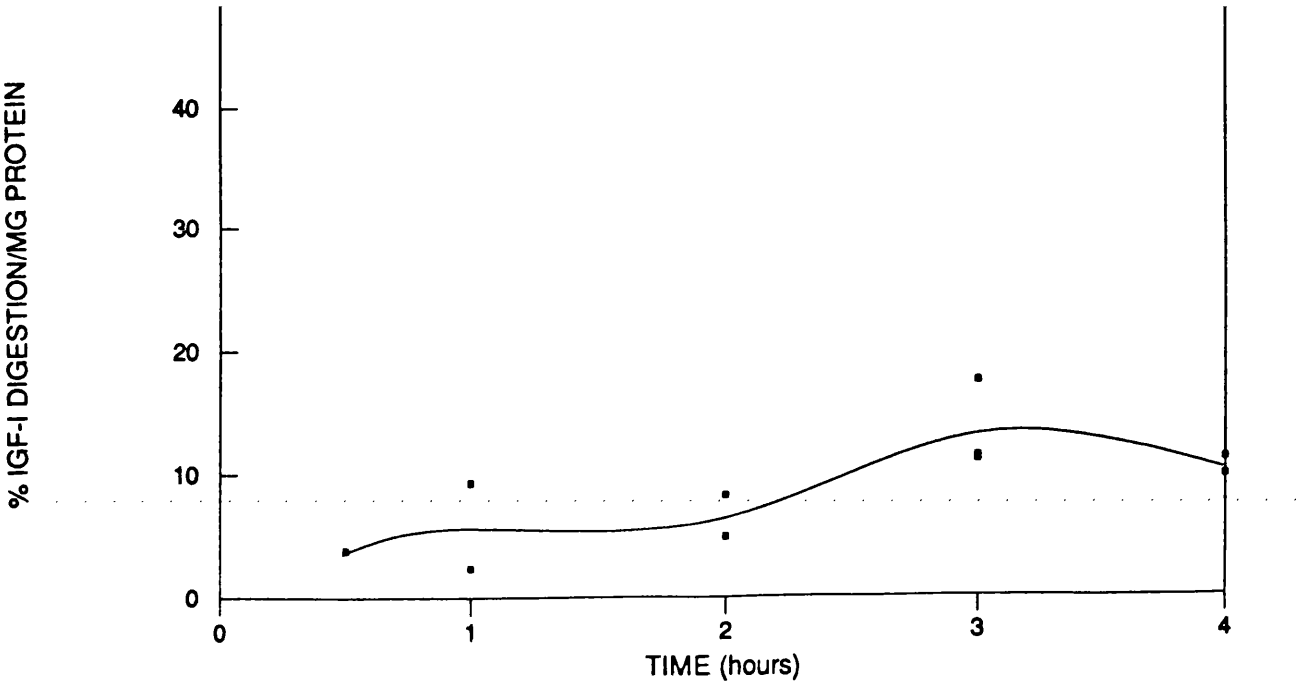

Fig 4.6b Processing of [^{125}I]-IGF-I by cultured anembryonic yolk sacs per mg yolk sac protein.

Yolk sacs were explanted from the mother on the 9th day of gestation, and cultured for the subsequent 8 days in a 50:50 mixture of M199 and rat serum. After this, the incubation medium was replaced with fresh M199 alone to wash the yolk sacs. This was removed and M199 containing [^{125}I]-IGF-I at 1μg/ml added. The yolk sacs were then incubated for between 30 minutes and 4 hours. At the end of the incubation period, medium surrounding the yolk sacs was removed, counted for radioactivity, subjected to acid precipitation, and then recounted. The values calculated are plotted here for 4 experiments, while the solid line represents the average calculated from each individual measurement.

As with the processing of this ligand by 17.5 day explanted yolk sas, it is also possible to examine the levels of radioactivity associated with the yolk sac tissue as previously described. This is shown in Fig. 4.7, from which it can be seen that that the level of tissue associated radioactivity falls from the initial value of 10% after 30 minutes, only to fall to a level around 1% at the 4 hour time point. This pattern suggests that, as with the 17.5 day explanted yolk sacs, there is no accumulation of radioactivity within the tissue. The pattern of percentage tissue-association is very similar to that seen when cultured anembryonic yolk sac are exposed to [^{125}I]-insulin.

These cultured yolk sacs have developed as a closed vesicle, which allows an examination of the fluid within the yolk sac. The proportion of radioactivity associated with this component is calculated from the total c.p.m. measured in the fluid collected when the yolk sacs are burst, divided by the total number of counts in the experimental system. This can be further analysed by counting the proportion of these counts which are acid soluble. This is shown in fig 4.8, in which the solid line shows the percentage radioactivity which is associated with EEC fluid, while the dashed line below illustrates the proportion of these counts which are acid soluble.

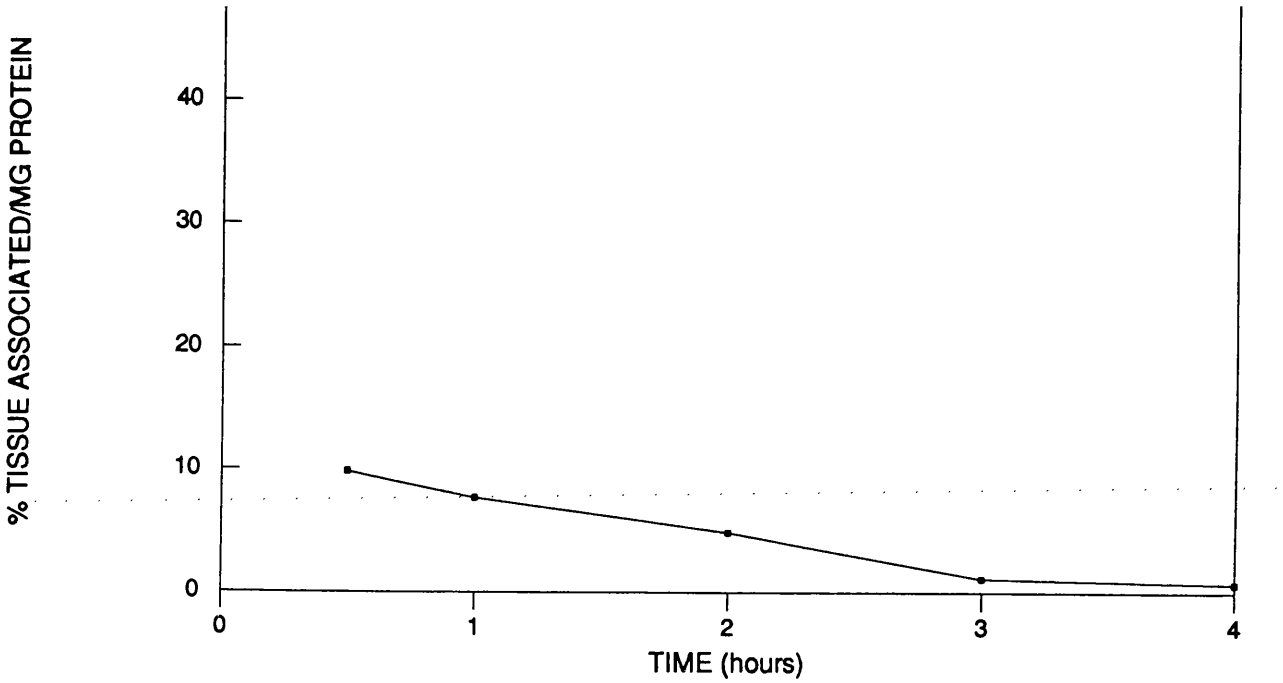

Fig 4.7 Percentage of the total radioactivity which is tissue-associated when cultured, anembryonic yolk sacs are exposed to [^{125}I]-IGF-I over a 4 hour incubation.

Yolk sacs were removed from the mother on the 9th day of gestation, and subsequently cultured for 8 days in 50:50 rat serum/M199. After this, the incubation medium was replaced with M199 containing 1µg/ml [^{125}I]-IGF-I. Yolk sacs were then harvested between 30 minutes and 4 hours later, and the EEC fluid within them carefully collected using a fine needle and syringe. The level of radioactivity associated with yolk sac tissue can be estimated by averaging the counts measured in each yolk sac. Each point is the average of between 4 and 6 yolk sacs.

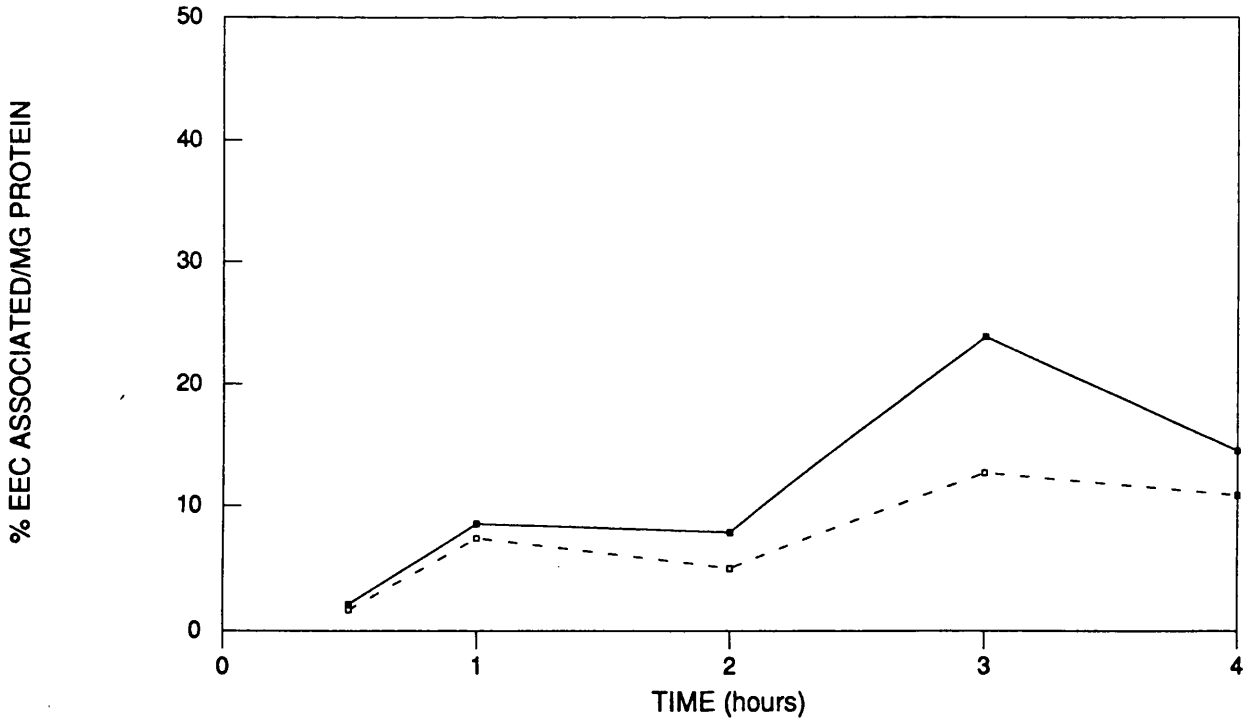

Fig 4.8. Percentage of the total radioactivity which is associated with the EEC fluid when 17.5 day cultured yolk sacs are exposed to [^{125}I]-IGF-I.

The level of radioactivity associated with yolk sac EEC fliud can be estimated by averaging the counts measured in each yolk sac. Each point is the average of between 4 to 6 yolk sacs. The EEC fluid was then acid precipitated to determine what proportion was associated with low molecular weight breakdown products, and this is shown in the dashed line.

The proportion of counts associated with this EEC compartment increases to reach a maximum of 24% of the total counts present after 3 hours of incubation. As was the case when [^{125}I]-insulin was investigated, the majority of the counts are associated with low molecular weight acid soluble material.

As with the [^{125}I]-insulin results, the proportion of acid soluble to insoluble counts within the EEC fluid was also analysed to establish how these varied over the course of the experiment. The fluid within these yolk sacs was again collected, weighed to give an estimate of its volume, counted for radioactivity, acid precipitated and then recounted. The difference between the acid soluble and insoluble counts was taken as an estimate of the amount of intact [^{125}I]-IGF-I which was present within this EEC fluid. The total counts in the medium at the beginning of the experiment was taken to equal 1μg/ml of intact IGF-I and using this figure, a concentration within the EEC was estimated from the number of counts and its volume.

This estimated amount of intact protein is plotted against time in fig 4.9, in which each point represents the amount of intact protein estimated within individual yolk sacs. The line on this graph has been fitted using linear regression analysis.

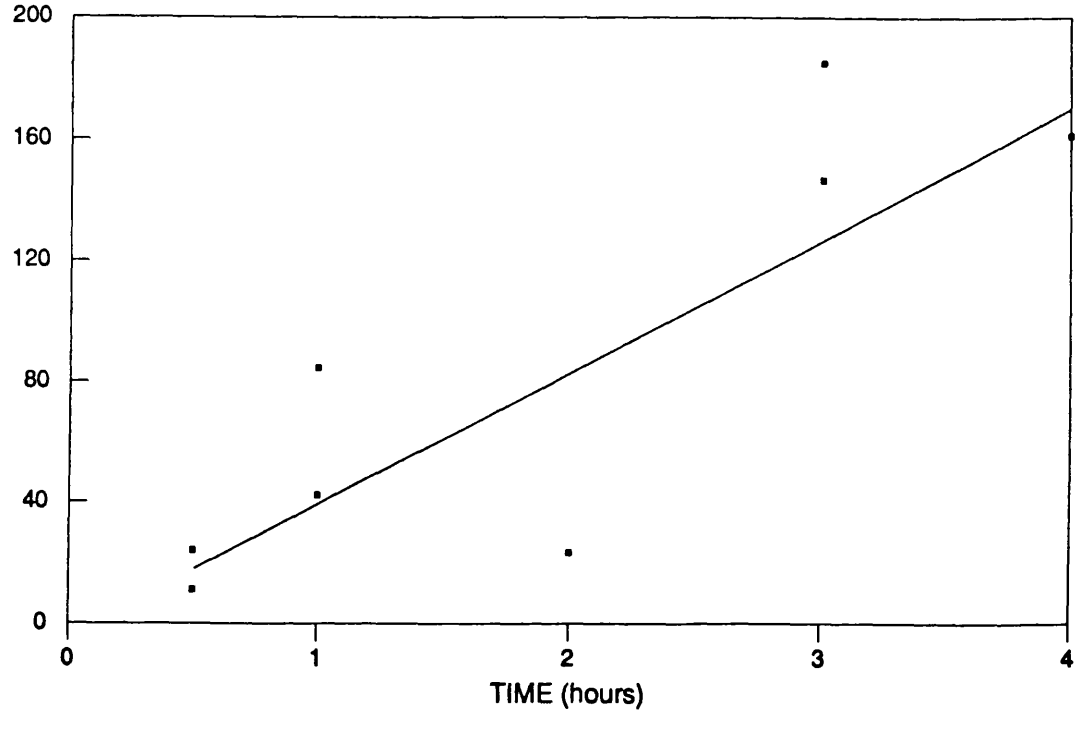

Fig 4.9 Accumulation of Intact [^{125}I]-IGF-I within cultured, anembryonic yolk sacs.

Yolk sacs were removed from the mother on the 9th day of gestation, and subsequently cultured for 8 days in 50:50 rat serum/M199. After this, the incubation medium was removed and replaced with M199 containing [^{125}I]-IGF-I at 1μg/ml. Yolk sacs were harvested between 30mins and 4 hours later, and the EEC fluid within them carefully collected using a fine needle and syringe. This fluid was then weighed to give an estimate of its volume. EEC fluid was then subjected to acid precipitation, and the estimated amount of intact [^{125}I]-IGF-I plotted to see how this varied with time.

In addition to the amount of intact protein which was accumulating within the EEC fluid, the amount of acid soluble material within this fluid has also been plotted against time (fig 4.10.)

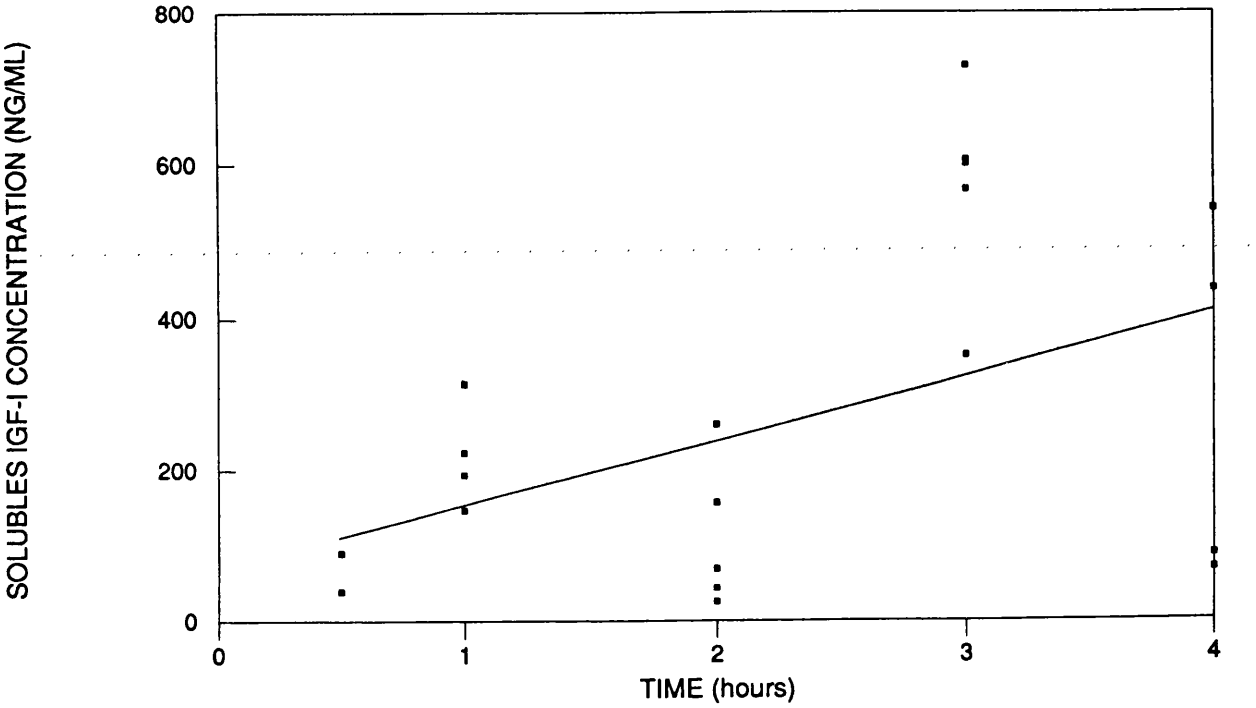

Fig 4.10 Accumulation of acid soluble material within cultured, anembryonic yolk sacs.

Yolk sacs were removed from the mother on the 9th day of gestation, and subsequently cultured for 8 days in 50:50 rat serum/M199. After this, the incubation medium was removed and replaced with M199 containing [^{125}I]-IGF-I at 1μg/ml. Yolk sacs were harvested between 30mins and 4 hours later, and the EEC fluid within them carefully collected using a fine needle and syringe. This fluid was then weighed to give an estimate of its volume. EEC fluid was then subjected to acid precipitation, and the acid soluble concentration (ie. ng of IGF-I digested per ml) was plotted to see how this varied with time.

In comparison with radioactive insulin results, far fewer yolk sacs were available here because of the higher cost of the purified protein. Overall the results are less consistent; however, the graphs here would indicate that a similar pattern of accumulation within the EEC fluid is occurring. Again there appears to be a linear accumulation of intact protein with time, so that after 4 hours approximately 160ng/ml of intact [^{125}I]-IGF-I is present within the EEC fluid. However, there is proportionally less acid soluble material than was the case with [^{125}I]-insulin, with only approximately twice as many radioactive counts associated with soluble digestion products than with the intact protein.

The gradual increase in intact protein over the 4 hours incubation may again suggest that some mechanism exists to transport IGF-I across the yolk sac epithelium intact.

Attempts to Elucidate the Mechanism of IGF-I Digestion.

Once again, the uptake of [^{125}I]-IGF-I was investigated at low temperature and in the presence of the glycolysis inhibitor sodium fluoride. Both systems are known inhibitors of pinocytosis, and should help elucidate the uptake mechanism in this system. For the low temperature experiment, yolk sacs were placed in a roller incubator at 4°C for 30 minutes, before the culture medium was replaced with M199 containing [^{125}I]-IGF-I at 1μg/ml. The bottles were then gassed with 95%O_2/5%CO_2, before being returned to the 4°C incubator, and harvested after 60, 120, 180 or 240 minutes later. In the case of the sodium fluoride experiment, yolk sacs were preincubated in the presence of the metabolic inhibitor before [^{125}I]-IGF-I was added. Yolk sacs were harvested after the same hourly intervals.

Results are shown in Figs 4.11 a and b.

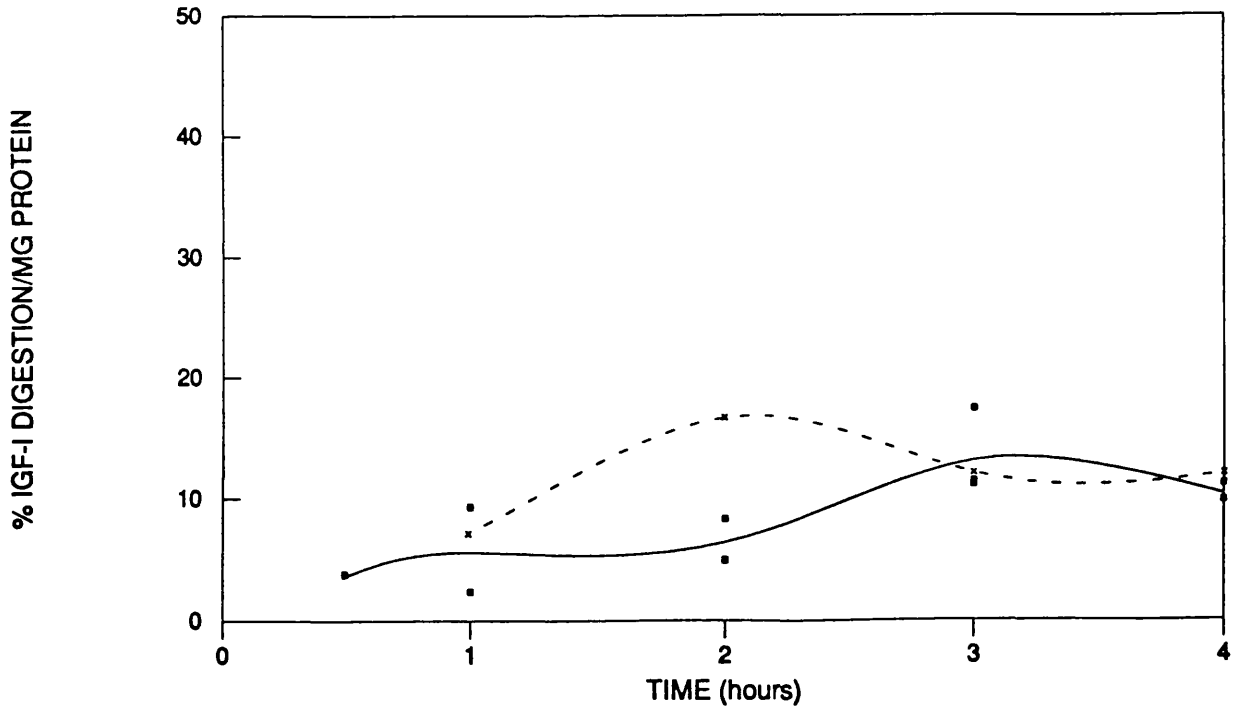

Fig. 4.11a Processing of [^{125}I]-IGF-I in the presence of sodium fluoride.
Yolk sacs were explanted and cultured from the 9th day of gestation at 37°C in a mixture of M199 and rat serum. On the day of the experiment, the culture medium was removed and replaced with M199 containing the glycolysis inhibitor sodium fluoride at 10μg/ml. After 30 minutes, [^{125}I]-IGF-I (at 1μg/ml) was added to the bottles. Tissue was harvested between 60 to 240 minutes later. Samples of incubation medium, EEC fluid and yolk sacs tissue were all collected for subsequent analysis. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that in the presence of sodium fluoride.

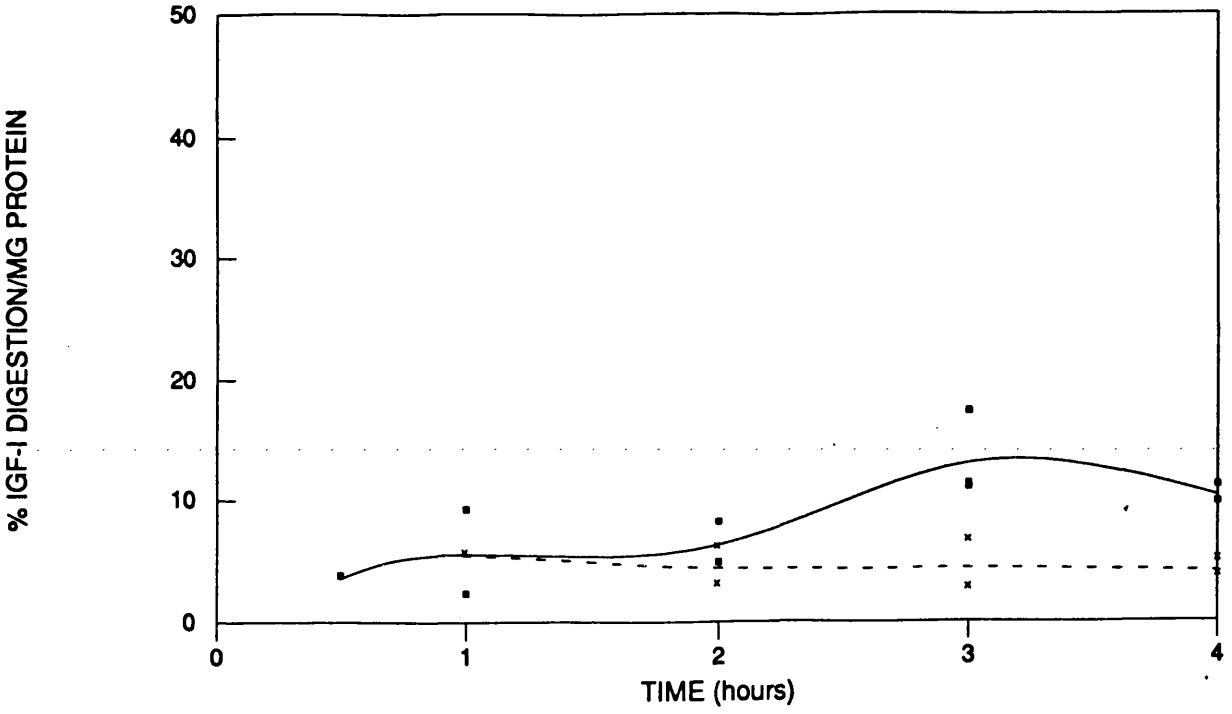

Fig. 4.11b Processing of [^{125}I]-IGF-I at low temperature.
Yolk sacs were removed from the mother on the 9th day of gestation, and cultured for the following 8 days at 37°C in M199/rat serum. On the day of the experiment, the yolk sacs were placed in a roller incubator at 4°C for 30 minutes before their culture medium was replaced with M199 containing [^{125}I]-IGF-I at 1μg/ml. Yolk sacs were then gassed with 95%O_2/5%CO_2, before being replaced in the 4°C incubator. Yolk sacs were harvested between 60 to 240 minutes later, when samples of incubation medium, EEC fluid and yolk sac tissue were collected for analysis. The solid line shows the average digestion seen per mg yolk sac protein in a series of control yolk sacs, while the dashed line shows that at 4°C.

When 10μg/ml sodium fluoride is present in the incubation medium it appears to increase the level of [^{125}I]-IGF-I over the first 2 hours of the experiment. After this, however, the rate of degradation is virtually identical to that seen in the controls.

When these yolk sacs are incubated at 4°C, a steady low level of protein digestion is seen. After 3 hours of the experiment only 36% of the degradation seen in the controls is observed.

The amount of radioactivity associated with the yolk sacs tissue, and with the EEC fluid within the yolk sac vesicle, can also be examined in the presence of sodium fluoride and at 4 C. These values are shown in Table 4.4. In the case of the EEC values, the figures in brackets show the proportion of the counts which were acid soluble.

The addition of sodium fluoride appears to increase the level of radioactivity associated with the tissue, while incubation at 4°C reduces this. Similarly, the level of radioactivity associated with the EEC fluid is reduced at low temperature, while sodium fluoride initially increases the level of EEC associated counts in comparison with the controls, before the level is reduces to that seen in the controls.

% TISSUE ASSOCIATED/MG PROTEIN

TIME (hrs)	CONTROL	+NaF	Low Temp
0.5	9.84	-	-
1	7.68	10.17	1.61
2	4.85	15.86	1.927
3	1.12	9.77	0.65
4	0.69	7.76	1.37

% EEC ASSOCIATED/MG PROTEIN

TIME (hrs)	CONTROL	+NaF	Low Temp
0.5	2.11 (1.67)	-	-
1	8.54 (7.36)	5.92	0.09
2	7.82 (4.95)	19.59	2.89
3	23.92 (12.68)	10.29	3.38
4	14.46 (10.84)	7.36	1.45

Table 4.4. Tables showing the percentage of [^{125}I]-IGF-I which was associated with the tissue and with the extraembryonic fluid per mg yolk sac protein when cultured anembryonic yolk sacs were exposed to the radiolabelled ligand for 4 hours. The equivalent values are also shown for yolk sacs in the presence of sodium fluoride and at low temperature. Each value has been expressed as a percentage by dividing the counts per minute/ml associated with that department by the total counts present in the experimental system.

Discussion

Although no work has previously been carried out on the processing of [^{125}I]-IGF-I by the rat visceral yolk sac, the structural similarly of this protein to insulin is so great as to suggest that IGF-I might be handled by a similar mechanism to that envisaged for insulin. One possibility is that [^{125}I]-IGF-I is taken up by a specific receptor-mediated process, although another possibility is that at this concentration, IGF-I could be interacting with insulin receptors. This could be partly elucidated by demonstrating that IGF-I receptors are present on the visceral yolk sac surface, using a specific marker such as a

monoclonal antibody.

In the case of the 17.5 day explanted yolk sacs, degradation is rapid over the first hour of incubation, after which the level of digestion is reduced. After 4 hours of incubation approximately 70% of the protein originally present has been degraded. When the total percentage degradation is considered in terms of the actual amount of protein present, then it is seen that around 10% of the protein present is degraded per mg protein. A similar pattern of protein degradation is seen in the cultured yolk sacs, except that after 4 hours of incubation only around 50% of the protein has been degraded.

The highest amount of radioactivity seen in both systems is clearly associated with soluble, lower molecular weight, digestion products. If the EEC samples within the cultured yolk sacs are analysed, the majority of the counts within this fluid are also acid soluble. This is again closely comparable with [^{125}I]-insulin. It would seem that the digestion products of both proteins are reaching the inner surface of the yolk sac membrane where they would be made available to the developing embryo for *de novo* protein synthesis. The largest proportion of the soluble digestion products is, however, found in the incubation medium.

The experiments carried out to try to inhibit the degradation of [^{125}I]-IGF-I by yolk sacs are clearly extremely limited. This was principally due to the extremely high cost of IGF-I in comparison to insulin, which meant that only a small amount was available exclusively for radiolabelling. As a consequence, the inhibition study results are incomplete. However, it can be determined that when 10μg/ml of the glycolysis inhibitor sodium fluoride was added to the incubation medium, it appeared to have very little effect upon the rate of protein degradation seen in either of the yolk sac systems. This may be at variance with the results seen with [^{125}I]-insulin degradation by the cultured yolk sacs, as sodium fluoride produced a slight reduction in the level of degradation seen, while the other glycolysis inhibitor tested, monoiodoacetate, produced a significant reduction in the level of IGF-I broken down. At each time point, however, the number of yolk sacs investigated was only three, which is not a large experimental sample population. This substance was chosen as the metabolic inhibitor for investigation as the experiments earlier described with [^{125}I]-insulin suggested that this compound may reduce protein degradation in one system of yolk sacs and not the other.

The experiments at low temperature indicate that degradation of [^{125}I]-IGF-I is severely reduced at 4°C. These results again indicate that the digestive process could be occurring via an enzyme mediated mechanism or equally by an intracellular process which requires an energy consuming internalisation step. Low temperature would be expected to reduce the amount of ATP

produced, as well as limit the number of uptake vesicles formed, or alternatively, inhibit some extracellular enzyme activity. As described in the previous chapter, it appears that pinocytosis is virtually eliminated below 20° C (clearly demonstrated in the cultured yolk sacs), as membrane fluidity is dramatically reduced (Haylett and Thilo, 1991). Below this temperature, membrane invagination does not occur and uptake is inhibited at one of the first points along the chain of events leading to uptake.

The results in this chapter demonstrate that the rat visceral yolk sac is extremely efficient at degrading [^{125}I]-IGF-I to which it has been exposed. How this degradation is achieved, however, is not clear. The limited experiments described in this chapter would suggest that this protein is processed in a manner similar to [^{125}I]-insulin. One important consideration in this series of experiments has been the expense of the purified protein. As a result, the number of experiments carried out has had to be reduced accordingly, and these experiments would clearly benefit from the availability of more IGF-I. In particular, it would be useful to examine uptake in the presence of all the inhibitors used to test [^{125}I]-insulin degradation, (monoiodoacetate, 2,4,dinitrophenol and EGTA) as this would add further evidence to the proposal that the handling of [^{125}I]-IGF-I is virtually identical to that of [^{125}I]-insulin. The next step would then be to investigate the effects of lysosomal enzyme inhibitors, which should establish whether IGF-I degradation is occurring via the endosome-lysosome pathway.

CHAPTER FIVE

USE OF FLUORESCENTLY LABELLED LIGANDS TO EXAMINE THE PROCESSING OF INSULIN AND INSULIN-LIKE GROWTH FACTOR I (IGF-I) BY THE RAT VISCERAL YOLK SAC.

Introduction

Although a great deal of information can be gleaned from the use of radiolabelled markers to examine the handling of a molecule by a cell, this information indicated principally whether or not the molecule is taken up by endocytic mechanisms, and whether it undergoes intracellular (or extracellular) digestion. It would also be of interest to discover the extent to which ligands were located inside the cell, and with which intracellular compartment they associate. This type of information can be acquired in part using the technique of fluorescence microscopy, which has been widely used as a means of visualising labelled components within the cell (reviewed Ploem and Tanke, 1987; Johnstone and Thorpe, 1987; and Ockleford, 1990).

Fluorescence is defined as the emission of light (or other radiation) from molecules bombarded with radiation from a separate source. The emission of photons detectable with a fluorescence microscope therefore, comes about solely as the result of this irradiation, and ceases as soon as the bombarding radiation is discontinued. Firstly, the molecule of interest is labelled with a fluorescent marker, or fluorophore. The most commonly used fluorophores are fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC), which are covalently bound to the molecule of interest prior to their addition to the cell. Fluorophores can then be excited by irradiation with light of a wavelength which spans the absorption maximum of that particular fluorophore. This light may be produced from a variety of sources- tungsten halogen lamps, or high pressure mercury or xenon bulbs, which vary in their intensity and expense. The light from this source excites the electrons in orbit around the fluorophore atoms, such that they absorb this energy and are raised to a higher energy, or excited, state. When these atoms later return to their original lower energy orbits around the fluorophore atoms, the energy they initially received from the incident light source is now released in the form of radiation emitted in the visible part of the spectrum.

Most fluorescent microscopes are of the incident light, or epifluorescence, variety. Here the specimen is illuminated by passing the excitatory light onto the upper surface of the specimen. The incident light can then be filtered from any unwanted reflected light, with the result that fluorescence in the specimen can be detected against a dark background, with low levels of background fluoresence.

A major advance in fluorescence microscopy has come with the recent developments in confocal imaging. Here, the light source consists of a laser beam. The principal feature of a confocal microscope is that the illumination and detection are confined to the same spot on the specimen at any one moment. Thus, every image detected is in focus, and any out of focus areas are detected as black. The resolution in a confocal microscope is greater than that

possible in any conventional light microscope, and the microscope is able to build up an image of the specimen either by scanning the specimen across the laser beam, or more conveniently, by scanning the beam over a fixed specimen. In addition to the greater resolution possible with a confocal microscope, another major advantage of this technique is that two or more ligands may be investigated simultaneously. This is a useful experimental tool brought about by the varying absorption and emission spectra of the different fluorophores. For example, FITC has an absorption maximum of 490nm and an emission maximum of 520nm. This fluorophore is then irradiated with blue light of a wavelength between 455-490nm, which produces an emission of light between 520-560nm, seen as a yellow/green fluorescence. TRITC on the other hand, has absorption and emission maxima of 554nm and 573nm respectively, and consequently is irradiated with green light, emitting a red fluorescence.

In this series of experiments, insulin has been covalently labelled with FITC, and insulin-like growth factor I (IGF-I) with TRITC. The results described in the previous two chapters suggest that both insulin and IGF-I are digested by the rat visceral yolk sac cells at a high rate. Additionally, it would also seem that after uptake, both insulin and IGF-I are digested to form products which are released at the inner surface of the yolk sac membrane, where they would be made available to the developing embryo. It would now be of interest to examine whether there are areas within the cell where these ligands are located or whether they remain at the cell surface. The technique of fluorescence labelling enables the visualisation of the molecules within the visceral yolk sac, and may indicate with which, if any, intracellular compartment they are associated with. The question of possible cross-reactivity between these ligands and the insulin and IGF-I receptors may also be examined, as the addition of both fluorophores together should indicate where the respective receptors are located, and whether both ligands are associated with this region. The position of the molecule can also be traced over a considerable time-course, which may give information concerning its eventual fate; for example, extracellular digestion may occur; intracellular lysosomal digestion may occur, or the ligand may be broken down following extra-lysosomal activity.

Processing of FITC-labelled insulin by the 17.5 day explanted rat visceral yolk sac.

Insulin was covalently linked to the fluorophore FITC as described below. A series of experiments were then carried out using 17.5 day explanted yolk sacs. The position of the ligand was examined at a series of differing time points, after which experiments at low temperature and in the presence of the metabolic inhibitor sodium fluoride were also performed to see if these altered the location, or reduced the level, of ligand seen.

FITC-labelling of insulin

Stock solutions used:

1) 0.01M HCl
2) 0.5M Bicarbonate buffer (0.5M sodium bicarbonate).
3) 0.05M Phosphate buffer (0.5M sodium dihydrogen orthophosphate and 0.5M disodium hydrogen orthophosphate mixed equally, and then diluted to give a 0.05M solution).
4) 145mM sodium chloride
5) Bovine serum albumin B.S.A (5mg/ml).

50mgs of insulin were dissolved in the minimum 0.01M HCl possible, before being made up to 4.5mls with 145mM sodium chloride. To this, 0.5mls of 0.5M Bicarbonate buffer (pH 9.0) containing 1mg Fluorescein isothiocyanate (FITC), were added. This solution was then mixed prior to incubation at 4°C overnight, after which the pH was adjusted to 7.0 using 0.1M HCl.

Sephadex G50 was rehydrated in phosphate buffer overnight at 4°C , and then degassed by bubbling helium through the solution for approximately 30 minutes. A 20ml disposable column (Biorad) was then set up in a retort stand, and carefully filled with the minimum of agitation with the Sephadex G50, until the gel reached the 20ml mark on the column. The column was then packed by running 0.05M phosphate buffer down it for two hours.

The non-specific protein sites on the column were then blocked by running 2mls of 5mg/ml B.S.A. (bovine serum albumin) down the column, and eluting with phosphate buffer.

Unbound FITC is separated from that conjugated to the insulin when it is run down the column, and the conjugated fraction is collected as the first to come off the column. This conjugated fraction was then freeze dried before resuspension in phosphate buffer to give a final concentration of 10mg/ml. The solution was stored at -20°C, and exposure to light was avoided as this may reduce the intensity of the FITC.

Experiments with Explanted Yolk Sacs

Yolk sacs were removed from the mother on the 17th day of gestation, and the amnions removed. The tissue was then washed in warmed M199 in a stoppered glass bottle, and gassed for approximately 1 minute with 95%O_2. Fresh M199 containing insulin labelled with FITC (FITC-insulin) was then added at a concentration of 20µg/ml, and the yolk sacs regassed. This concentration had been chosen because it was the lowest level at which fluorescence could be clearly determined. In a pilot series of experiments, concentrations of 5µg/ml and 10µg/ml had also been looked at, but only a minimal amount of fluorescence could be detected using a standard Zeiss epifluorescence microscope.

After regassing, the yolk sacs were then returned to the 37°C roller incubator. Yolk sacs were harvested either 15, 30 or 60 minutes later by removal of the tissue from the bottle, and immersion in 1% saline. The tissue was then washed by gentle agitation in 3 changes of 1% saline, before being blotted dry on tissue paper. The yolk sac was then arranged in OCT in an aluminium foil cup, and frozen down in a mixture of hexane and dry ice. Frozen blocks were then stored at -40°C before sectioning.

In addition, yolk sacs were also preincubated for 30 minutes at 4°C before 20μg/ml FITC-insulin was applied for 30 minutes; similarly, tissue was also preincubated for 30 minutes in the presence of 10μg/ml sodium fluoride before FITC-insulin was applied for 30 minutes. Tissue was then washed and frozen down as previously described.

Frozen blocks were cut using a Leitz cryostat. Sections were cut between 6 or 7μm and mounted on glass slides. These slides were then washed in distilled water, followed by phosphate buffered saline (PBS) to remove the OCT. Tissue was then fixed in 3% formal saline for three minutes, before being rinsed in PBS and distilled water once more, and allowed to air dry. The slides were then coverslipped using an antiphotobleaching agent, Citifluor, which is a mixture of glycerol and PBS. The edges of the coverslips were then secured using nail varnish, which prevents leakage of the Citifluor from beneath the coverslip.

The sections were then examined using either a Zeiss epifluorescence microscope or an inverted Zeiss epifluorescence microscope in conjunction with a Biorad MRC-600 laser scanning confocal imaging system.

Results

Results are shown in Figs 5.1 a-d of tissue-association of FITC-insulin in the 17.5 day explanted yolk sac. Figs 5.1.a and b show the results seen after 15 and 30 minutes incubation, as viewed under a standard Zeiss epifluorescence microscope. After only 15 minutes incubation (Fig 5.1a), a considerable amount of fluorescence is detectable at the apical surface of the tissue. After 30 minutes, (Fig 5.1b) the fluorescence is brighter and FITC-insulin is also deeper in the tissue, although most of this fluorescence can still be seen lying near the apex of the cells.

In comparison with this last photograph, Fig 5.1c shows the effect of incubation with FITC-insulin for 30 minutes at low temperature. In this case, uptake has been virtually eliminated, and no fluorescence can be seen.

Fig 5.1d shows the effects of addition the metabolic inhibitor sodium fluoride at 10μg/ml. The sections show that a large amount of fluorescence has become tissue-associated, and that, once again, this fluorescence is most strongly detectable beneath the apical surface of the yolk sac.

Fig 5.1a

Fig 5.1b

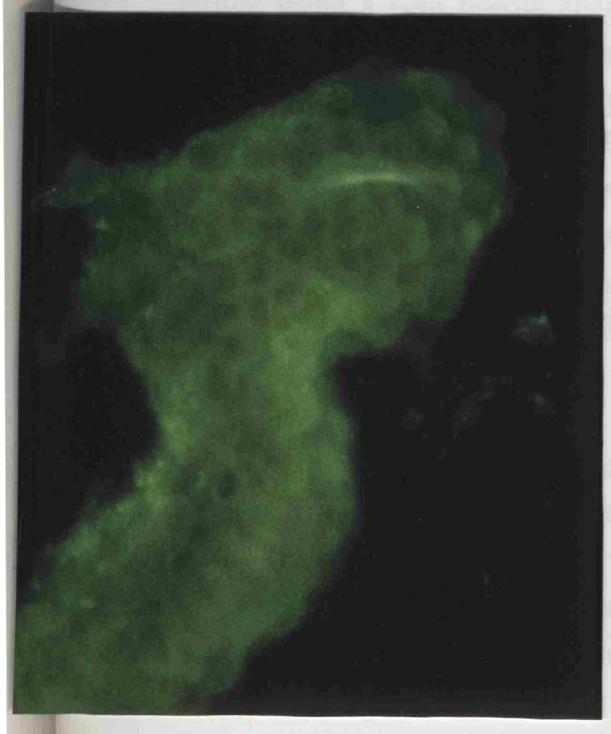

Fig 5.1c

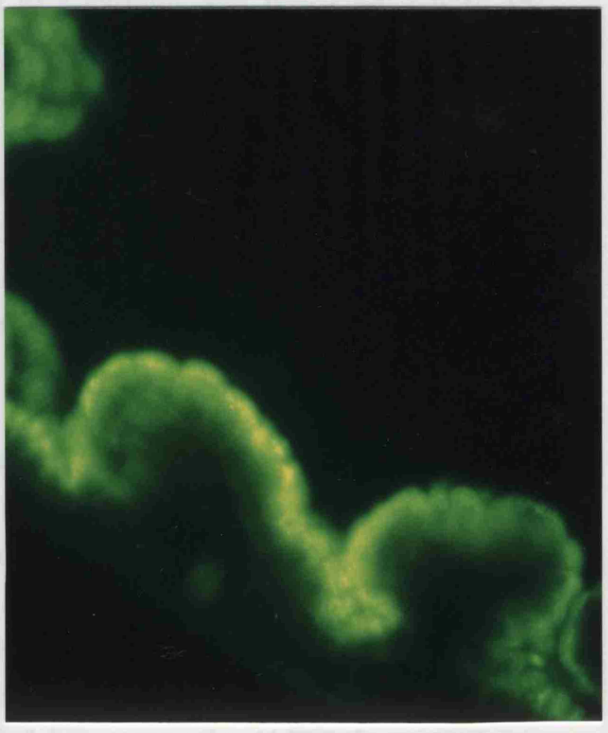

Fig 5.1d

Fig 5.1 Tissue-association of FITC-labelled insulin by 17.5 day explanted rat visceral yolk sacs.
Tissue is shown after a)15 and b)30 minutes incubation, and also after c)30 minutes incubation at low temperature and d)in the presence of 10µg/ml sodium fluoride. (Mag:120x).

Processing of FITC-labelled insulin by the cultured visceral yolk sac.

In parallel with the series of experiments described above, the handling of FITC-insulin was also examined in cultured, anembryonic rat visceral yolk sacs.

Method

Rat conceptuses were removed from their mother on the 9th day of gestation, and their yolk sacs cultured as previously described in Chapter 2. On the 8th day of culture, yolk sacs were carefully washed by removal of their existing culture medium using a bent needle attached to a syringe, and replacing it with fresh M199. Yolk sacs were washed in this way 3 times, before M199 containing FITC-insulin (again at 20μg/ml) was introduced to the bottle.

Yolk sacs were then gassed for approximately 1 minute with 95%O_2, before bottles were returned to a roller incubator, and the experiment started. Time points were taken after 15 and 30 minutes. The effects of low temperature and sodium fluoride were also examined by incubating yolk sacs either at 4° C, or in the presence of 10μg/ml sodium fluoride for 30 minutes, before FITC-insulin was added.

Incubation was terminated by removal of the M199 containing FITC-insulin using a bent needle attached to a syringe, and replacing it with 1% saline. Tissue was washed in this way 3 times, before the yolk sac was floated out into a large beaker containing 1% saline. The yolk sac was then burst, and the tissue was then arranged in OCT embedding compound in an aluminium foil cup, and frozen down in a mixture of hexane and dry ice. Frozen blocks were then stored at -40° C before sectioning.

Sections were cut, fixed and mounted as described for explanted tissue, and again examined using an epifluorescence microscope.

Results

Fig 5.2a shows the tissue-association of FITC-insulin by cultured, anembryonic yolk sacs after 15 minutes incubation, as viewed using a Zeiss epifluorescence microscope. As with the equivalent explanted tissue, a large amount of fluorescence is detectable after only 15 minutes. This fluorescence once again appears to be concentrated just beneath the surface of the tissue. After 30 minutes incubation (Fig 5.2b), the fluorescence is brighter, indicating that more tissue-association of the label has occurred. Although most of this FITC is detectable close to the apical surface of the yolk sac, this section reveals that a small amount of fluorescence has moved deeper into the cell. This may represent transport of FITC-insulin across the yolk sac epithelium.

At low temperature, (Fig. 5.2c), the amount of fluorescence visible is reduced, while the addition of sodium fluoride (Fig 5.2d) appears to have no effect on uptake.

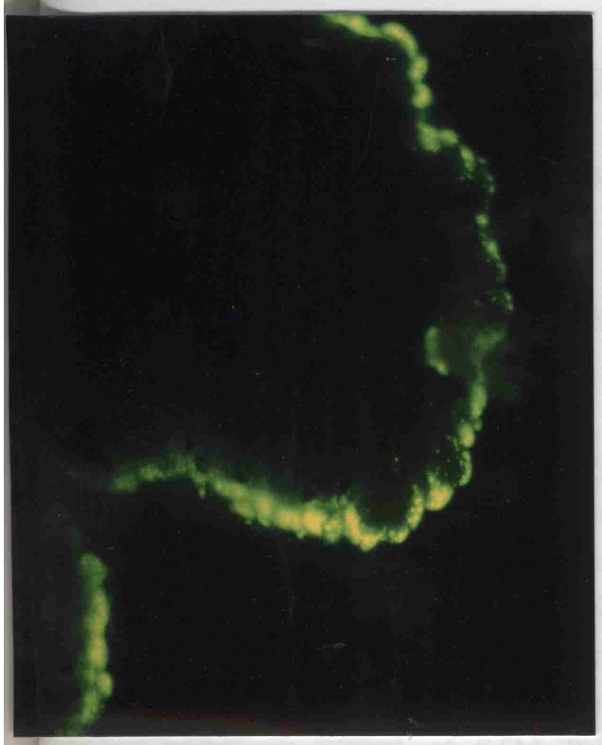

Fig 5.2a

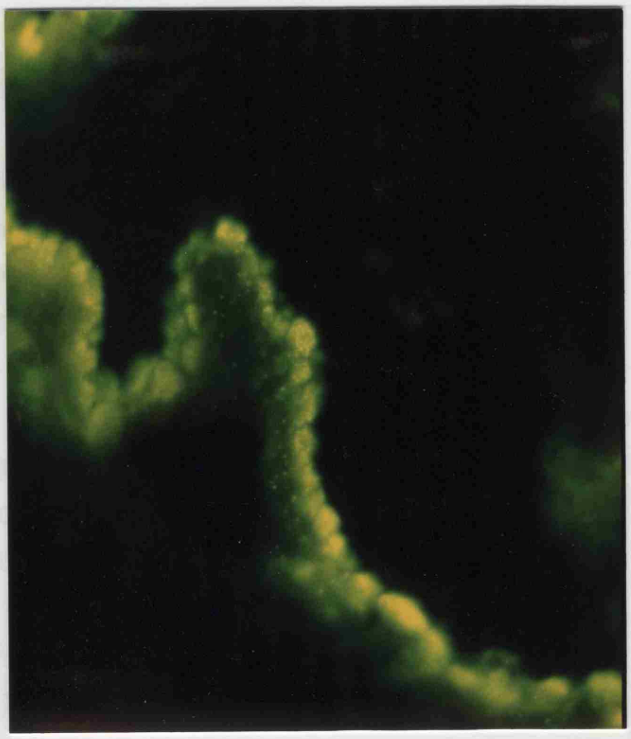

Fig 5.2b

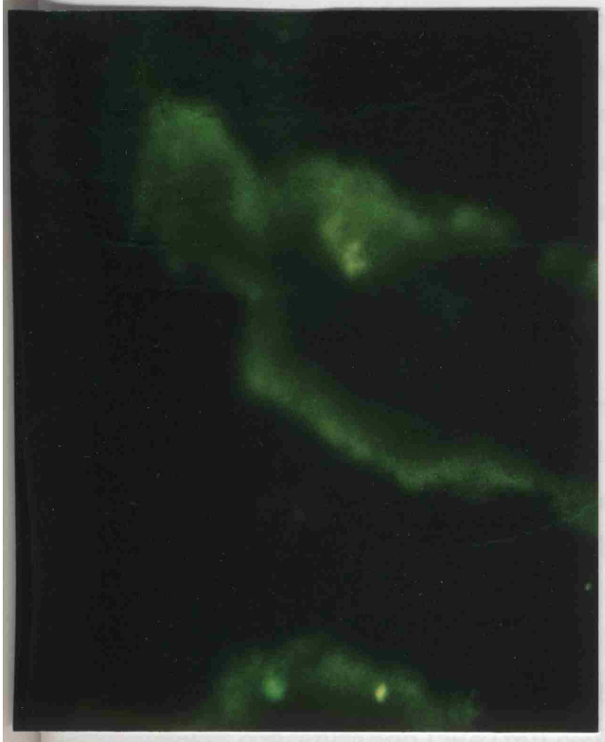

Fig 5.2 c

Fig 5.2d

Fig 5.2 Tissue-association of FITC-labelled insulin by cultured, anembryonic rat visceral yolk sacs.
Tissue is shown after a)15 and b)30 minutes incubation, and also after c)30 minutes incubation at low temperature and d)in the presence of 10µg/ml sodium fluoride. (Mag:120x).

Processing of TRITC-labelled IGF-I by the 17.5 day explanted rat visceral yolk sac.

To complement the series of FITC-insulin experiments previously described, the tissue-association of IGF-I labelled with TRITC (TRITC-IGF-I) was also examined in 17.5 day explanted yolk sacs. This was examined at several time points, while the effects of low temperature and sodium fluoride were also examined.

Tetramethyl Rhodamine Isothiocyanate (TRITC) Labelling of IGF-I

The procedure used for labelling this protein was essentially that used for the FITC labelling of insulin, except on a smaller scale. This time, 2.25mg of IGF-I (Ciba-Geigy) had previously been dissolved in 225µl of 0.1M acetic acid, and aliquoted into 25µl (250µg) samples prior to freezing. Two of these aliquots were resuspended in 2.25mls of 145mM sodium chloride, which was then added to 0.25ml of 0.5M Bicarbonate buffer (pH 9.0) containing 1mg tetramethyl rhodamine isothiocyanate (TRITC), and the mixture incubated at 4°C overnight.

A disposable 20ml column packed with rehydrated Sephadex G50, had previously been prepared, and the non-specific binding sites blocked, before the TRITC-IGF-I mixture was run down the column. The first fraction to come off the column contains the conjugated TRITC-IGF-I, and was collected, freeze dried, and resuspended in 1ml of 50mM phosphate buffer to give a concentration of 500µg/ml.

Method

Yolk sacs were removed from the mother on the 17th day of gestation, and the amnions removed. The tissue was then washed in warmed M199 in a stoppered glass bottle, and gassed for approximately 1 minute with 95%O_2. Fresh M199 containing IGF-I labelled with TRITC (referred to hereafter as TRITC-IGF-I) was then added at a concentration of 5µg/ml, and the yolk sacs regassed. TRITC is detectable at lower levels than is FITC, and because of the high cost of IGF-I it was decided to conjugate this protein with TRITC, so that a lower concentration (5µg/ml) could be used.

After regassing, the yolk sacs were then returned to the 37°C roller incubator. Yolk sacs were harvested either 15, or 30 minutes later by removal of the tissue from the bottle, and plunging it into 1% saline. The tissue was then washed by gentle agitation in 3 changes of 1% saline, before being blotted dry on tissue paper. The yolk sac was then arranged in OCT in an aluminium foil cup, and frozen down in a mixture of hexane and dry ice. Frozen blocks were then stored at -40°C before sectioning.

Sections were cut between 6-8μm, and fixed and mounted as previously described for the FITC-insulin samples. Sections were then examined using a Zeiss epifluorescence microscope.

Results

Fig 5.3a shows uptake of TRITC-IGF-I by the 17.5 day explanted rat visceral yolk sac. A large amount of fluorescence is detectable at the apical surface of the yolk sac. After a further 15 minutes incubation (Fig 5.3b), the fluorescence is much greater, and appears to have spread throughout the entire tissue. In this section, fluorescence is clearly detectable at the basolateral surface, and so must have been transported across the entire yolk sac epithelium.

In comparison with Fig. 5.3b, which shows TRITC-IGF-I uptake after 30 minutes incubation at 37°C, Fig 5.3c shows the uptake over the same incubation period at only 4°C. The level of fluorescence is dramatically reduced at low temperature. This photograph clearly illustrates the large nuclei which are present within yolk sac epithelial cells.

When the glycolysis inhibitor sodium fluoride is added to the incubation medium for 30 minutes at 10μg/ml (Fig. 5.3d), it appears that there is a reduction in uptake.

Fig 5.3a

Fig 5.3b

Fig 5.3c

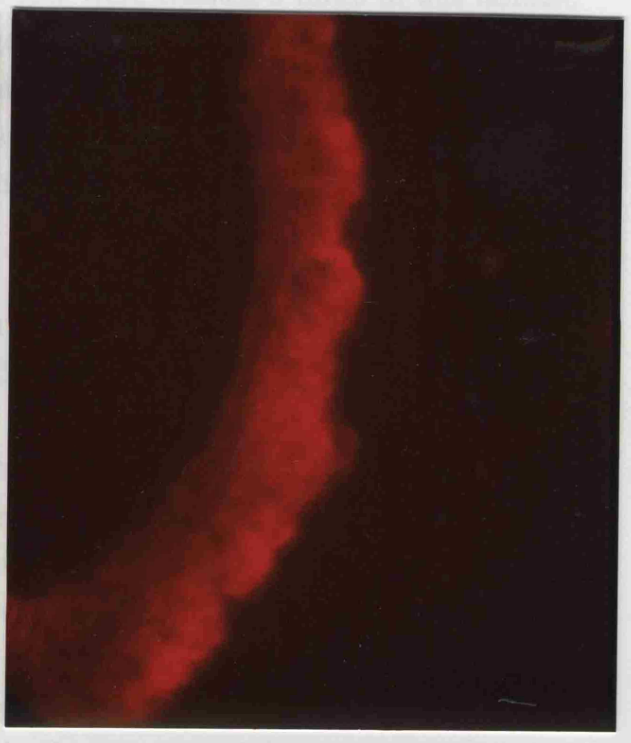

Fig 5.3d

Fig 5.3 Tissue-association of TRITC-labelled IGF-I by 17.5 day rat visceral yolk sacs.
Tissue is shown after a)15 and b)30 minutes incubation, and also c)after 30 minutes incubation at low temperature and d)in the presence of 10µg/ml sodium fluoride. (Mag:120x).

Processing of TRITC-labelled IGF-I by the cultured, anembryonic rat visceral yolk sac.

In parallel, with the series of experiments investigating the location of ligand in the 17.5 day explanted yolk sac, experiments were also carried out using the equivalent cultured tissue.

Method

Rat embryos were explanted from the mother on the 9th day of gestation, and cultured for the next 8 days in a 50:50 mixture of heat-inactivated rat serum and M199 as previously described (Chapter 2). On the 8th day of culture, these yolk sacs were washed with fresh, warm M199, before M199 containing 5µg/ml TRITC-IGF-I was added. Yolk sacs were then gassed with 95%O_2 before being returned to the roller incubator. Again, a series of time points were collected, when yolk sacs had been incubated with TRITC-IGF-I for 15, or 30 minutes. When the required incubation period was reached, the fluid surrounding the yolk sacs was removed using a bent needle and syringe, and 1% saline introduced to the bottle. The yolk sacs were washed 3 times in this fashion, before being floated out into a large container filled with 1% saline. The yolk sac was then burst and the tissue washed thoroughly once more in saline. After blotting dry, the yolk sac was then arranged in OCT and frozen down as described for the explanted yolk sacs.

Results

Fig. 5.4a shows a cultured, anembryonic rat visceral yolk sac which has been exposed to TRITC-IGF-I for 15 minutes. Fluorescence has been taken up into the cells, and appears to have penetrated quite deeply. After 30 minutes incubation (Fig 5.4b), there seems to be more fluorescence, although the general pattern of distribution seems similar to Fig 5.4a.

Fig 5.4c shows yolk sac tissue incubated at 4°C for 30 minutes in the presence of TRITC-IGF-I. The level of fluorescence is dramatically reduced, and very little fluorescence is detectable throughout the tissue.

When the glycolysis inhibitor sodium fluoride is added, (Fig 5.4d) there appears to be a slight reduction in the amount of fluorescence detectable, though not as noticeable as in Fig 5.4c.

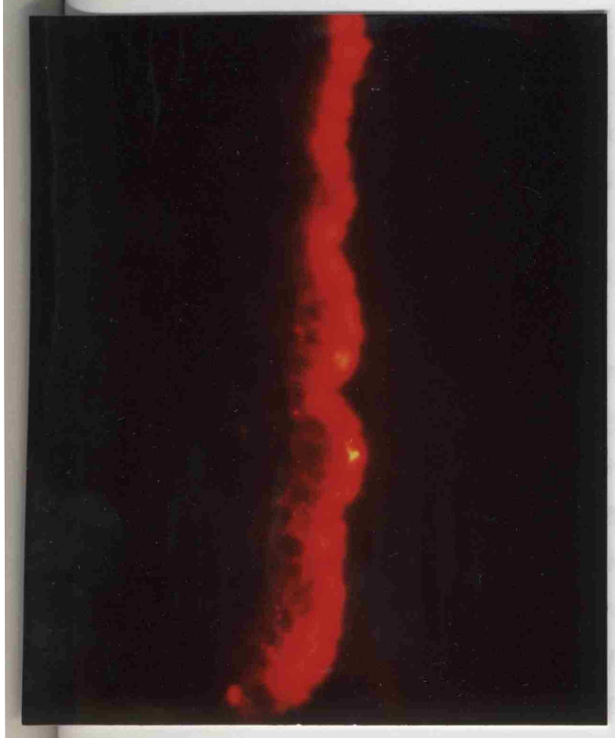

Fig 5.4a

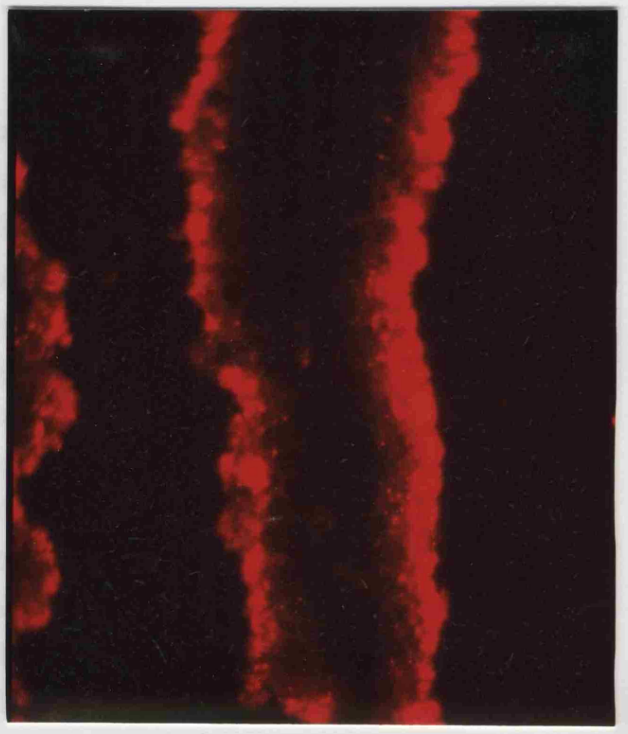

Fig 5.4b

Fig 5.4c

Fig 5.4d

Fig 5.4 Tissue-association of TRITC-labelled IGF-I by cultured, anembryonic rat visceral yolk sacs.
Tissue is shown after a)15 and b)30 minutes incubation, and also after c)30 minutes incubation at low temperature and d)in the presence of 10µg/ml sodium fluoride. (Mag:120x).

Dual labelling experiments

One of the most useful features of a confocal laser-scanning microscope is its ability to digitally record visual information. This now makes it possible to examine the localisation of two differing fluorophores within the same tissue section, as the results seen when the tissue is examined at one wavelength can be stored, and then superimposed upon the image seen when that same section is examined at a differing wavelength. Such "dual labelling" experiments, as they are referred to, are extremely useful as it becomes possible to determine how differently labelled molecules exist in relation to each other within the cell.

In the case of studying insulin and IGF-I uptake, this should prove a particularly useful technique, because the similarity between these two molecules may mean that both are taken up at the same receptor sites. It should now be possible to visually determine whether this is occurring, and whether these proteins co-exist within the yolk sac epithelial cells.

Method

Insulin had previously been conjugated to the fluorescent marker fluorescein isothiocyanate (FITC), and IGF-I to tetramethyl rhodamine isothiocyanate (TRITC) as described earlier in this chapter. These fluorescently labelled proteins were then added together to either 17.5 day explanted yolk sacs, or to the corresponding cultured, anembryonic tissue

For the 17.5 day explanted yolk sac experiments, rat conceptuses were removed on the 17th day of gestation, and the yolk sacs removed (see Chapter 2). Tissue was then placed in glass bottles which contained M199 with both FITC-insulin (at 20µg/ml) and TRITC-IGF-I (at 5µg/ml) added. The tissue was then gassed for 1 minute with 95%O_2, and the bottles then placed in roller incubators at 37°C. Yolk sacs were removed after various periods of incubation, and washed 3 times in 1% saline. After this, tissue was blotted dry on tissue paper, and then frozen down in embedding compound placed in a hexane/ dry-ice mixture. Sections were then cut between 6-8µm, fixed and mounted (as described earlier in this chapter) to allow examination of the tissue with an inverted Zeiss epifluorescence microscope with a Biorad MRC-600 laser scanning confocal imaging system attached.

In parallel, rat conceptuses were removed from the mother on the 9th day of gestation, and the yolk sacs cultured for 8 days as previously described (Chapter 2). On the day of the experiment, these yolk sacs were washed with fresh, warmed M199 which was introduced to the bottle using a bent needle attached to a syringe. After 3 such washes, all medium was removed to be replaced with M199 which contained both FITC-insulin (at 20µg/ml) and TRITC-IGF-I (at 5µg/ml) at 1ml per yolk sac. Tissue was then gassed with

95%O_2 for 1 minute before the bottles containing yolk sacs were replaced in the roller incubators at 37° C.

The incubation medium was removed after differing periods of time, again using a bent needle attached to a syringe. The yolk sacs were then washed with 1% saline, before being frozen down in embedding compound immersed in a hexane/ dry ice freezing mixture. Sections were cut at 6-8μm width, fixed and mounted as earlier described, and examined using the confocal microscope imaging system.

Results

Fig. 5.5a-d shows uptake of FITC-insulin and TRITC-IGF-I over a 1 hour incubation period in the 17.5 day explanted rat visceral yolk sac when both fluorescently labelled ligands are added simultaneously. The images show uptake after 10, 20, 30 and 60 minutes incubation. It is important to remember that these fluorphores have not been added at the same concentrations, and so it is impossible to make any comparison between the varying amounts of fluorescence seen. However, the localisation of these labels within the yolk sac epithelia is interesting.

It appears that when these fluorophores are added simultaneously, staining is detectable within differing areas of the yolk sac. This is noticeable after only 10 minutes incubation (Fig 5.5a), when already the TRITC-IGF-I is detectable in vesicles deeper within the tissue than FITC-insulin. After 30 minutes, (Fig 5.5c) there is still strong FITC fluorescence present close to the apical surface, while there is a more diffuse pattern of red TRITC staining throughout the tissue. This pattern is also apparent after 60 minutes incubation (Fig 5.5d), when there is even stronger FITC staining close to the apical surface.

Using the confocal microscope, the tissue resolution is much better than that seen under a standard epifluorescence microscope. In particular, it is now possible to see that the fluorescence is present within discrete vesicles.

Fig 5.6a-d show the uptake of FITC-insulin and TRITC-IGF-I again over a 1 hour incubation period, this time in 17.5 day cultured, anembryonic yolk sac tissue. Although the tissue definition is not quite as good in these images, a similar pattern of uptake is seen, with a diffuse, red stain detectable deeper within the tissue than the FITC. Figs 5.6 c and d are, unfortunately, somewhat over-exposed, which is a result of the automatic processing which the transparency negatives undergo. However, Fig 5.6b, which shows uptake after 20 minutes continuous incubation, reveals this pattern of uptake.

Fig 5.5a

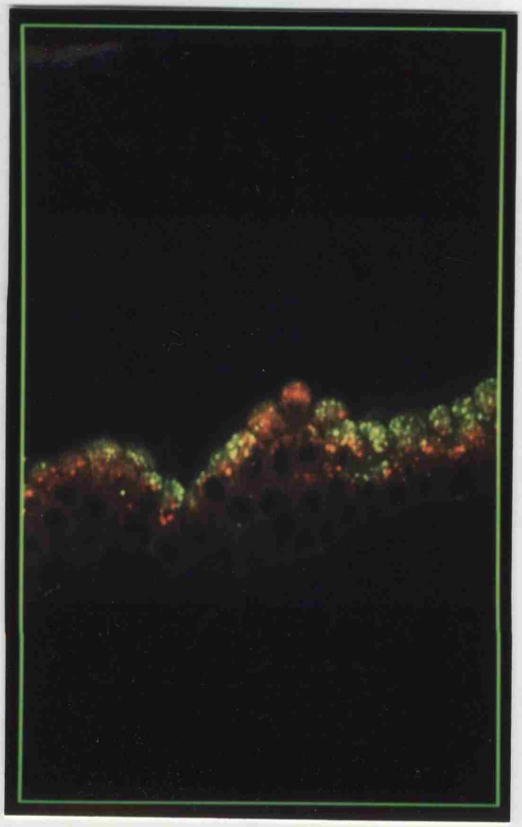

Fig 5.5b

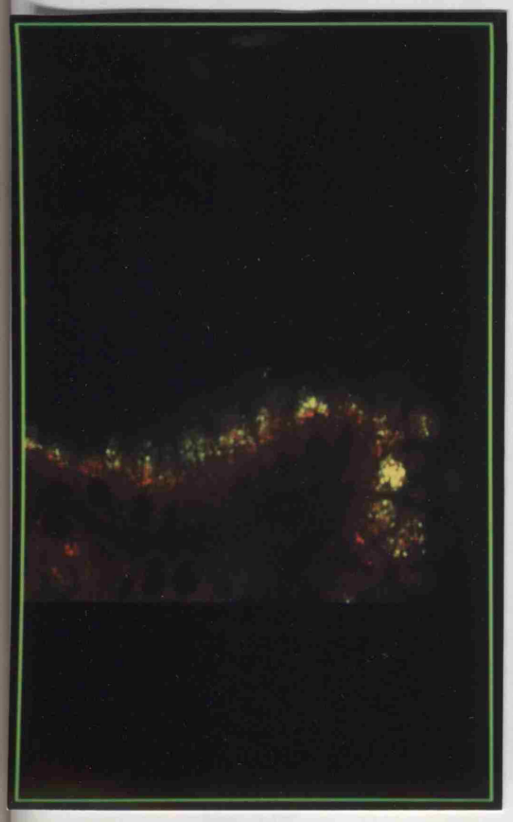

Fig 5.5c

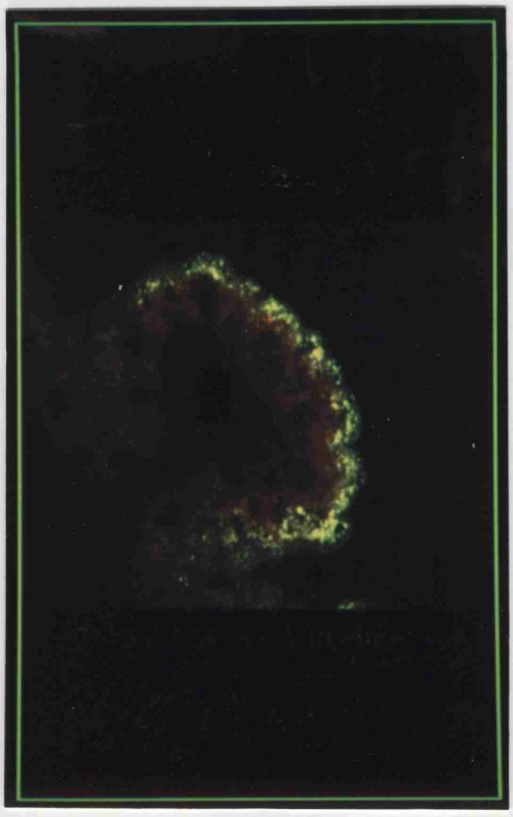

Fig 5.5d

Fig 5.5 Dual labelling of 17.5 day explanted rat visceral yolk sacs with FITC-insulin and TRITC-IGF-I after 10, 20, 30 and 60 minutes incubation. (Mag:120x).

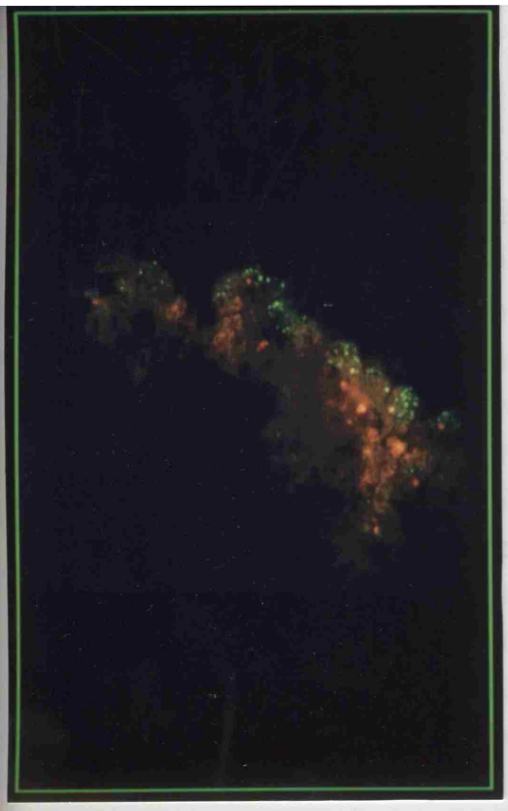

Fig 5.6a

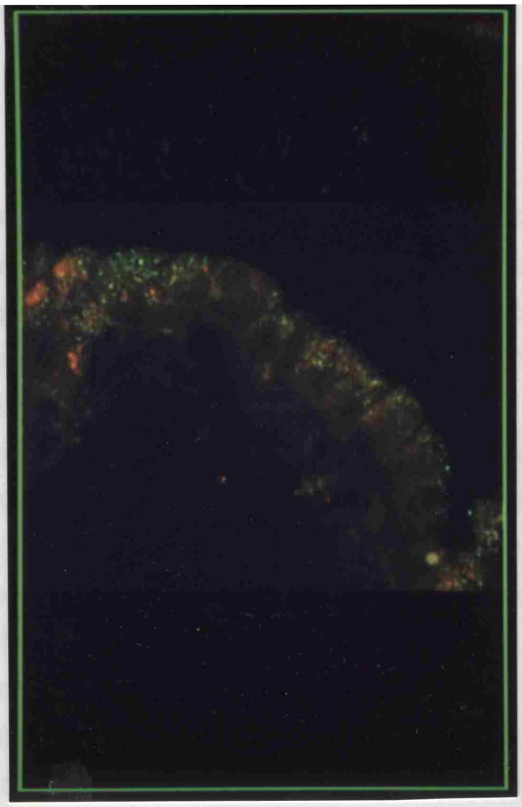

Fig 5.6b

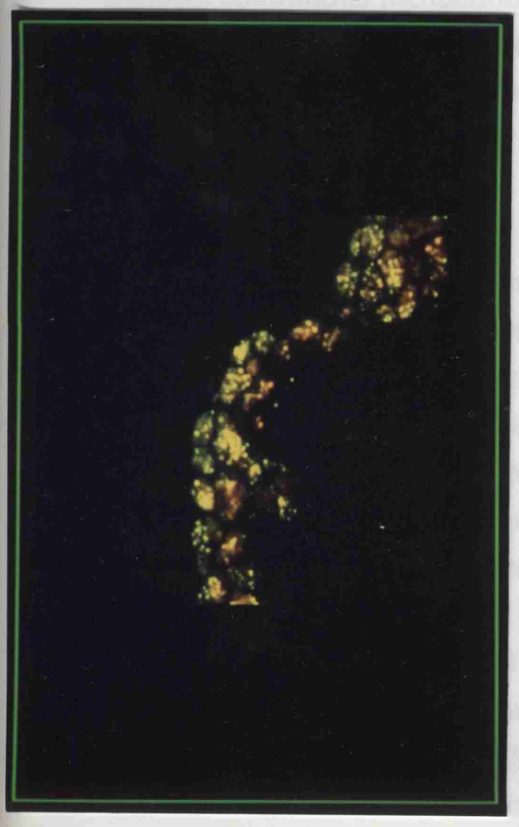

Fig 5.6c

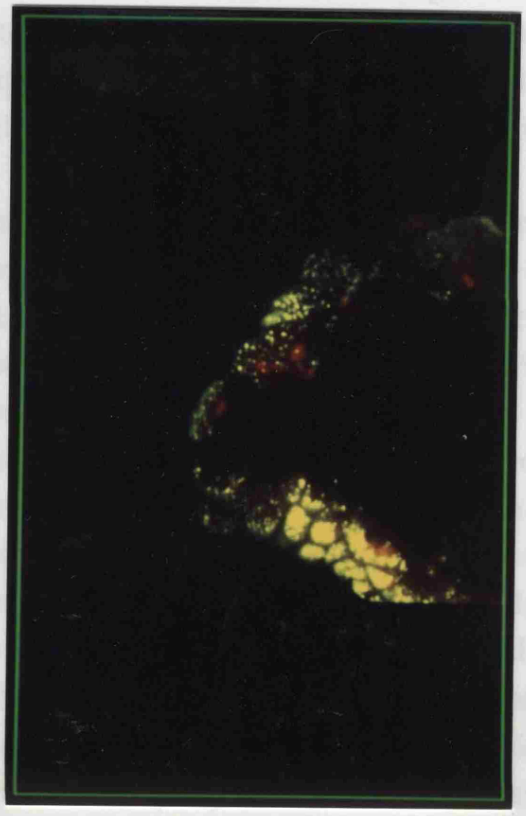

Fig 5.6d

Fig 5.6 Dual labelling of 17.5 day cultured, anembryonic rat visceral yolk sacs, with FITC-insulin and TRITC-IGF-I after 10, 20, 30 and 60 minutes incubation. (Mag:120x).

Discussion

The results presented in this chapter would appear to add considerably to the radiolabelled studies previously described, providing insights into the intracellular nature of the processing of these hormones. They provide direct morphological evidence that when yolk sac tissue is exposed to either of these fluorescently labelled hormones, this results in uptake into that tissue. This strongly suggests that the high levels of protein degradation seen in Chapters 3 and 4 using radiolabelled substrate, occurs as a result of some intracellular processing mechanism rather than an enzyme present at the cell surface. Further, significant differences do become apparent when FITC-labelled insulin and TRITC-labelled IGF-I intracellular localisation are compared, which may reflect a difference in intracellular sorting mechanisms.

If FITC-insulin is first considered, a large amount of fluorescence is first detectable after a short period of incubation (10 minutes in the dual labelling confocal images). This fluorescence is detectable within small, discrete vesicles lying close to the periphery of the cell, and this vesicular distribution is seen in both the explanted, and the corresponding cultured, yolk sac tissue. After 30 minutes continuous incubation, the amount of fluorescence is so large that this vesicular pattern is lost to a great extent, possibly due to the close proximity of these vesicles, so that the entire apical surface of the yolk sac epithelium appears to fluoresce. These vesicles are probably part of the endosomal complement of the cell.

Following binding of a number of hormones to their cell surface receptors, the entire hormone-receptor complex is internalised, and this internalisation has in fact been demonstrated for insulin in lymphocytes (Carpentier, 1989). Some receptors, such as the low-density lipoprotein or LDL receptor, are known to be selectively concentrated at the cell surface into clathrin coated pits, while in the case of insulin, is seems that the initial binding of hormone to receptor occurs over the entire cell surface, after which the complex appears to accumulate within coated pits in a variety of cultured cells (reviewed Bergeron *et al*, 1985; Gruenberg and Howell, 1989). These coated pits then invaginate forming a pit into the cell cytoplasm, so that eventually a closed, coated vesicle is formed when this pit pinches off and a continuous plasma membrane is formed (reviewed Rodman *et al*, 1990). Coated vesicles, once formed, are short lived structures which rapidly lose their clathrin coats to become uncoated vesicles. Uptake into these coated vesicles is most commonly associated with receptor-mediated endocytosis, and uptake may also occur directly via uncoated vesicles.

Once a vesicle is within the cell, it may then fuse with a population of intracellular vesicles known as endosomes. Early endosomes, the first

population with which the internalised hormone-receptor complex is associated, are the site where intracellular processing begins. These have also been referred to as the compartment for uncoupling receptor and ligand (or CURLs), which describes one of the principal functions of these organelles. Once the receptor becomes dissociated from the ligand, it may be recycled back to the cell surface. Alternatively, receptors may be transported to the lysosomes for breakdown (eg, the epidermal growth factor receptor); to the Golgi apparatus (eg small amounts of the transferrin receptor); or even to the surface of the opposing membrane (eg the polymeric immunoglobulin receptor). It is believed that in cultured fibroblasts at least, insulin receptors are recycled back to the cell surface (Knutson, 1992).

Following sorting in the early endosomes, internalised ligand is frequently detected in a population of late endosomes which differ morphologically from the early endosomes. These still do not contain the enzymes required for the breakdown of ligand, so these endosomes then fuse with a population of lysosomes, located in the perinuclear region of the cell, to form secondary lysosomes. Lysosomes contain acid hydrolases, so that ligand is then broken down to low molecular weight products which then pass out of the secondary lysosome either by passive diffusion or active transport (Docherty *et al*, 1983). Any molecule which cannot be broken down by the actions of lysosomal enzymes remains within the compartment as a residual body.

From these morphological data, processing of FITC-insulin appears to result in the transport of fluorescent label to a population of early endosomes. The passage after this is less clear, as fluorescence appears to remain concentrated at this apical surface, so that even after one hour of incubation, fluorescence is not detectable deep within the cell. The radioactive data presented in Chapter 3 suggests that a significant proportion of insulin is broken down into smaller molecular weight products, although the site where this occurs is unclear. This breakdown cannot be visualised using such fluorescence labelling techniques. Only one image (Fig 5.2b) appears to show vesicles lying deep within the yolk sac epithelium, which may demonstrate passage of FITC-insulin across the yolk sac, although generally, FITC remains localised at the apical surface of the cells.

One possible reason for the apparent lack of FITC-insulin deeper within the yolk sac cells, is that FITC-insulin is not stable in the acid pH present within these vesicles. To test whether this was true, FITC-insulin was incubated for 20 minutes at 37°C at either an acidic or a neutral pH (0.1M citrate buffer, pH 5.0 and 50mM phosphate buffer pH 7.2). It was then spun at 5000rpm for 1 hour at 4°C in a Filtron microconcentrator with a 3000 Dalton filter. Any free FITC

(MW 389.4) would pass through the filter membrane and be detectable as fluorescence within the filtrate, while that bound to insulin would remain within the sample reservoir. There was no appreciable dissociation of FITC from insulin at either neutral or acidic pH, so it is unlikely that dissociation is occurring *in vivo*. FITC-insulin, therefore, is detected within the early endosomes, and appears to remain within these vesicles.

One possibility is that after transport of insulin to the endosomes, it may be subject to attack by an enzyme present within these organelles, rather than one active at the acid pH present within lysosomes. This enzyme may initially have existed in an inactive form on the plasma membrane, only to become active upon its internalisation with the ligand-receptor complex. Alternatively, insulin present within the endosomes may then be subject to attack by a cytosolic enzyme, although the mechanism by which such an enzyme could gain access to the hormone remains obscure.

A third possibility is that some insulin is transported intact across the yolk sac epithelia. In some cases, internalised ligands (and receptors) may be transported intact from endosomes to the surface of the cell opposite to that at which they entered. This process is termed transcytosis (reviewed Mostov and Simister, 1985), and is known to occur in the case of immunoglobulin transport across epithelia; for example, transport of IgG across the small intestine of the neonatal rat, when a specific IgG receptor binds IgG at pH6 (the pH of the internal lumen of the gut) but releases it at pH 7.5 (blood pH). This process has also been reported to occur in the case of insulin in bovine aortic endothelial cells, which internalise radiolabelled insulin and release 80% of the hormone intact at the opposite cell surface (King and Johnson, 1984). This mechanism, therefore, allows one way transport of protein, and may be occurring within this yolk sac system to transfer insulin intact from the endosomes lying close to the apical surface of the yolk sac, to the basolateral surface, where it could be released to act upon the developing embryo. It must be stressed however, that there is little morphological evidence that such transport is occurring is this tissue.

If the processing of TRITC-IGF-I by rat visceral yolk sac tissue is next considered, then a large amount of fluorescence is detectable within the tissue after only 15 minutes incubation. Again, if the confocal images are studied, the label appears to be present within vesicles inside the tissue rather than adhered to the cell surface. A reduction in the level of fluorescence is seen once again at low temperature, with sodium fluoride producing little effect.

In the dual labelling experiments, the location of both fluorescent markers can be directly compared, and it seems that TRITC-IGF-I penetrates the cells more deeply than fluorescently labelled insulin. Although both ligands

initially appear to be present within a population of endosomes, FITC and TRITC do not seem to coexist extensively within the same vesicles. Over the 60 minutes of continuous incubation with both ligands, TRITC appears to move deeper into the cell in both the 17.5 day explanted, and corresponding cultured tissue. This may represent the passage of labelled IGF-I through an intracellular system, either to lysosomes or some other site of digestion. Alternatively, TRITC-IGF-I may be transported from the early endosomes to deep within the cell via a system of transport vesicles not connected with an intracellular breakdown mechanism.

CHAPTER SIX

USE OF A MONOCLONAL ANTIBODY TO BLOCK IGF-I RECEPTORS ON RAT EMBRYONIC TISSUES.

Introduction

Monoclonal antibodies are produced by the fusion of spleen cells from an animal previously immunized by repeated injection of an antigen, with malignant myeloma cells, which are cells capable of growth and division in culture. The resulting hybrid cell is then capable of producing antibody against the original antigen. The antibody so produced is derived therefore from a single clone and is highly specific for the antigen it was raised against.

A mouse monoclonal IgG1 antibody to the human IGF-I receptor was originally prepared by fusing spleen cells from mice, previously injected with a partially purified preparation of insulin receptors from human placental membranes, with Fo myeloma cells (Kull *et al*, 1983). The placental preparation must also have contained some IGF-I receptors, as subsequent characterisation of the antibody (called αIR-3) revealed that it immunoprecipitated [^{125}I]-labelled IGF-I bound to solubilized IGF-I receptors. The antibody appeared to immunoprecipitate two polypeptides, of apparent molecular weight 135,000 and 90,000, which correspond to the α and β subunits of the IGF-I receptor, with both the reduced and denatured [^{125}I]-labelled α-subunit (the larger molecular weight subunit) being recognized. The conclusion of this work was that the αIR-3 antibody had only a minimal cross reaction with insulin receptors, though in some gels the β-subunit appears as a doublet. This may be due to a small amount of cross-reactivity with insulin receptors or alternatively slight differences in the β-subunit structure due to proteolysis of the receptor during the membrane preparation. The antibody has also been reported to have no cross-reactivity with the IGF-II receptor (Germain-Lee *et al*, 1992).

The production of this antibody provided an impetus for study in this field. Flier and Moses (1985) demonstrated that αIR-3 blocked the IGF-I receptor, completely inhibiting binding of IGF-I to its receptor in placental preparations. It thus provided a useful tool, as the actions of insulin and IGF-I could now be examined separately.

The addition of this antibody has been shown to block IGF-I mediated DNA synthesis in human skin fibroblasts (Flier *et al*, 1986), demonstrating that the mitogenic response to IGF-I in these cells is mediated via the IGF-I receptor. This work also demonstrated that even when IGF-I binding is completely inhibited, the αIR-3 antibody does not act as an agonist, producing DNA synthesis. Jacobs *et al* (1986) further showed that [^{125}I]-labelled αIR-3 binding was inhibited by the presence of unlabelled αIR-3 at concentrations far lower than those required to inhibit IGF-I binding. Together, these two facts suggest that the epitope to which the antibody binds is distinct from the IGF-I binding site, with the antibody acting as a competitive antagonist, as inhibition is reversible with the addition of excess IGF-I (Arteaga and Osborne, 1989).

Use of this antibody has also led to an explanation for why many cultured cell lines require supraphysiological concentrations of insulin, that is concentrations which would saturate the insulin receptors present, for their continued growth. DNA synthesis has been shown to be inhibited in cultured human fibroblasts in the presence of the αIR-3 antibody (Van Wyk *et al*, 1985). Thus, the mitogenic response of insulin is such cell lines must be partly mediated through the IGF-I receptor.

As this antibody has been reported to block the IGF-I receptor and thereby inhibit IGF-I activity (Flier and Moses, 1985; Rohlik *et al*, 1987), it was decided to culture embryos from the early head fold stage, that is approximately 9.5 days of gestation, in the presence of this antibody. Culture of rat embryos from this stage until 11.5 days is extremely useful for examining the requirements for embryonic growth and development, and also for identifying potential teratogens (substances harmful to developing embryos). This is because these 48 hours cover a major proportion of organogenesis in the rat, which is the period during which all the major organ systems of the body are developed. If any morphological abnormalities did become apparent in the presence of this antibody it would be strong evidence that not only was the IGF-I receptor present, but also that IGF-I is necessary for development in the rat during this critical period.

One potential problem with this antibody was that it had originally been raised against human tissue, and no information had been published concerning the cross-reactivity of this human antibody with tissues from other species. However, in the absence of any commercially available antibody which would block the rat IGF-I receptor, it was decided to use this antibody, and it was assumed that some cross-reactivity would occur. This assumption is based upon the finding that there is a high degree of homology between the human and rat IGF-I receptors. Sequencing of the signal peptide and 333 amino acids of the α subunit (the external subunit which contains the antibody binding site) reveal over 94% homology between the human and rat sequences (Werner *et al*, 1989), which strongly suggests that some degree of cross-reactivity should occur.

In parallel with this investigation into the effect of this antibody on embryo culture, further experiments were performed in an attempt to demonstrate the presence of this receptor using a more direct approach, and also to locate where this receptor was situated. The method chosen to address the first question was a very simple immunostaining procedure, while immunoprecipitation was chosen to try to directly establish the existence of the receptor.

Effects of a Monoclonal Antibody against the IGF-I Receptor on Embryonic Development.

Method

Rat embryos were explanted from the mother at 9.5 days gestation as described in Chapter 2. Five embryos were placed in a glass bottle, with 1ml medium per embryo. In this experiment, the medium used was 75% heat inactivated rat serum and 25% M199, the extra serum being required for the survival and intact development of the embryos. In the first bottles, 5 embryos were placed in 5mls of the culture medium, and gassed with 5%O_2 mixture for 1 minute. To half of these bottles, the antibody was then added at 1μg/ml, the other series of bottles acting as a control.

In addition, the effects of a non-specific antibody was also examined. This was to ensure that any effects seen with the monoclonal against the IGF-I receptor were specific, rather than a general effect seen when mouse antibodies were added to this system. The rat visceral yolk sac is known to have extensive IgG receptors on its surface (Weisbecker *et al*, 1983: Roberts *et al*, 1990), so it is important to determine that the αIR-3 antibody (which is an IgG molecule) is not acting on these IgG receptors. Here, embryos were placed in glass bottles with 1ml medium per embryo. One of these tubes also contained the purified mouse IgG at the same concentration as αIR-3 (namely 1μg/ml). These embryos were then also gassed with 5%O_2, before being returned to the roller incubator.

The embryos were subsequently cultured for 48 hours at 37°C, being gassed after 24 hours with a 20%O_2 mixture, and with a 40%O_2 mixture approximately 4 hours before harvesting.

After the 48 hour culture period, the 11.5 day embryos were removed for scoring.

Morphological Assessment of Embryos

A morphological scoring system has been developed by Brown and Fabro (1981), and can be used routinely to evaluate the development of rat embryos *in vitro*. Thirteen criteria which are relatively easy to assess morphologically are examined; these include limb, heart and brain development, the number of somites apparent, and the state of the yolk sac circulation. Each feature is awarded a score, as recorded in Table 6.1, and the sum of the individual scores equals the morphological score. These, together with a parallel estimation of the total protein content of the embryo, provide an accurate estimation of the embryonic age. The possibility that a substance may produce a dysmorphic effect may, therefore, be examined with a quantitative comparison of growth and development possible using this system.

		0	1	2	3	4	5	SCORE
A	YOLK SAC CIRCULATORY SYSTEM	no visible, or scattered, blood islands	Corona of blood islands w or w/o anastamoses	Vitelline vessels with few yolk sac vessels	Full yolk sac plexus of vessels	Yolk stalk obliterated, vitelline artery & vein well separated		
B	ALLANTOIS	Allantois free in exocoelome	Allantois fused with chorion	Umbilical vessels	Separate aortic origins of umbilical and vitelline vessels			
C	FLEXION	Ventrally convex	Turning	Dorsally convex	Dorsally convex with sacral torsion			
D	HEART	Endocardial rudiment not visible, or visible but not beating	Beating 's' shaped cardiac tube	Convoluted cardiac tube	Bulbus cordis, atrium commune and ventriculus communis	Dividing atrium commune		
E	CAUDAL NEURAL TUBE	Neural plate or neural folds	Closing, but unfused neural folds (groove)	Neural folds fused at level of somites 4/5	Posterior neuropore formed but open	Posterior neuropore closed		
F	HIND BRAIN	Neural plate	Rhombomeres A and B	Anterior neuropore formed but open	Anterior neuropore closed, rhombencephalon formed	Pronounced pontine flexure with transparent roof of 4th ventricle		
G	MID BRAIN	Neural plate	Mesencephalic brain folds	Closing or fusing mesencephalic folds	Completely fused mesencephalon	Visible division between mesencephalon & diencephalon		
H	FORE BRAIN	Neural plate or no visible prosencephalon	Prosencephalic brain folds	Completely fused prosencephalon	Visible telencephalic evaginations	Well elevated telencephalic hemispheres		
J	OTIC SYSTEM	No sign of otic development	Flattened or indented otic primordium	Otic pit	Otocyst	Otocyst with dorsal recess	Otocyst with endolymphatic duct	
K	OPTIC SYSTEM	No sign of optic development	Sulcus opticus	Elongated optic primordium	Primary optic vesicle with open optic stalk	Indented lens plate	Lens pocket or lens vesicle	
L	OLFACTORY SYSTEM	NOT ROUTINELY ASSESSED						
M	BRANCHIAL BARS	None visible	I visible	I and II visible	I, II and III visible	II overgrowing and obscuring III		
N	MAXILLARY PROCESS	NOT ROUTINELY ASSESSED						
P	MANDIBULAR PROCESS	NOT ROUTINELY ASSESSED						
Q	FORE LIMB	No sign of fore limb development	Distinct evagination of wolffian crest at level of somites 9-13	Fore limb bud	Paddle shaped fore limb bud	Distinct Apical ridge on fore limb bud		
R	HIND LIMB	No sign of hind limb development	Distinct evagination of wolffian crest at level of somites 26-30	Hind limb bud	Paddle shaped hind limb bud			
S	SOMITES	0 - 6	7 - 13	14 - 20	21 - 27	28 - 34	35 - 41	

Table 6.1 Morphological Score Sheet for the Assessment of Growth and Differentiation.

Results

Results are given for the primary control experiments, when no antibody was added; the secondary control experiments, when the non-specific antibody mouse IgG was added; and finally for the series of experimental embryos, when the αIR-3 antibody was added.

The results are given in Table 6.2 and also in the form of histograms in Figs 6.1 a,b,c,d, and e.

	CONTROL (no antibody) n=22	CONTROL (+ mouse IgG) n=12	EXPT. (+ αIR-3) n=37
Morphological Score	41.63 ± 0.49 S.D.= 2.34	40.42 ± 0.72 S.D.= 2.50	29.92 ± 0.69* S.D.= 4.25
Yolk sac Diameter (mm)	3.98 ± 0.06 S.D.= 0.29	3.97 ± 0.11 S.D.= 0.37	3.42 ± 0.048* S.D.=0.29
Crown Rump Length (mm)	3.63 ± 0.07 S.D.= 0.35	3.62 ± 0.09 S.D.= 0.31	2.81 ± 0.043* S.D.=0.26
Somite mumber	26.55 ± 0.23 S.D.= 1.405	25.83 ± 0.55 S.D.= 1.89	20.08 ± 0.56* S.D.= 3.42
Protein content (μg)	296.14 ± 14.03 S.D.= 65.8	250.59 ± 4.40# S.D.= 15.25	174.97 ± 5.74* S.D.= 34.92

Table 6.2 Addition of antibodies to rat embryos grown in culture in comparison with controls.

Rat embryos were explanted on the 9th day of gestation and either cultured in the presence of a monoclonal antibody to the IGF-I receptor, in the presence of a non-specific antibody mouse IgG, or without the addition of an antibody. Results were analysed for significance using Student's t-test.

Significance: * = significant to control at p<0.001%
= significant to control at p<0.01%.

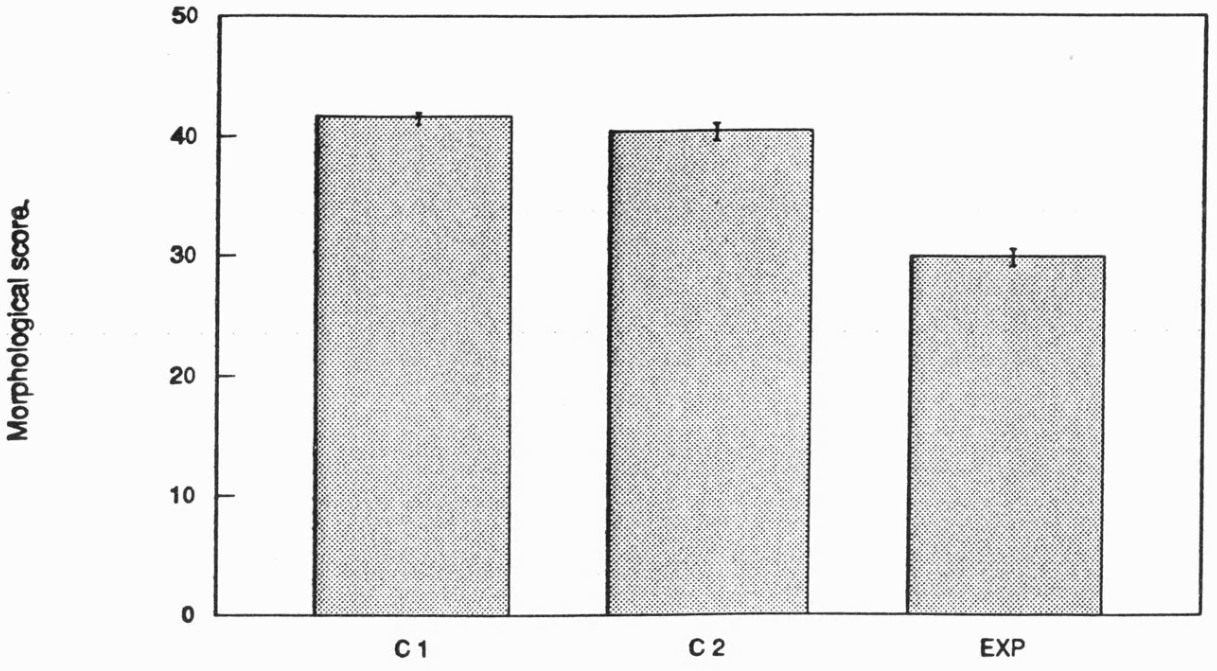

Fig. 6.1a. Morphological Scores of Embryos
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3), cultured in the presence of mouse IgG, or cultured without the addition of an antibody. Results shown are the mean ± S.E.M.
Key: C1 = no antibody (n=22); C2 = + mouse IgG (n=12); EXP = + αIR-3 (n=37).

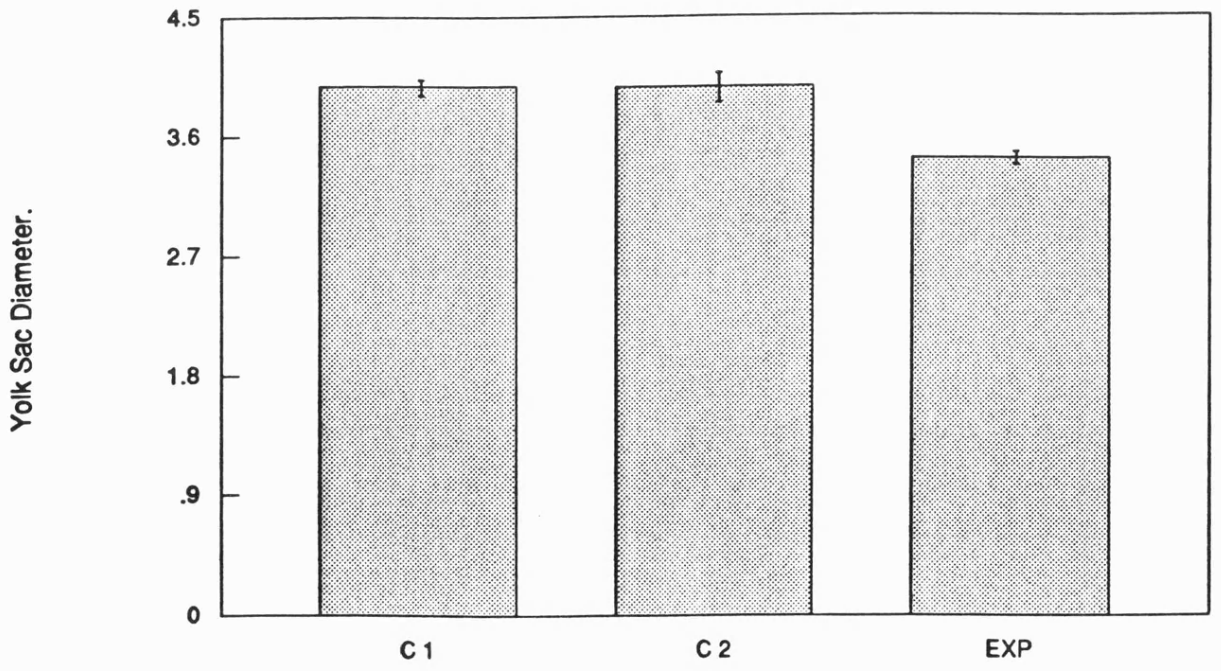

Fig. 6.1b Yolk sac diameter.
Embryos explanted on the 9th day of gestation were cultured for 48 hours either in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3); in the presence of mouse IgG; or without antibody. Results shown are the mean ± S.E.M.
Key: C1 = no antibody (n=22); C2 = + mouse IgG (n=12); EXP = + αIR-3 (n=37).

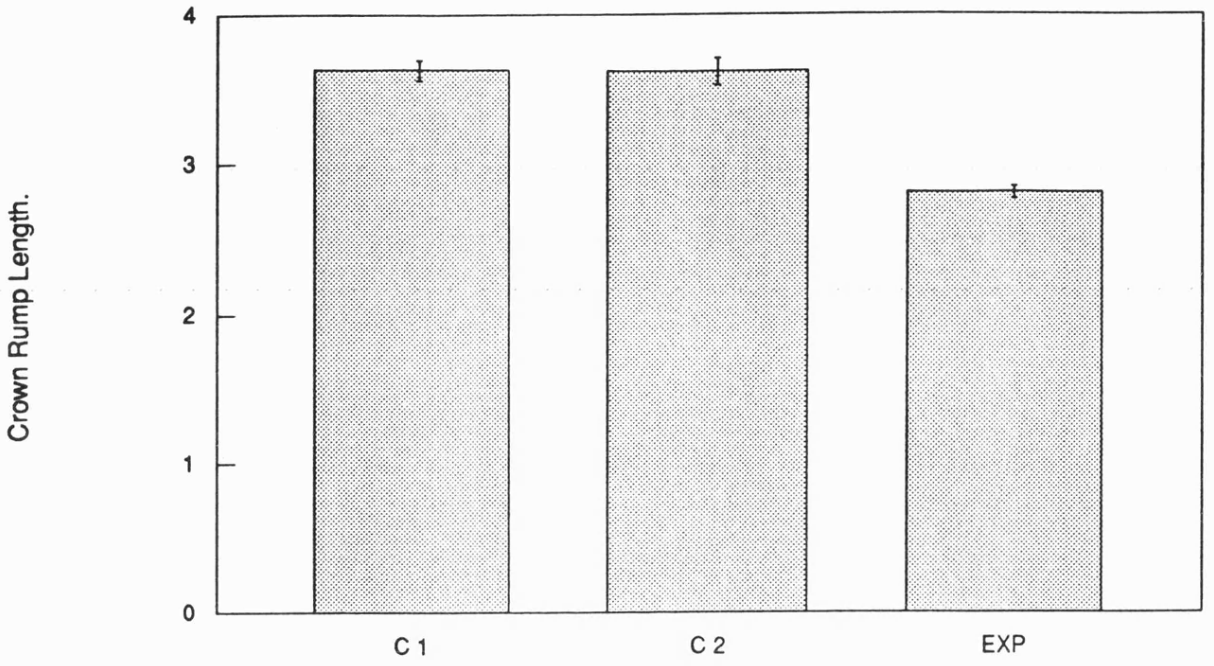

Fig 6.1c. Crown Rump Length of Embryos.
Yolk sacs were explanted on the 9th day of gestation and cultured for 48 hours either in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3); in the presence of mouse IgG; or without antibody. Results are the mean ± S.E.M.
Key; C1 = no antibody (n=22); C2 = + mouse IgG (n=12); EXP = + αIR-3 (n=37).

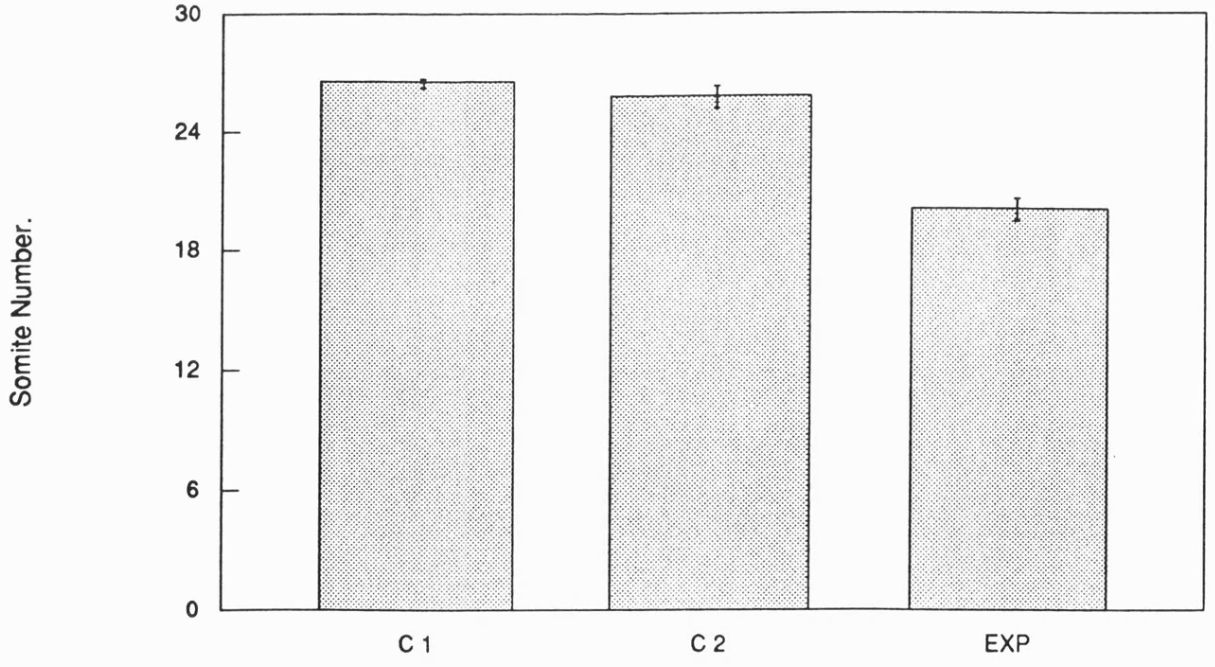

Fig. 6.1d. Number of Somites.
Yolk sacs were explanted on the 9th day of gestation, and cultured for the following 48 hours either with a monoclonal antibody to the IGF-I receptor, with mouse IgG, or with no antibody present. Results plotted are the mean ± S.E.M.
Key; C1 = no antibody (n=22); C2 = + mouse IgG (n=12); EXP = + αIR-3 (n=37).

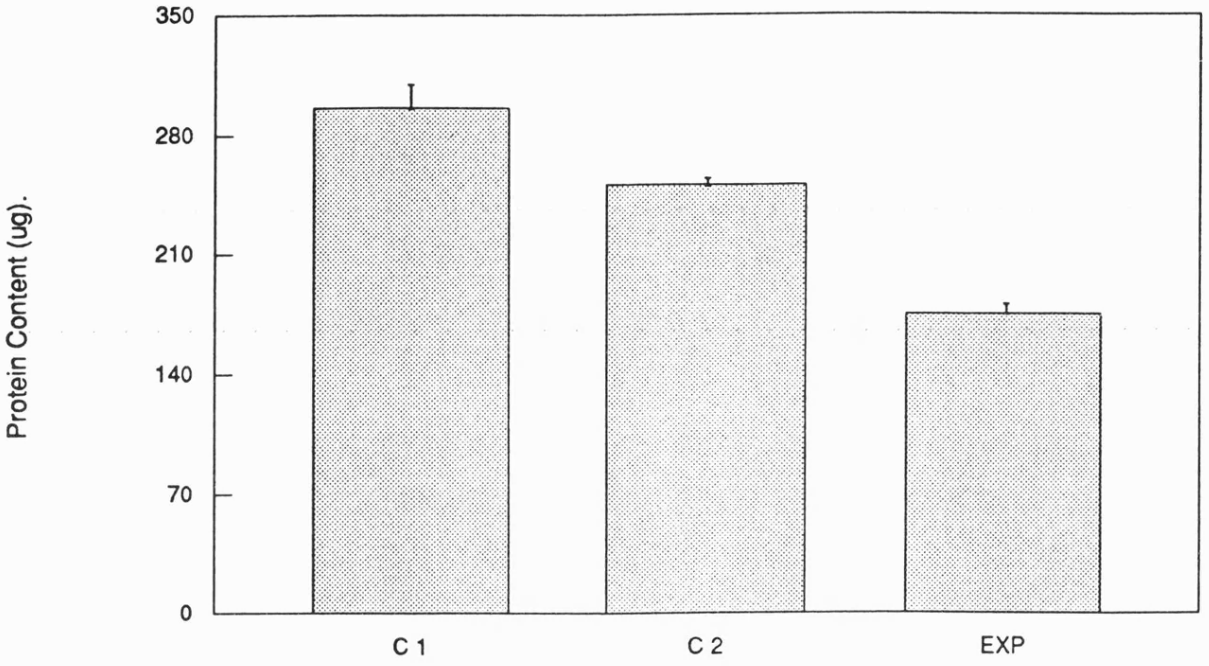

Fig. 6.1e. Total Protein Content of Embryos.
Yolk sacs explanted on the 9th day of gestation and cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3), in the presence of mouse IgG, or without an antibody. The total protein was estimated using the Lowry technique previously described. Results are the mean ± S.E.M.
Key; C1 = no antibody (n=22); C2 = + mouse IgG (n=12); EXP = + αIR-3 (n=37).

It appears that significant decreases in morphological score, yolk sac diameter, crown rump length, number of somites, and the total protein content are observed when embryos are cultured in the presence of the IGF-I receptor antibody, while the addition of mouse IgG produced no effect on the embryos, except in the case of total protein content, when a significant decrease is recorded in the presence of mouse IgG in comparison with the controls.

The addition of a wide number of teratogenic agents the rat embryos at this stage of development may result in several of the abnormalities seen here. For example, neural tube defects are common because the period between 9.5 and 11.5 days of gestation is of particular importance in formation of the brain. Similarly, during these 48 hours the embryo also turns from being ventrally to dorsally convex. Examples of embryos cultured in the presence of this antibody are shown in comparison with a control in Figs 6.2a to 6.2e. Overall, a general decrease in growth was apparent, suggesting that blockage of the IGF-I receptor in this manner produced an reduction of growth (Fig 6.2a and b). Generally, the yolk sac circulation was poor in the experimental embryos. In addition to the general decrease in overall body growth seen by the above criteria, the experimental embryos exhibited a number of more specific abnormalities. In particular, embryonic blood patches were seen on a large number of embryos examined (23 out of the total 37 or 62%). These were mainly present on the

head and tail area, but occasionally seen all over the embryo (Fig 6.2c). Open neural tubes were observed in 9 of the embryos (Fig 6.2b), with one also possessing a fusion line on the telencephalon. Failure of the embryonic tail to turn to its correct position was observed in 19 cases, while 9 embryos demonstrated a kink in their tails (Fig. 6.2d). Finally, several of the experimental embryos also displayed uneven somites (Fig 6.2e).

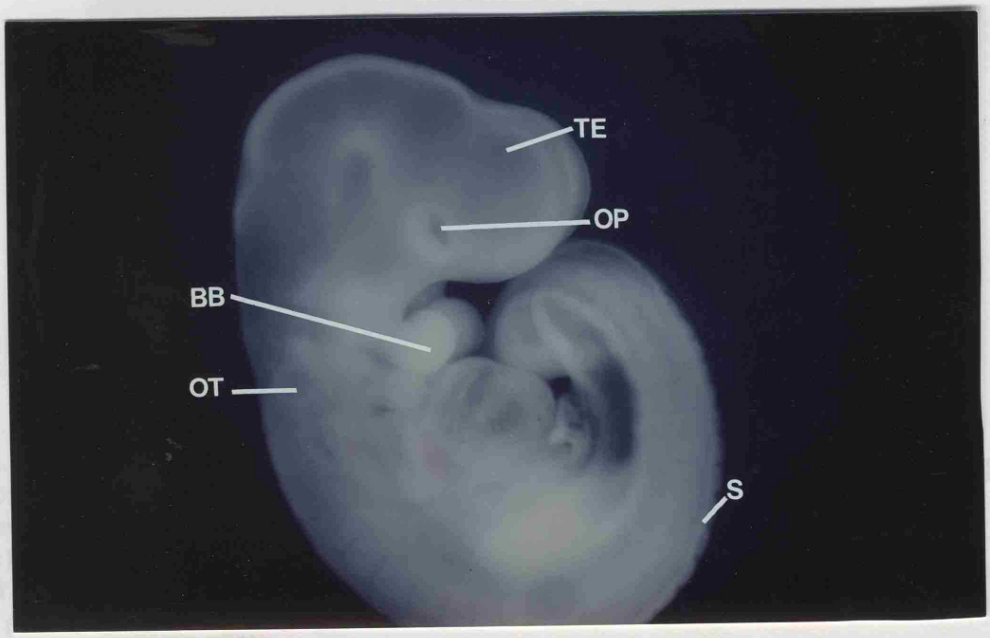

Fig 6.2a Control embryo.
Rat embryos were removed from the mother on the 9th day of gestation, and cultured for the following 48 hours in a 75% rat serum/ 25% M199 mixture. Note the overall size of the embryo and the position of the tail. (TE-telencephalon; OT-otocyst; OP-optic vesicle; BB-branchial bars; S-somites) Magnification: x20.

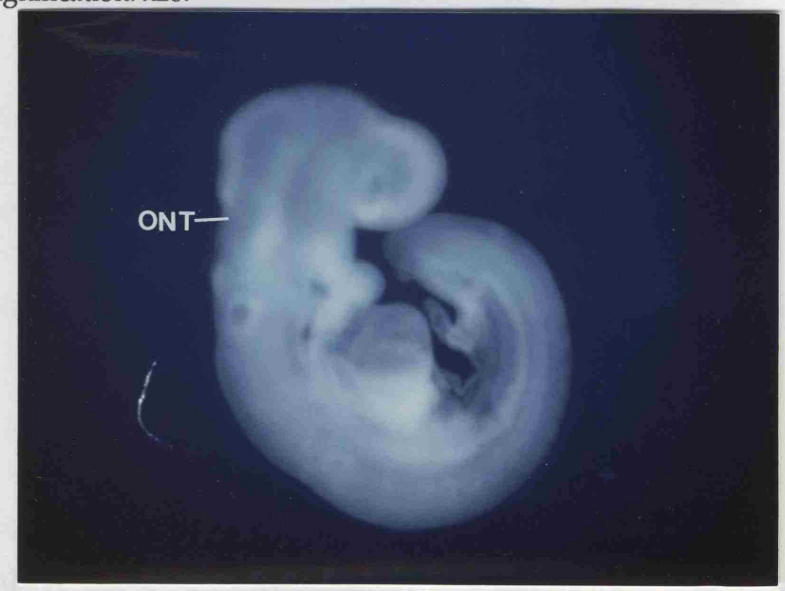

Fig 6.2b Embryo cultured for 48 hours in the presence of a monoclonal antibody to the IGF-I receptor.
Rat embryos explanted from the mother on the 9th day of gestation were cultured for 48 hours in a 3:1 mixture of rat serum and M199 which had a monoclonal antibody to the IGF-I receptor added at 1µg/ml. The overall size of the embryo is greatly reduced in comparison with 6.2a, and the head in particular appears not as well developed with uneven contours. There is also an open neural tube (ONT).Magnification: x20.

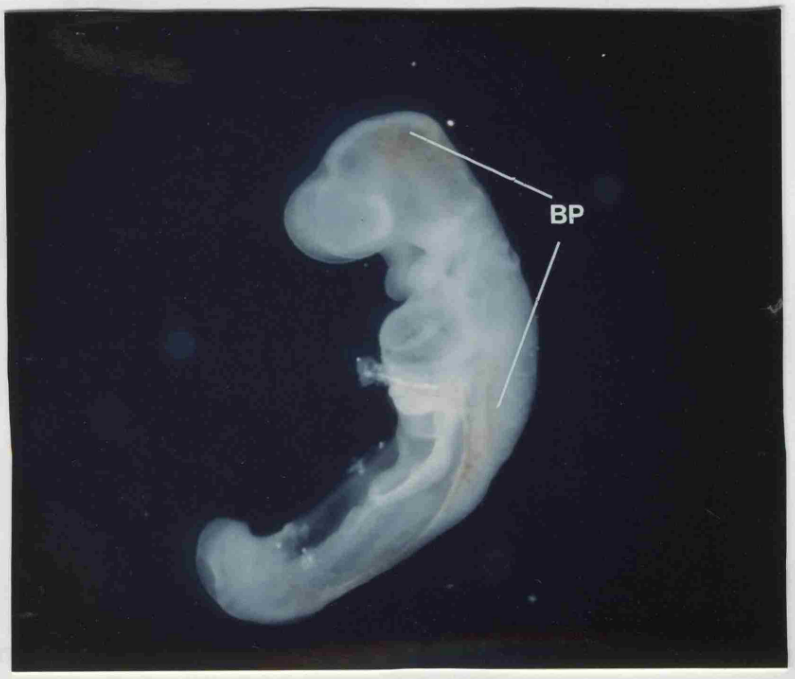

Fig 6.2c Embryo cultured in the presence of a monoclonal antibody to the IGF-I receptor.
Rat embryos explanted from the mother on the 9th day of gestation were cultured for 48 hours in a 3:1 mixture of rat serum and M199 which had a monoclonal antibody to the IGF-I receptor added at 1µg/ml. The tail has failed to turn to its correct position, and there are also extensive blood patches (BP) over the surface.
Magnification: x20.

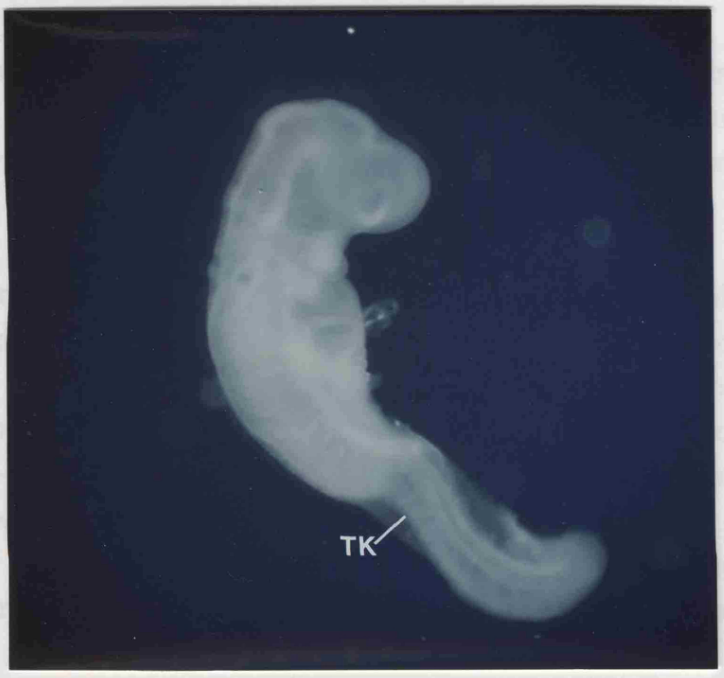

Fig 6.2d Embryo cultured in the presence of a monoclonal antibody to the IGF-I receptor.
Rat embryos explanted from the mother on the 9th day of gestation were cultured for 48 hours in a 3:1 mixture of rat serum and M199 which had a monoclonal antibody to the IGF-I receptor added at 1µg/ml. This embryo shows the failure of the tail to turn correctly, which was a common feature seen in the presence of this antibody. In addition, there is also a kink present in the tail (TK).
Magnification: x20.

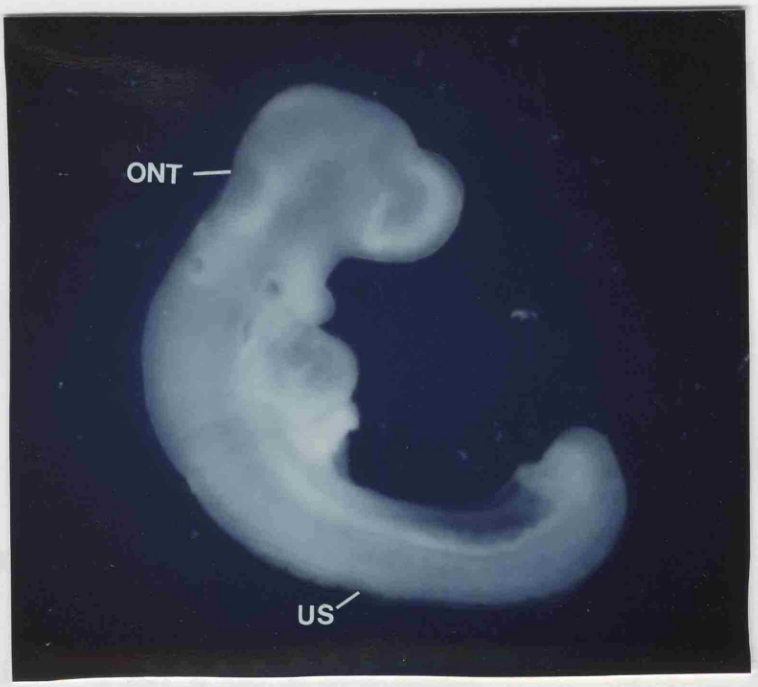

Fig 6.2e Embryo cultured in the presence of a monoclonal antibody to the IGF-I receptor.
Rat embryos explanted from the mother on the 9th day of gestation were cultured for 48 hours in a 3:1 mixture of rat serum and M199 which had a monoclonal antibody to the IGF-I receptor added at 1μg/ml. This embryo displays the uneven somites regularly seen in the experimental embryos (US). The profile of the head also appears abnormal with general dysmorphology. There is also the suggestion of an open neural tube (ONT).
Magnification: x20.

Addition of insulin, IGF-I and IGF-II to the incubation medium.

A further series of experiments was set up to examine whether the addition of IGF-I, and the related growth factors, insulin and IGF-II, could improve the rate of growth seen when the αIR-3 antibody was added.

Method

Rat embryos were explanted from the mother on the 9th day of gestation, and cultured for the following 48 hours as previously described. Rat embryos were either cultured in the presence of the monoclonal antibody to the IGF-I receptor alone (at 1μg/ml), or in the presence of this antibody plus either insulin, IGF-I or IGF-II (also at a concentration of 1μg/ml). A control series was also set up which had none of the above components added.

After 48 hours, the embryos were assessed for growth and development using system set out on Table 6.1, and were then assayed for protein.

Results

The results given are for this series of experiments in Table 6.3. The results consist of the control series, which had no antibody added (control); those which had the monoclonal antibody to the IGF-I receptor (αIR-3) at 1μg/ml added (+Ab); and those which had the antibody (at 1μg/ml) plus either 1μg/ml IGF-I (Ab+IGF-I); 1μg/ml IGF-II (Ab+IGF-11); or 1μg/ml insulin

(Ab+INS) added. These results are again plotted in histogram form in Figs 6.3 a,b,c,d,and e.

	CONTROL (n=5)	+Ab (n=5)	Ab +IGF-1 (n=5)	Ab +IGF-11 (n=4)	Ab +INS (n=5)
Morphological score	40.40±0.2 SD=0.5	28.80±1.4* SD=3.2	32.60±1.3 SD=2.9	29.60±1.8 SD=3.7	29.60±1.0 SD=2.4
Yolk sac diameter (mm)	4.34±0.06 SD=0.13	3.42±0.09* SD=0.19	3.88±0.10# SD=0.25	3.5±0.10 SD=0.30	3.5±0.30 SD=0.25
Crown rump length (mm)	3.88±0.07 SD=0.15	2.66±0.15* SD=0.34	2.9±0.18 SD=0.40	2.65±0.07 SD=0.15	2.98±0.09 SD=0.20
Somite Number	26.4±0.36 SD=0.80	19.0±1.02* SD=2.28	21.8±0.66$ SD=1.47	18.7±1.24 SD=2.49	22.4±0.36# SD=0.80
Protein content (µg)	326.0±18.5 SD=41.3	105.5±5.7* SD=30.6	127.4±13.6 SD=12.8	68.0±7.3# SD=14.7	97.6±9.1 SD=20.3

Table 6.3 Addition of IGF-I, IGF-II and insulin to embryos cultured in the presence of a monoclonal antibody to the IGF-I receptor.
Rat embryos were explanted on the 9th day of gestation and cultured for 48 hours either in the presence of a monoclonal antibody to the IGF-I receptor alone, or in the presence of this antibody plus either IGF-I, IGF-II or insulin (all at 1µg/ml). Results were analysed for significance using Student's t-test. Significance *=Significant to control at p<0.001%; #=Significant to antibody at p<0.01%; $=Significant to antibody at p<0.05%.

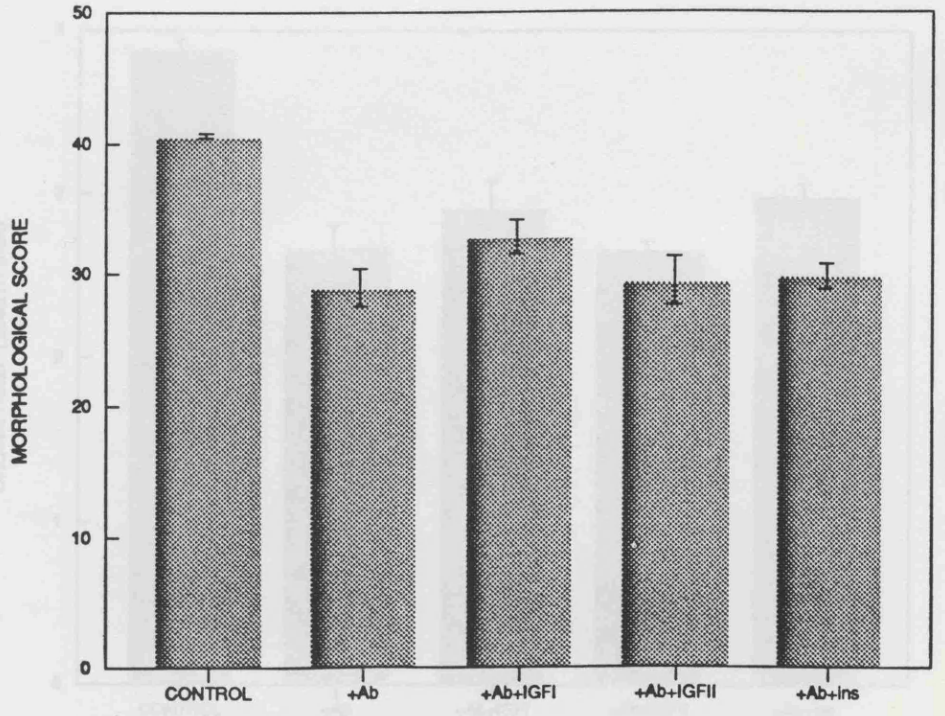

Fig 6.3a Morphological Scores of Embryos.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either IGF-I, IGF-II or insulin added back to the medium. Embryos were also cultured without the addition of antibody or growth factor. Results shown are the mean ±one S.E.M. (n=5 except Ab+IGF-II series, where n=4).

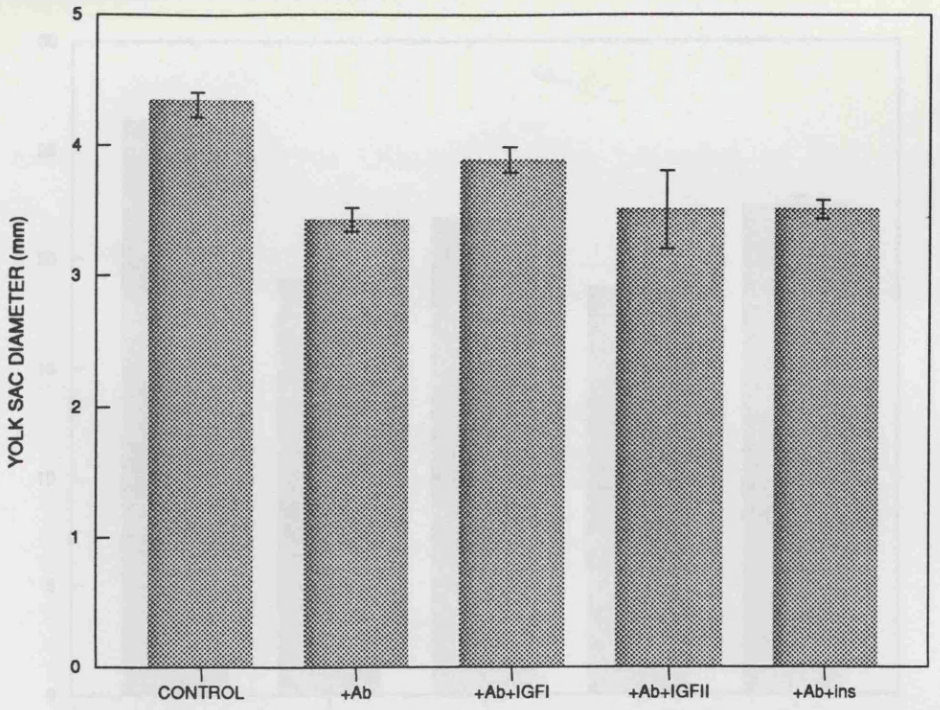

Fig 6.3b Yolk sac diameter.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either IGF-I, IGF-II or insulin added back to the medium. Embryos were also cultured without the addition of antibody or growth factor. Results shown are the mean ±one S.E.M. (n=5 except Ab+IGF-II series, where n=4).

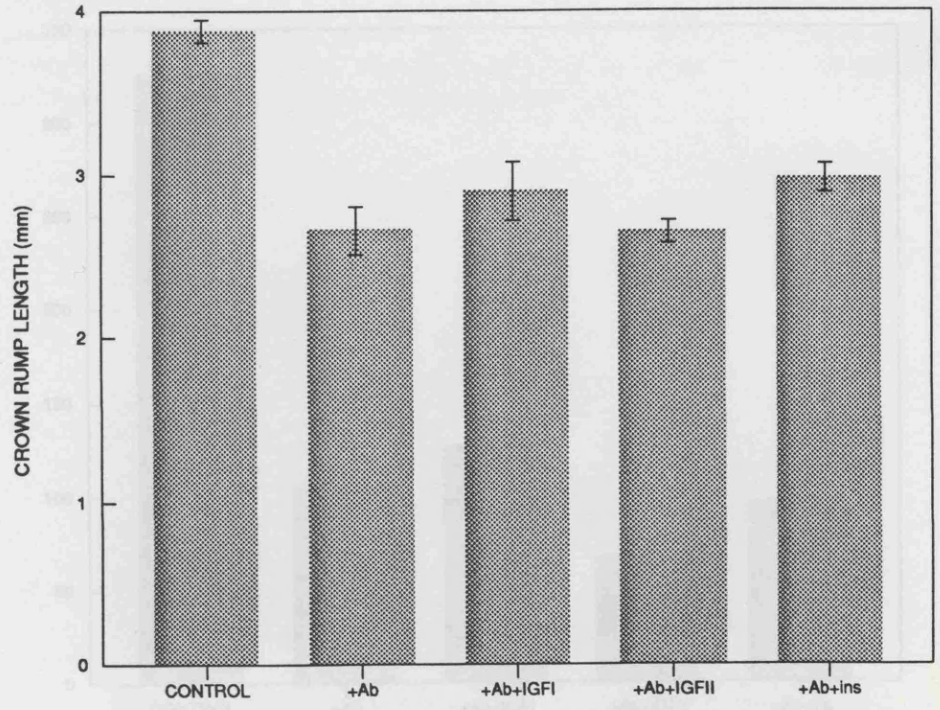

Fig 6.3c Crown Rump Length.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either IGF-I, IGF-II or insulin added back to the medium. Embryos were also cultured without the addition of antibody or growth factor. Results shown are the mean ±one S.E.M. (n=5 except Ab+IGF-II series, where n=4).

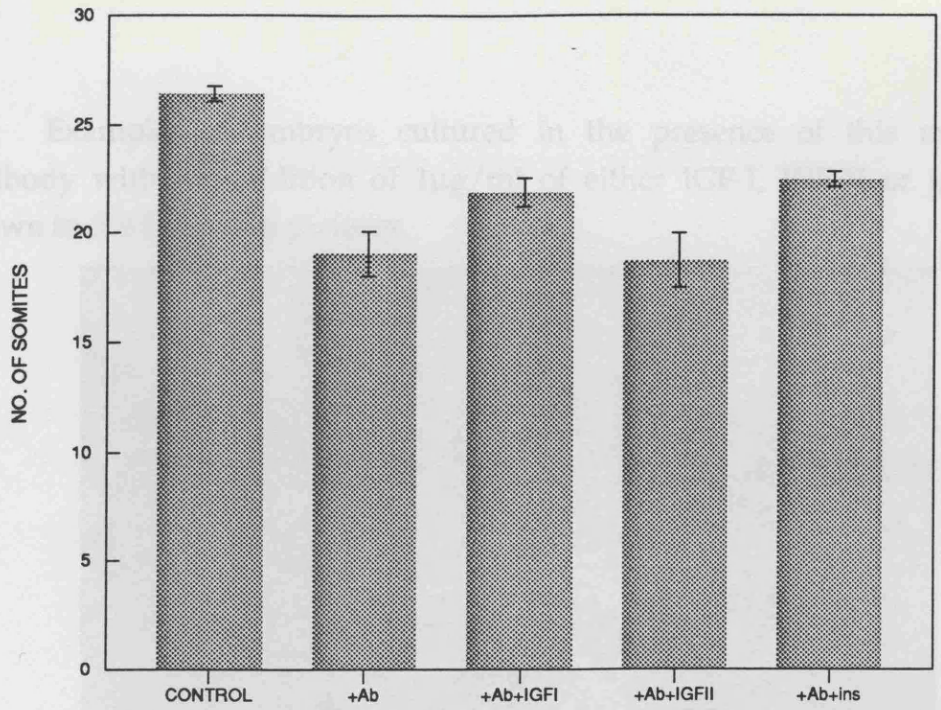

Fig 6.3d Number of Somites.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either IGF-I, IGF-II or insulin added back to the medium. Embryos were also cultured without the addition of antibody or growth factor. Results shown are the mean ±one S.E.M. (n=5 except Ab+IGF-II series, where n=4).

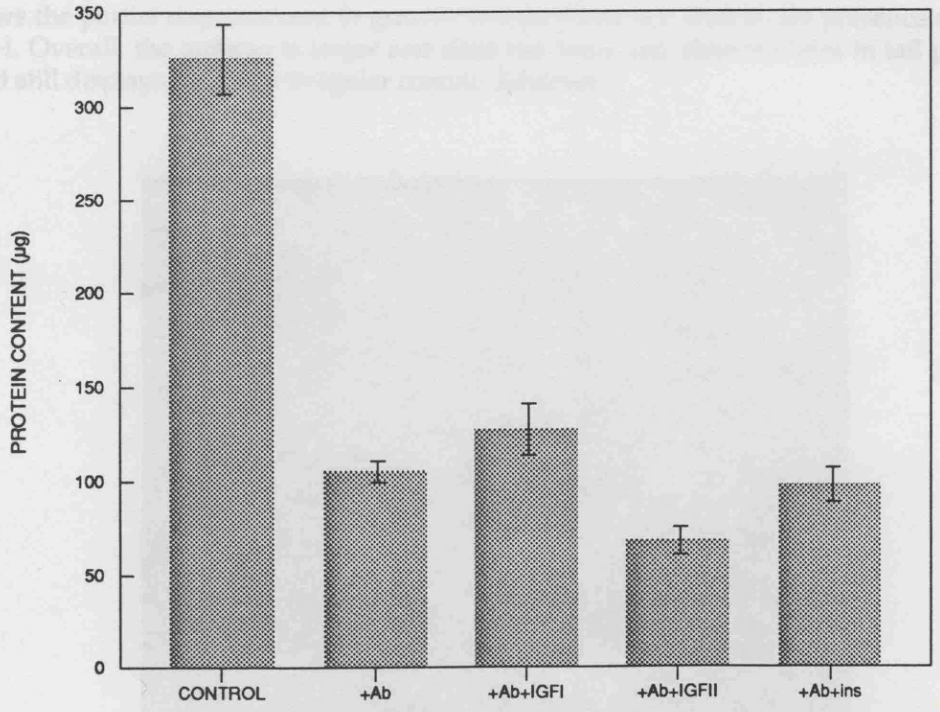

Fig 6.3e Total Protein Content of Embryos.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either IGF-I, IGF-II or insulin added back to the medium. Embryos were also cultured without the addition of antibody or growth factor. Results shown are the mean ±one S.E.M. (n=5 except Ab+IGF-II series, where n=4).

Examples of embryos cultured in the presence of this monoclonal antibody with the addition of 1μg/ml of either IGF-I, IGF-II or insulin are shown in the following pictures.

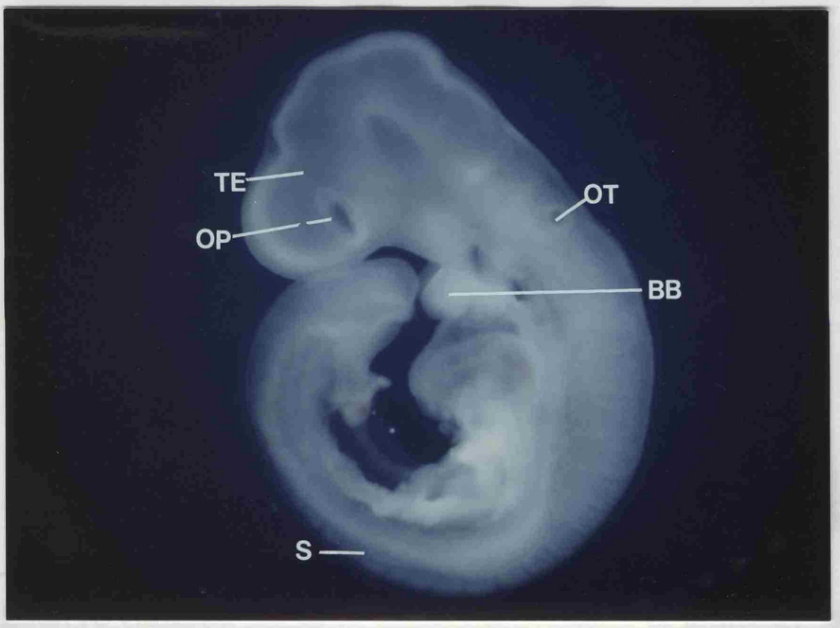

Fig 6.4a Embryo cultured for 48 hours in the presence of antibody to the IGF-I receptor plus additional IGF-I.
(TE-telencephalon; OT-otocyst; OP-optic vesicle; BB-branchial bars; S-somites). This embryo shows the partial improvement in growth and development seen in the presence of additional IGF-I. Overall, the embryo is larger and does not show any abnormalities in tail position. The head still displays a slightly irregular contour however.

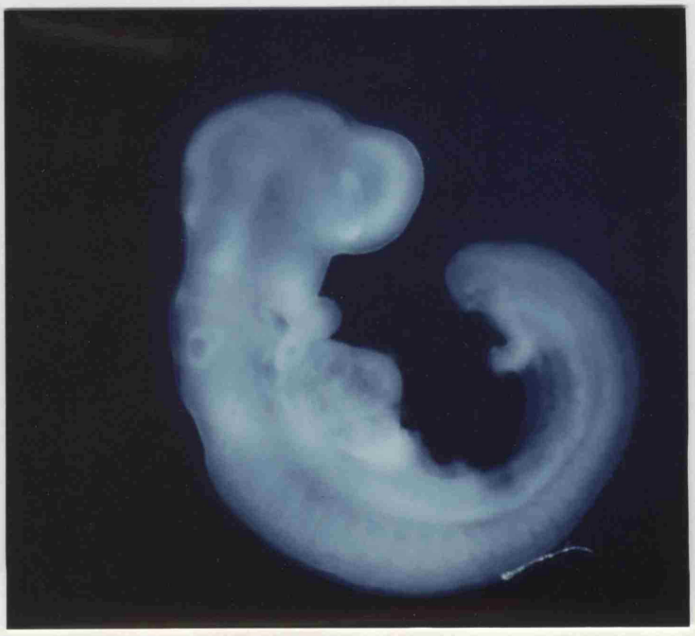

Fig 6.4b Embryo cultured for 48 hours in the presence of antibody to the IGF-I receptor plus additional IGF-I.
Again this embryo shows the improvement in growth seen when the antibody is added with additional IGF-I. The general appearance of the embryo is more normal than was observed when the antibody alone was added, although dysmorphology of the head is noticeable.

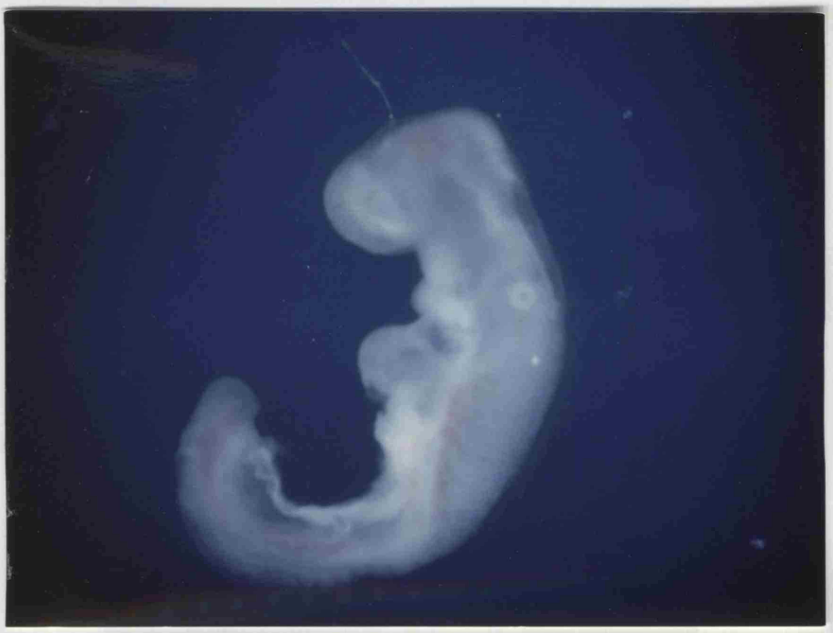

Fig 6.4c Embryo cultured for 48 hours in the presence of antibody to the IGF-I receptor and additional IGF-II.
The head of this embryo is underdeveloped and there are extensive blood patches over the whole embryo. The tail has also failed to turn correctly, a feature commonly seen in the presence of this antibody.

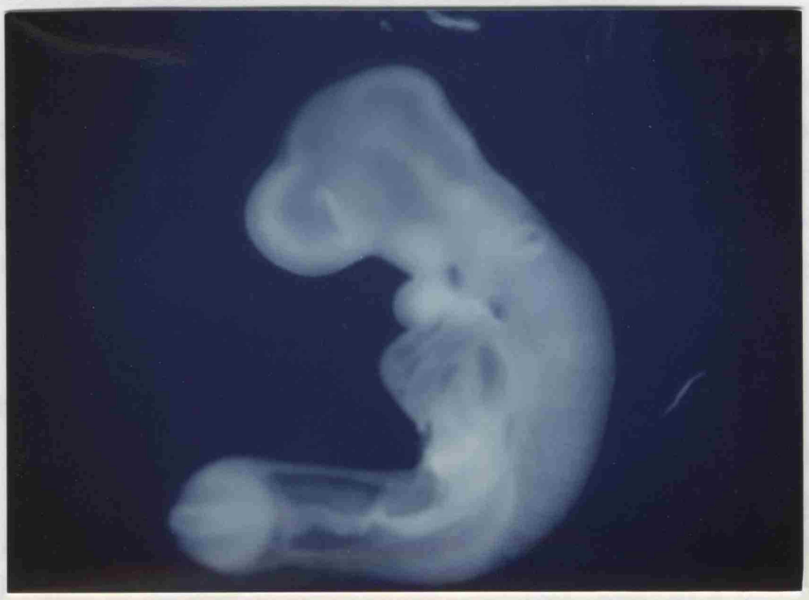

Fig 6.4d Embryo cultured for 48 hours in the presence of antibody to the IGF-I receptor plus additional insulin.
Although this embryo is somewhat larger than those seen when the antibody alone is added, it is still growth retarded. The tail has folded in the wrong direction and the area at the back of the head appears irregular.

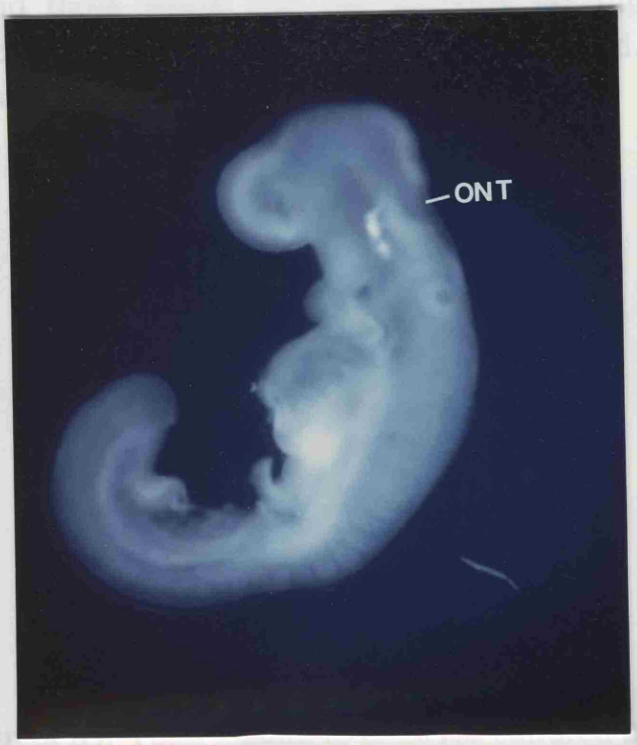

Fig 6.4e Embryo cultured for 48 hours in the presence of antibody to the IGF-I receptor and additional insulin.
This embryo is smaller than the controls embryos seen and has not turned correctly. In addition, its somites appear uneven and the area at the back of the head is irregular. There is also an open neural tube (ONT).

Effects of doubling the concentration of insulin and IGF-I.

As it appears that the addition of 1μg/ml of IGF-I to the incubation medium improves growth and development of embryos cultured for 48 hours in the presence of this antibody, while insulin has little effect, it was decided to investigate whether this apparent improvement could be further increased by the addition of double the concentration of hormone. It was not possible to further investigate IGF-II effects due to the high cost of the purified protein.

Method

Rat embryos were again removed from the mother on the 9th day of gestation, and cultured for the following 48 hours as described earlier in this chapter. Embryos were either cultured in the presence of 1μg/ml αIR-3 antibody alone, or in the presence of the antibody at this concentration with additional IGF-I or insulin present in the medium, both at a concentration of 2μg/ml.

Results

The results are given for this series of experiments in Table 6.4. The results consist of a control series, which had no antibody added (control); those which had the monoclonal antibody to the IGF-I receptor (αIR-3) at 1μg/ml

added (+Ab); and those which had the antibody (at 1μg/ml) plus either 2μg/ml IGF-I (Ab+IGF-I); or 2μg/ml insulin (Ab+INS) added. These results are again plotted in histogram form in Figs 6.5 a,b,c,d,and e.

	CONTROL (n=8)	+Ab (n=10)	Ab+IGF-I (n=9)	Ab+INS (n=10)
Morphological Score	40.8±0.45 S.D.=1.3	31.3±0.49* S.D.=1.6	33.44±1.2 S.D.=3.7	29.8±1.22 S.D.=3.8
Yolk. Sac Diameter.(mm)	3.49±0.08 S.D.=0.2	3.34±0.05* S.D.=0.2	3.39±0.08 S.D.=0.2	3.27±0.09 S.D.=0.3
Crown. rump Length (mm)	3.09±0.06 S.D.=0.16	2.39±0.05* S.D.=0.05	2.73±0.09# S.D.=0.09	2.50±0.09 S.D.=0.29
Somite Number.	25.38±0.25 S.D.=0.7	18.5±0.80* S.D.=2.5	21.11±0.43$ S.D.=1.29	18.20±0.95 S.D.=2.99
Protein Content (μg)	230.5±9.39 S.D.=26.5	136.7±14.1* S.D.=44.6	163.67±6.64# S.D.=19.9	160.5±10.9 S.D.=34.5

Table 6.4 Addition of IGF-I and insulin to embryos cultured in the presence of a monoclonal antibody to the IGF-I receptor.
Rat embryos were explanted on the 9th day of gestation and either cultured in the presence of a monoclonal antibody to the IGF-I receptor alone, or in the presence of this antibody with either 2μg/ml IGF-I or 2μg/ml insulin to see if this improved growth. Results were analysed for significance using Student's t-test.
Significance: * = significant to control at p<0.001%; # = significant to antibody at p<0.001%; $ = significant to antibody at p<0.01%.

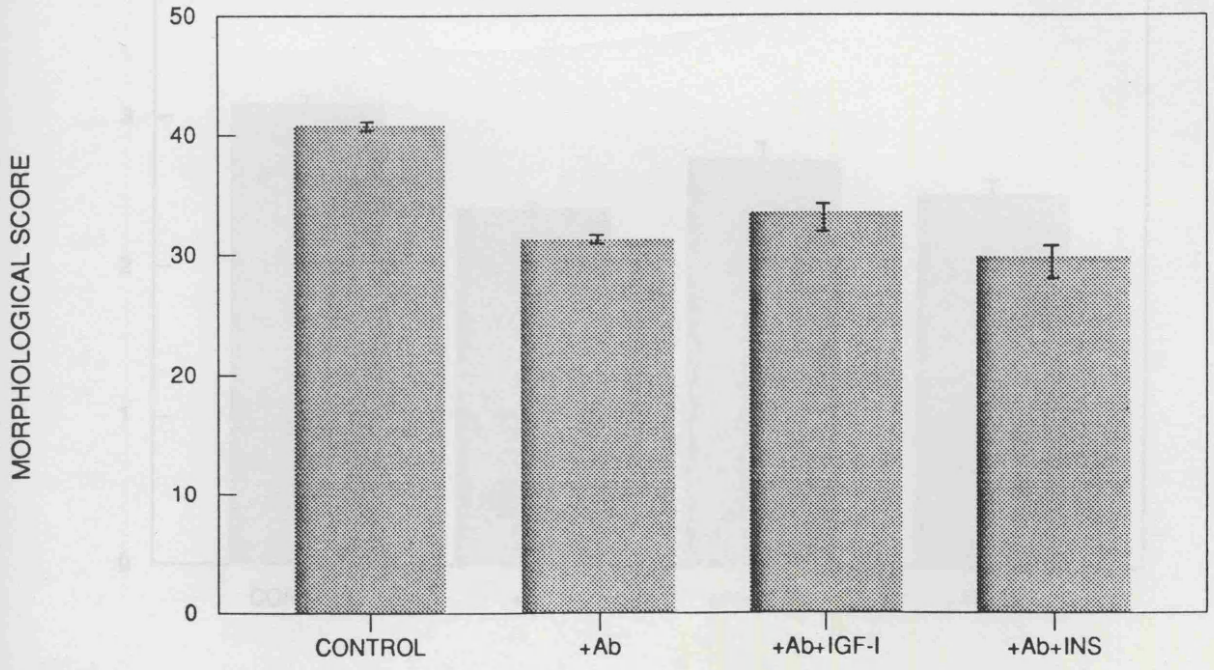

Fig 6.5a Morphological Scores of Embryos.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either 2μg/ml IGF-I, or 2μg/ml insulin added back to the medium. Embryos were also cultured without the addition of antibody (controls). Results shown are the mean ± one S.E.M. (n=8-10).

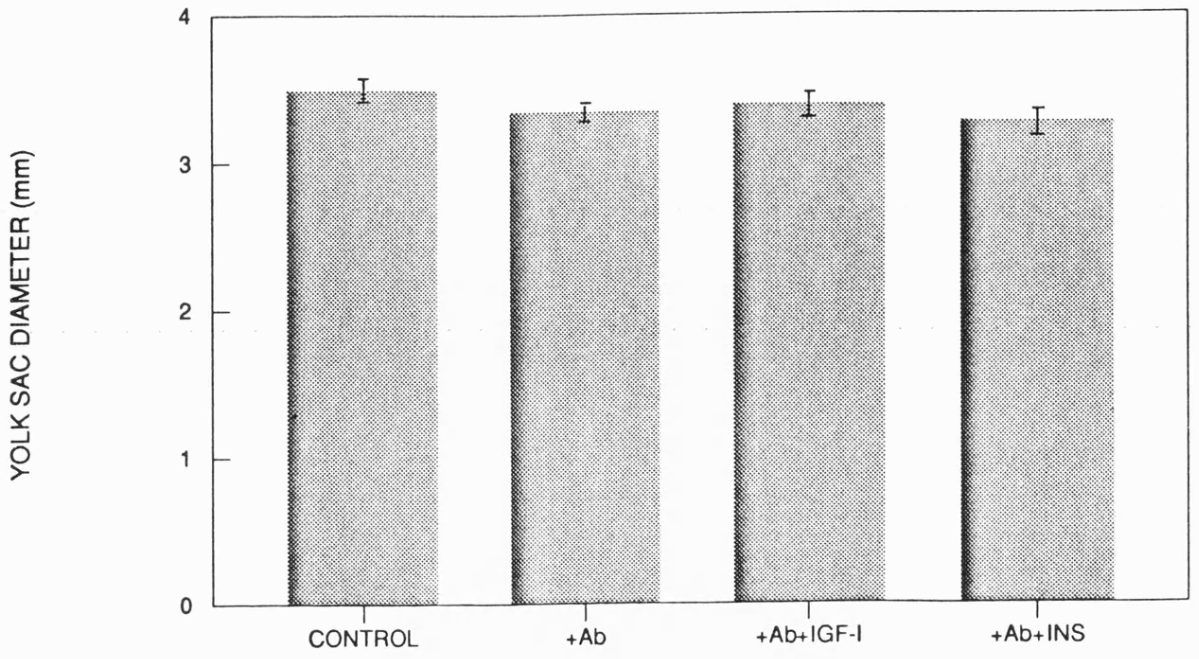

Fig 6.5b Yolk sac diameter.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either 2μg/ml IGF-I, or 2μg/ml insulin added back to the medium. Embryos were also cultured without the addition of antibody (controls). Results shown are the mean ± one S.E.M. (n=8-10).

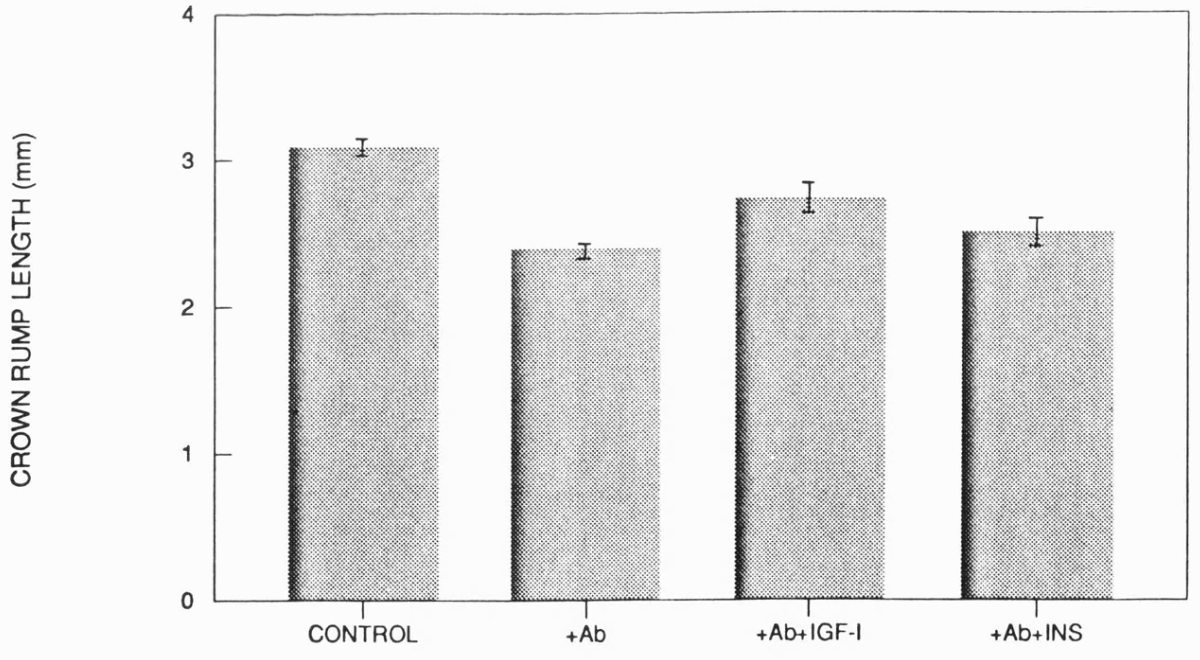

Fig 6.5c Crown Rump Length.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either 2μg/ml IGF-I, or 2μg/ml insulin added back to the medium. Embryos were also cultured without the addition of antibody (controls). Results shown are the mean ± one S.E.M. (n=8-10).

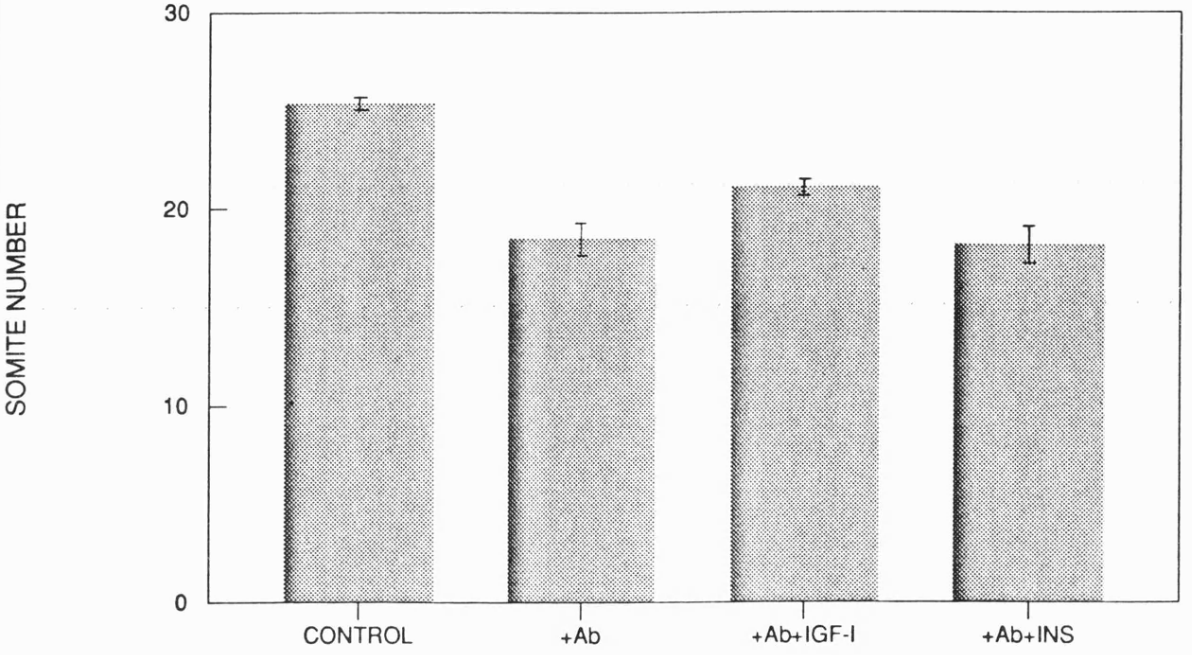

Fig 6.5d Number of Somites.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either 2μg/ml IGF-I or 2μg/ml insulin added back to the medium. Embryos were also cultured without the addition of antibody (controls). Results shown are the mean ± one S.E.M. (n=8-10).

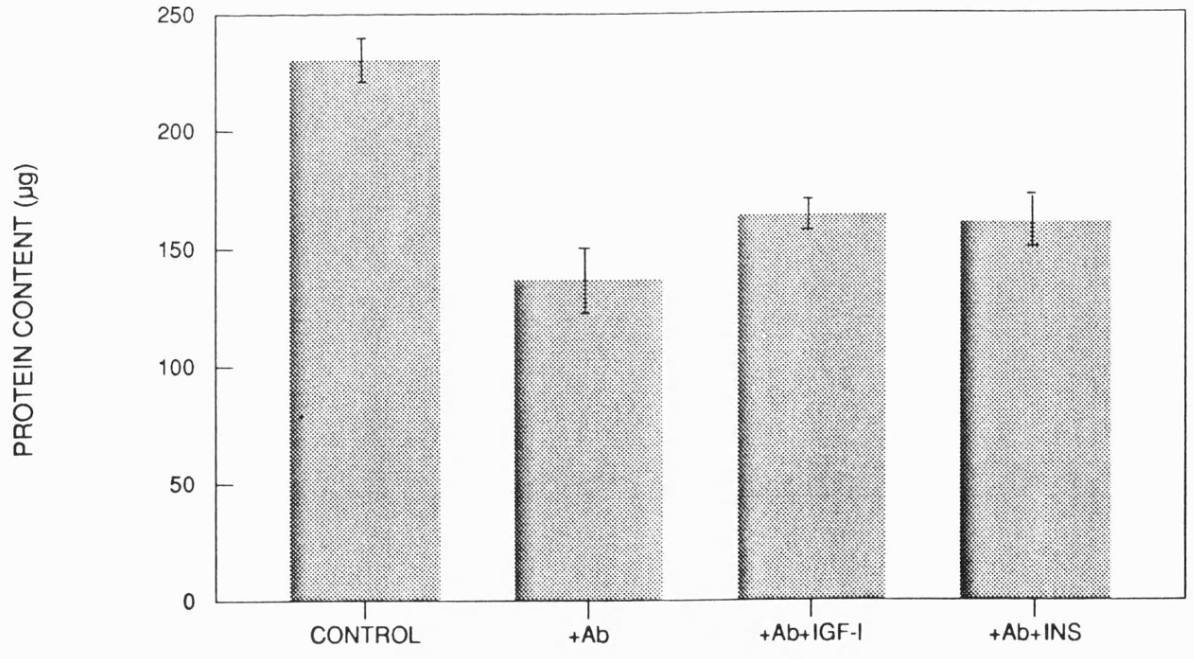

Fig 6.5e Total Protein Content of Embryos.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) alone, or in the presence of this antibody with either 2μg/ml IGF-I or 2μg/ml insulin added back to the medium. Embryos were also cultured without the addition of antibody (control). Results shown are the mean ± one S.E.M. (n=8-10).

Investigation into antibody activity.

The final series of experiments described in this chapter concern whether the effects of the αIR-3 antibody upon the embryonic development of the rat are produced by this antibody in a dose-dependant manner. This was examined by looking at the effects produced by three differing concentrations of antibody upon development during the 9.5 to 11.5 day period. The effects of adding insulin and IGF-I were also examined at these varying concentrations of antibody, while the addition of IGF-I and insulin to embryos which had not been antibody treated were also looked at. This was to ensure that the addition of these growth factors was not inducing supraphysiological growth in developing embryos, which might partially account for the improvement in growth and development seen in the presence of additional IGF-I seen in earlier experiments.

Method

Rat embryos were removed from the mother on the 9th day of gestation as previously described, and were cultured for the subsequent 48 hours in 75% rat serum containing either no αIR-3 antibody, or in the presence of this antibody at either 0.25μg/ml, 0.5μg/ml or 1μg/ml. Embryos were again assessed for morphological development and assayed for protein.

The results are given in Table 6.5, which shows the morphological score, yolk sac diameter, crown rump length, number of somites and total protein content of embryos explanted from 9.5 days and cultured for 48 hours. The results of the varying antibody concentrations are shown in comparison with a series of control embryos. In addition, results are also given when either 1μg/ml IGF-I or insulin were also added to the incubation medium to examine whether the addition of insulin or IGF-I alone results in increased embryonic growth.

Results

The results for these experiments are shown in Table 6.5. and also plotted in histogram form in Figs 6.6 a,b,c,d,and e.

When the antibody is added at one quarter concentration, there appears to be no adverse effect on embryonic development. When the antibody is added at half concentration (0.5μg/ml), an intermediary effect is seen, with all the criteria for development reduced in comparison with the controls, though not as severely as with 1μg/ml of this monoclonal. Again, the addition of 1μg/ml IGF-I to the incubation medium appears to partially overcome this growth inhibition, though 1μg/ml insulin also seems to produce a significant effect in all the parameters measured, except yolk sac diameter.

The addition of insulin and IGF-I without the antibody does not result in an excess of growth in the embryos, suggesting that the improvements in growth and development seen when IGF-I is added to the antibody treated embryos is a result of competition for the antibody.

ANTIBODY CONC.	MORPHOLOGICAL SCORE	YOLK SAC DIAMETER	CROWN RUMP LENGTH	NO. SOMITES	TOTAL PROTEIN
CONTROL	41.63±0.49 S.D.=2.34	3.98±0.06 S.D.=0.29	3.63±0.35 S.D.=0.35	26.55±0.23 S.D.=1.405	296.14±14.03 S.D.=65.8
+ IGF-I	41.67±0.27 S.D.=0.47	3.57±0.09 S.D.=0.17	3.23±0.03 S.D.=0.05	25.33±0.27 S.D.=0.47	235.67±12.9 S.D.=22.37
+ INSULIN	41.25±0.41 S.D.=0.83	3.78±0.40 S.D.=0.23	2.98±0.14 S.D.=0.29	25.5±0.25 S.D.=0.50	289.25±16.38 S.D.=32.77
0.25µg/ml	41.33±0.3 S.D.=0.745	3.75±0.04 S.D.=0.09	3.48±0.03 S.D.=0.07	26.5±0.204 S.D.=0.5	278.0±7.29 S.D.=17.85
+ IGF-I	41.17±0.36 S.D.=0.89	3.47±0.107 S.D.=0.26	3.3±0.09 S.D.=0.23	26.17±0.28 S.D.=0.69	245.16±16.77 S.D.=41.07
+ INSULIN	40.17±0.15 S.D.=0.89	3.5±0.04 S.D.=0.26	3.28±0.05 S.D.=0.23	25.33±0.19$ S.D.=0.69	238.33±9.17$ S.D.=22.47
0.5µg/ml	31.28±0.80* S.D.=2.12	3.37±0.04* S.D.=0.10	2.48±0.04* S.D.=0.09	22.4±0.45* S.D.=1.18	184.43±5.60* S.D.=14.84
+ IGF-I	38.43±0.39£ S.D.=1.049	3.38±0.08 S.D.=0.209	3.05±0.05£ S.D.=0.145	25.27±0.18£ S.D.=0.49	232.0±7.16£ S.D.=18.95
+ INSULIN	36.43±0.63£ S.D.=1.68	3.38±0.08 S.D.=0.216	2.95±0.05£ S.D.=0.139	24.28±0.39 S.D.=1.03	198.14±9.96 S.D.=26.3
1µg/ml	28.8±1.42* S.D.=3.18	3.42±0.08* S.D.=0.19	2.66±0.15* S.D.=0.34	19.0±1.02* S.D.=2.28	105.6±5.72 S.D.=30.59
+ IGF-I	32.6±1.31 S.D.=2.94	3.88±0.11# S.D.=0.25	2.9±0.18 S.D.=0.4	21.8±0.66# S.D.=1.47	127.4±13.6* S.D.=12.78
+ INSULIN	29.6±1.01 S.D.=2.42	3.50±1.35 S.D.=0.25	2.98±0.89~ S.D.=0.20	22.4±0.36 S.D.=0.80	97.6±9.07 S.D.=20.3

Table 6.5 Effects of varying the concentration of a monoclonal antibody to the IGF-I receptor, plus the addition of insulin and IGF-I alone, and in the presence of this antibody.

Rat embryos were explanted on the 9th day of gestation and either cultured in the presence of a monoclonal antibody to the IGF-I receptor at three different concentrations, or in the presence of this antibody with either 1µg/ml IGF-I or 1µg/ml insulin to see if this improved growth. Insulin and IGF-I were also tested without the antibody, to examine whether they affected growth. (n lies between 6 and 10). Results were analysed for significance using Student's t-test. *=Sign. to control at p<0.001%: $=Sign. to 0.25µg/ml Ab at p<0.01%: £= Sign. to 0.5µg/ml Ab at p<0.001%: #=Sign. to Ab at p<0.05%: ~=Sign. to 1µg/ml Ab at 0.001%.

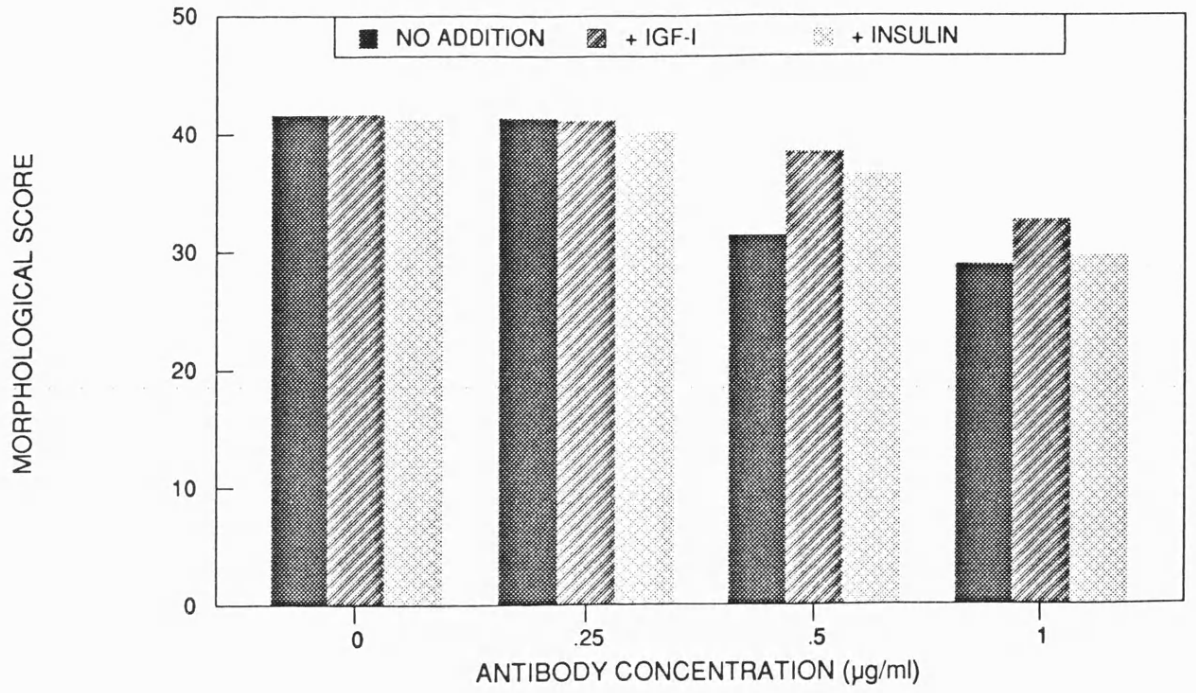

Fig 6.6a Morphological Scores of Embryos.
Embryos explanted on the 9th day of gestation were cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) at three differing concentrations (0.25µg/ml, 0.5µg/ml and 1µg/ml), and also without the antibody. Embryos were also cultured with additional IGF-I or insulin added back to the medium.

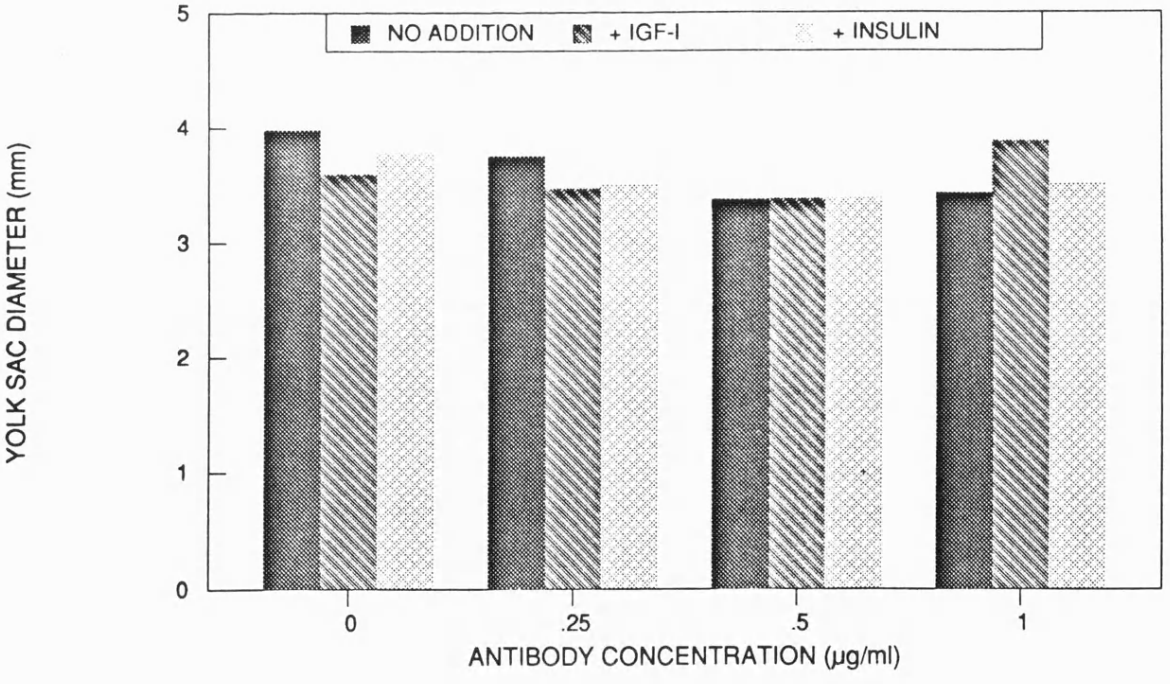

Fig 6.6b Yolk sac diameter.
Embryos explanted on the 9th day of gestation were cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) at three differing concentrations (0.25µg/ml, 0.5µg/ml and 1µg/ml), and also without the antibody. Embryos were also cultured with additional IGF-I or insulin added back to the medium.

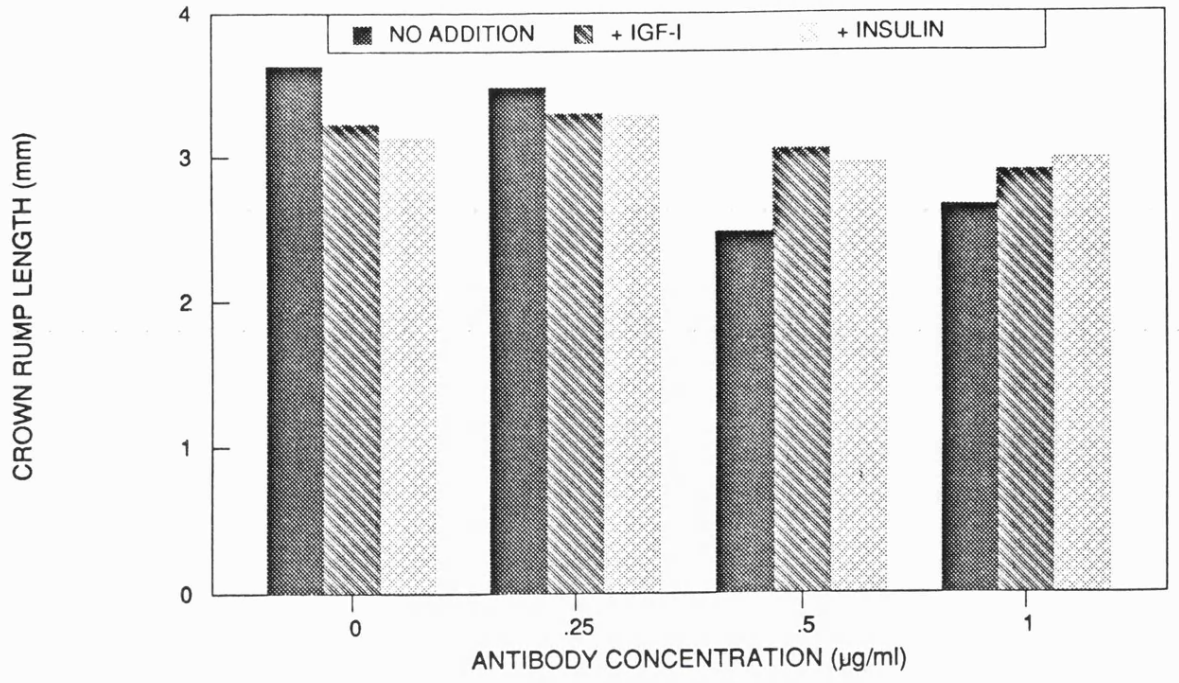

Fig 6.6c Crown Rump Length.
Embryos explanted on the 9th day of gestation were cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) at three differing concentrations (0.25µg/ml, 0.5µg/ml and 1µg/ml), and also without the antibody. Embryos were also cultured with additional IGF-I or insulin added back to the medium.

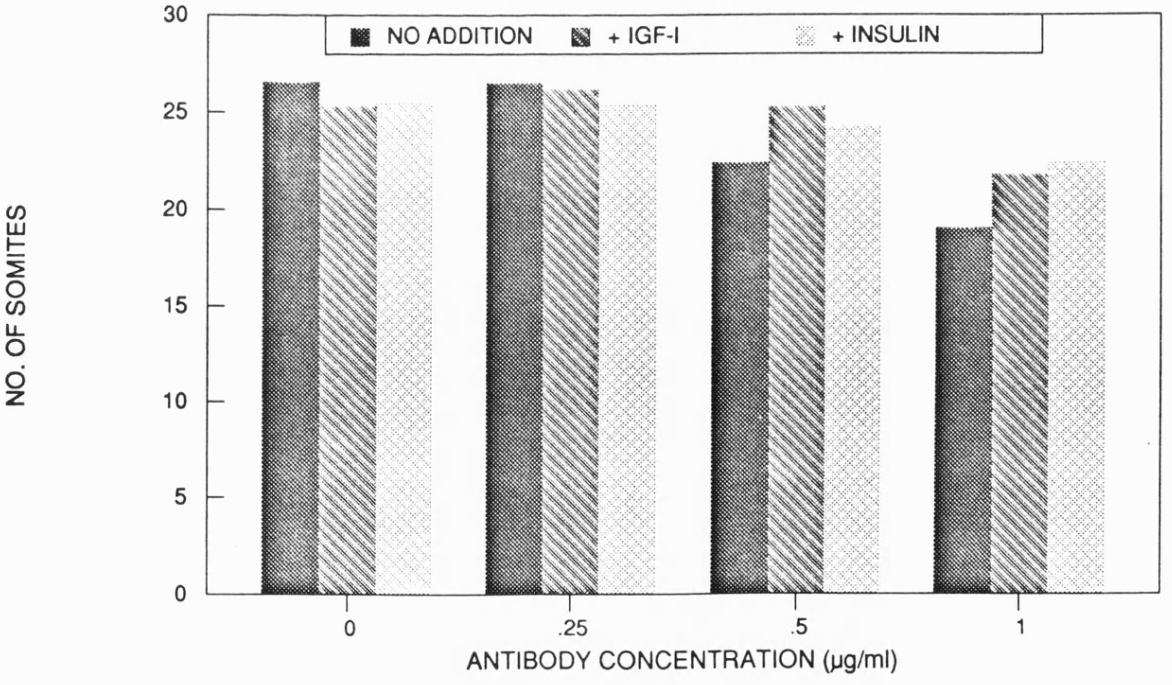

Fig 6.6d Number of Somites.
Embryos explanted on the 9th day of gestation were cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) at three differing concentrations (0.25µg/ml, 0.5µg/ml and 1µg/ml), and also without the antibody. Embryos were also cultured with additional IGF-I or insulin added back to the medium.

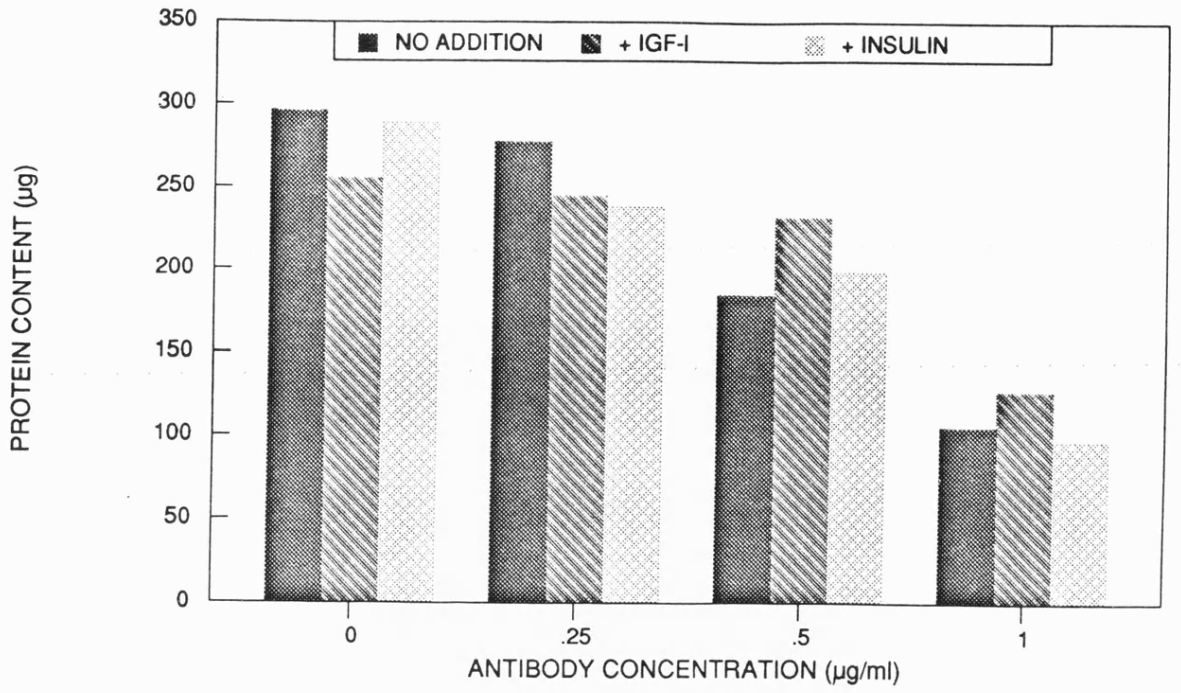

Fig 6.6e Total Protein Content of Embryos.
Embryos explanted on the 9th day of gestation were cultured in the presence of a monoclonal antibody to the IGF-I receptor (αIR-3) at three differing concentrations (0.25µg/ml, 0.5µg/ml and 1µg/ml), and also without the antibody. Embryos were also cultured with additional IGF-I or insulin added back to the medium.

Discussion

The radio- and fluorescent-labelling experiments presented in Chapters 4 and 5 of this thesis may indicate that a specific, high affinity binding site for IGF-I exists on the 17.5 day visceral yolk sac surface to mediated pinocytic uptake of this growth factor. This yolk sac system was originally chosen for investigation because of the large quantity of data available regarding uptake of other molecules, enabling comparisons to be made. Such a high affinity binding site may represents a receptor for IGF-I. In these experiments a monoclonal antibody, previously reported to block this receptor (Flier and Moses 1985), has been used in an attempt to block any IGF-I receptors present on the 11.5 day yolk sac/embryonic surface and so examine whether this has an effect upon the normal development of the rat. Although the [^{125}I]-IGF-I and TRITC labelled IGF-I has not been used to examine the 11.5 day receptor complement, this stage in development approaches the limit at which rat embryos may be grown in culture so that their *in vitro* development precisely mimics that seen *in vivo*. The period from 9.5 to 11.5 days gestation is of particular interest in the rat because it covers the majority of organogenesis,

while beyond 11.5 days the increasing importance of the chorio-allantoic placenta means that it is not possible to maintain an adequate supply of blood and nutrients to the embryo in culture, leading to growth retardation (New, 1978).

The initial experiment examines the growth and development of embryos cultured in the presence of this monoclonal antibody in comparison with controls. The addition of the αIR-3 produces a significant reduction in the size of both yolk sac and the embryo it contains (15% and 22.5% reduction respectively when compared to control yolk sac diameter and crown rump length), as well as a reduction in differentiation (28% reduction in morphological score and 24.5% in the number of somites measured). A pattern of developmental abnormalities is also apparent when the antibody is applied at this stage. This would indicate that this anti-human antibody can cross-react with rat tissue, but more importantly that blockage of this receptor results in growth retardation with an associated increase in morphological abnormalities. The most striking feature of the embryos cultured in the presence of 1μg/ml of the antibody is that they were noticeably smaller when compared to the controls: possibly this is what would be expected of an antibody reported to block a growth factor receptor.

The addition of a non-specific antibody, mouse IgG, does not produce any significant difference in comparison with the control embryos, except in the case of total protein content. The addition of 1μg/ml mouse IgG appears to significantly reduce the total protein content as calculated by a Student's t-test. Protein content is a general, overall measure reflecting the size of the embryo, and it is odd that a series of embryos (where n=12) which show no significant difference in any other of the criteria measured, be they specific measures of growth (ie yolk sac diameter and crown rump length) or of differentiation (somite number and morphological score) should show any effect by this criterion. Indeed, it is impossible to imagine that an embryo with similar measures by all these other criteria could possibly contain significantly less protein, and the reason for this result remains unclear. However, the effect seen with mouse IgG is not as great as that seen with the addition of αIR-3 so this result does not affect the overall conclusions we can draw from this series of experiments.

Such monoclonal antibodies as αIR-3 are produced to act specifically against the antigen against which they were raised, and there is no obvious reason to believe that the results of these experiments do not demonstrate the effects of direct blockage of the IGF-I receptor. However, one possibility is that the reduction in growth seen is as a result of some other component of the antibody solution, such as a toxic effect produced by some component of the

buffer containing αIR-3 which is not present in the mouse IgG samples. This possibility is partially eliminated by the second series of experiments described, in which the effects of αIR-3 appear to be partially reversed by the addition of IGF-I to antibody treated embryos, while insulin and IGF-II produce no effect. Improvements could be seen with the additional IGF-I in 4 out of the 5 criteria measured, the exception being total embryonic protein content which shows a slight decrease. Once again, there is no apparent reason for this anomaly, as embryos which show improvements in all the other areas measured should logically contain more protein. This improvement in development is most strongly seen in the measurements of yolk sac diameter and morphological score, while the addition of insulin and IGF-II does not appear to alter growth significantly from the antibody treated group. In two of the other parameters measured (number of somites and crown rump length) the addition of 1μg/ml IGF-I improves growth, though in these cases the addition of insulin also has a positive effect. The overall appearance of the embryos given the additional IGF-I was that they were larger than those which were antibody treated. Interestingly, the morphological abnormalities seen in the presence of the antibody are virtually eliminated by the addition of an excess of IGF-I, while they were still apparent when the same concentration of insulin or IGF-II was applied.

Although these results would clearly be improved by larger sample populations (n is only 4 in the case of the IGF-II samples), this was made impossible by the high cost of purified IGFs.

The addition of 1μg/ml IGF-I to the culture may partially overcome the effect of the antibody by competing for binding sites on the receptor. Presumably, the binding sites for both the natural ligand and the antibody must be fairly close on the IGF-I receptor molecule, as otherwise it would not be possible to overcome the effect of the antibody by competition.

It is possible to speculate that at these relatively high concentrations, insulin may be cross-reacting with the IGF-I receptor and also competing with αIR-3 for binding to the receptor. Alternatively, at 1μg/ml insulin the high degree of insulin to insulin receptor interactions may partially compensate for inhibition of the IGF-I receptor and so lead to a partial improvement of growth and development. There is no direct evidence for either of these suggestions. In contrast to IGF-I, IGF-II has no effect on improving development and the results seen are not significantly different from the antibody treated alone.

However, when the effects of doubling the concentration of IGF-I and insulin added to the medium of the antibody-treated embryos are examined, the results are somewhat surprising. There is no significant difference between the results seen at these concentrations and those at the lower concentration of

1μg/ml. This is surprising since if the effects of the antibody were being competitively inhibited by IGF-I, it would be expected that the addition of more IGF-I would further inhibit the antibody and so improve the level of growth and differentiation seen to a greater extent.

The final set of embryo culture experiments using αIR-3 describe the effects of various concentrations of antibody on embryonic development. Generally, 0.25μg/ml of the antibody produced no effect when compared to controls, while 1μg/ml produces the significant effects already described. An intermediate concentration, 0.5μg/ml, produces an intermediary effect. Thus, αIR-3 appears to act in a dose-dependent manner. Again, the addition of IGF-I appears to partially overcome the inhibitory effect of the antibody, while insulin has a lesser effect.

The results presented with this antibody indicate that blocking the IGF-I receptor with a monoclonal antibody leads to a reduction in growth and development, from which it may be inferred that IGF-I is a requirement for normal embryonic development.

Although these data represent the effects of blocking this receptor type, they can give no indication as to where these receptors are situated. Initially, the antibody is exposed to any IGF-I receptors present on the external surface of the visceral yolk sac , and so one possibility is that this is the principal site of antibody action. This monoclonal could therefore be preventing binding of IGF-I at this surface. IGF-I could be taken up and transported intact across the membrane to be released at the internal surface from which it could then act on the embryo. However, another important possibility is that this antibody itself may be transported intact across the yolk sac epithelium, to then act directly on embryonic IGF-I receptors.

Antibodies are immunoglobulins (in this case a mouse IgG molecule) and must be considered as such in the case of the visceral yolk sac. The yolk sac is known to be capable of transporting maternal immunoglobulins intact to the fetus, which is the principal mechanism of conferring passive immunity in the rat before birth (reviewed Rodewald, 1980). Transfer of rat IgG has been reported across the 11.5 day yolk sac (Huxham and Beck, 1981) and was believed to be a receptor-mediated process in which the IgG is taken up at the apical yolk sac surface and transported in coated vesicles which protect the protein from attack. The contents of these vesicles are then discharged when they fuse with the basal membrane, releasing them into the fetal circulation. So, the yolk sac appears to possess IgG receptors during organogenesis. The IgG receptor appears to specifically recognise the Fc portion of the IgG molecule (Brambell *et al*, 1960), and recently an IgG-Fc receptor has been reported on the rat yolk sac (Roberts *et al*, 1990). This has an apparent molecular weight of

between 54-58kD, and it seems that this receptor binds IgG not at the yolk sac surface, but within apical compartments.

Whether IgG receptors are present directly on the yolk sac surface or within apical compartments such as the endosomal complex, receptors would seem to be present to mediate the transport of the αIR-3 antibody across the yolk sac to act directly on the embryo. It would be ideal if this antibody could now be applied directly to the fluid immediately surrounding the embryos in culture, to try to separate the effects of any IGF-I receptors on the yolk sac surface from those on the embryonic surface, and so determine where this antibody is producing its principal effects. This is would prove impossible as the survival of the embryo is linked to the functioning of the yolk sac and the two cannot be separated for this purpose.

CHAPTER SEVEN

USE OF A MONOCLONAL ANTIBODY TO INVESTIGATE THE PRESENCE AND LOCALISATION OF THE IGF-I RECEPTOR PROTEIN

Introduction

The results presented in the previous chapter strongly suggest that a receptor for IGF-I is present between 9.5 and 11.5 days of gestation in the rat, and that blockage of this receptor results in a reduced development in comparison to control embryos. Such experiments, however, do not provide information concerning the localisation of the receptor. In an attempt to establish the location of this receptor, and also to demonstrate its presence morphologically, the monoclonal antibody to the IGF-I receptor previously described (the αIR-3 antibody) was used an a primary antibody in an immunofluorescence experiment, and also in an immunoprecipitation experiment.

Visualisation of the IGF-I receptor using immunofluorescent staining

Method

Rat conceptuses were removed from the mother on the 9th day of gestation, and the egg cylinders carefully dissected out. Two egg cylinders were then placed in 2mls of M199 which contained 10μl/ml (ie 1μg/ml) of the monoclonal antibody to the IGF-I receptor, αIR-3. These cylinders were incubated at 37°C in roller incubators for 1 hour. After this, they were washed in 1% saline before being exposed to the secondary antibody. As the monoclonal antibody being used had been raised in the mouse, the secondary antibody chosen must be anti-mouse in order to bind to it. The antibody chosen was a fluorescein isothiocyanate (FITC) labelled $F(ab')_2$ rabbit anti-mouse (Serotec). The egg cylinder was then placed in M199 containing the secondary antibody at a dilution of 1:500 for 1 hour at 37°C.

A series of negative control experiments were also carried out. In these, egg cylinders from the same mother were incubated either in the presence of the primary antibody alone, with the secondary antibody alone, or in the presence of mouse IgG (at 1μg/ml) for 1 hour, followed by the addition of the secondary antibody.

After this period of incubation, the egg cylinders were removed and washed thoroughly in 1% saline. They were then positioned within the wells of bored, glass microscope slides, and secured by placing preprepared nutrient agar (Oxoid) around the tissue. This agar had been dissolved in M199, boiled, and then maintained at 60°C before use. Once secure, the egg cylinders were examined using an inverted Zeiss epifluorescence microscope in conjunction with a Biorad MRC-600 laser scanning confocal imaging microscope.

Results

The results of these experiments are shown in Fig 7.1 a,b,c and d.

Fig. 7.1a shows a 9.5 day egg cylinder incubated for 1 hour in M199 containing αIR-3 antibody at 1μg/ml, after which the FITC labelled secondary was applied. The resulting image is a montage produced by pasting together three separate images recorded on the confocal microscope.

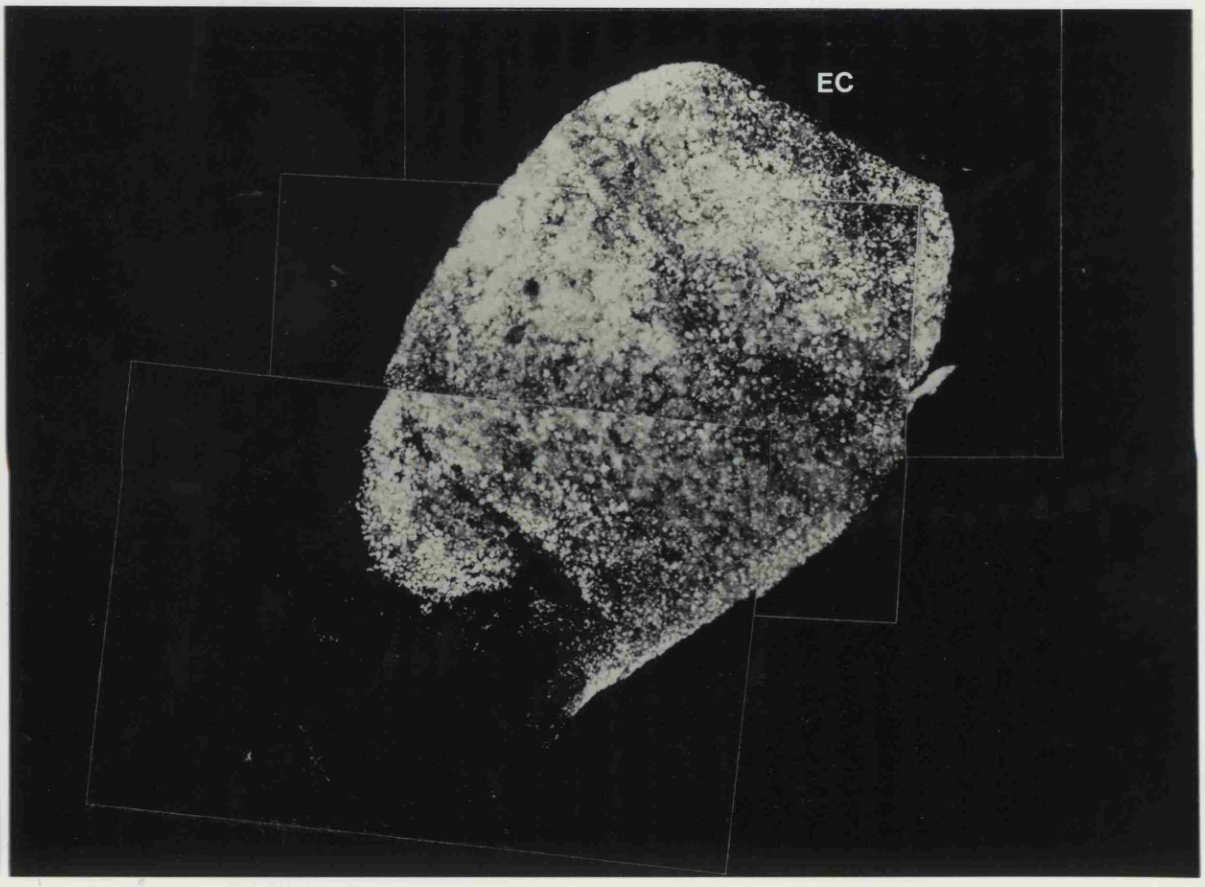

Fig 7.1a. A 9.5 day egg cylinder stained with an FITC labelled secondary against the αIR-3 antibody.

The 9.5 day egg cylinder was incubated with the αIR-3 antibody (1μg/ml) for 1 hour in a roller incubator. After this, an FITC-labelled rabbit anti-mouse secondary antibody was applied at 1:500 dilution for a further 60 minutes incubation. The egg cylinder was then washed with saline, and examined using an epifluorescence microscope attached to a confocal laser-scanning system.

The majority of the body of the egg cylinder is heavily stained with immunofluorescence. In contrast, the ectoplacental cone area (marked EC) is not stained at all. Similarly, only a very small amount of staining can be detected in the embryonic pole of the cylinder. This is the area which contains

the embryonic endoderm, which then inverts into the yolk sac tissue (the area heavily stained) to produce the embryo.

Fig 7.1 b,c,and d show the various negative controls carried out. Fig 7.1b shows an egg cylinder incubated for 1 hour at 37°C in the presence of the primary antibody (αIR-3 at 1μg/ml) alone, while Fig 7.1c shows another egg cylinder from the same mother, incubated for 1 hour with the secondary (FITC-labelled rabbit anti-mouse) alone. Similarly, Fig 7.1d shows an egg cylinder incubated for an hour with a non-specific antibody (mouse IgG at 1μg/ml), followed by the fluorescently labelled secondary for a further hour. After their various incubations, all controls were washed and mounted in bored glass slides in the same manner as the experimentals. In all three controls, only a very small amount of immunofluorescent staining can be detected, which is in no way comparable to that seen when the anti-IGF-I receptor antibody was applied.

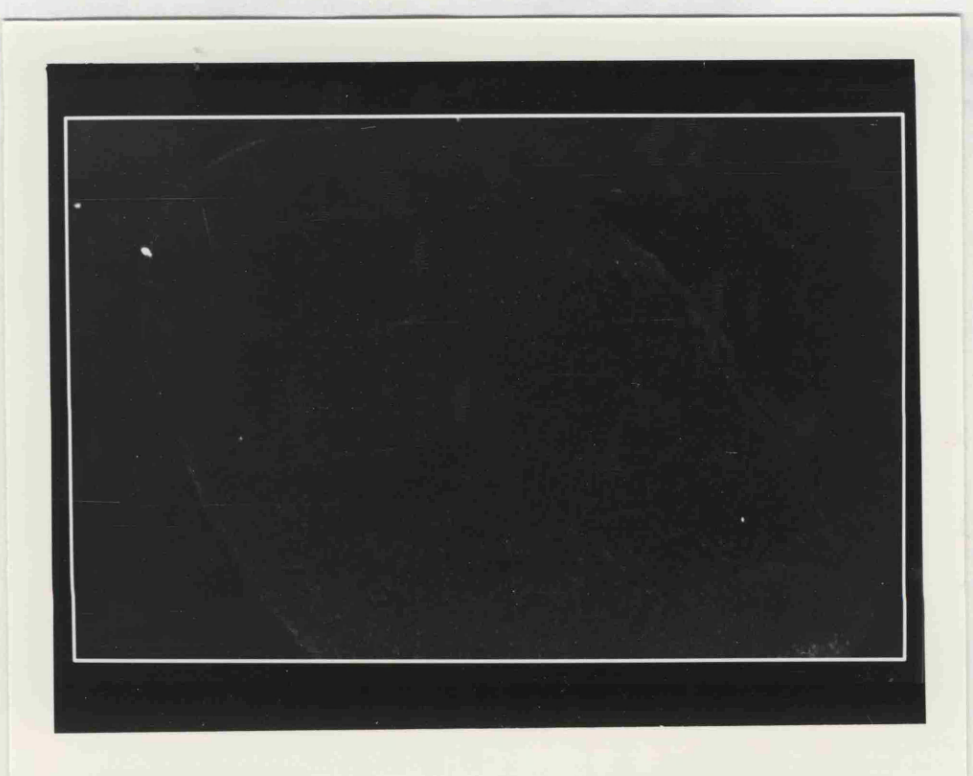

Fig 7.1b Negative control (omission of secondary antibody)
A 9.5 day egg cylinder was incubated for 1 hour in the presence of the αIR-3 (anti-IGF-I receptor) antibody at 37°C. After this, the tissue was washed in 1% saline, and positioned in a bored, glass slide and secured with nutrient agar, before examination using an epifluorescence microscope attached to a confocal laser-scanning system.

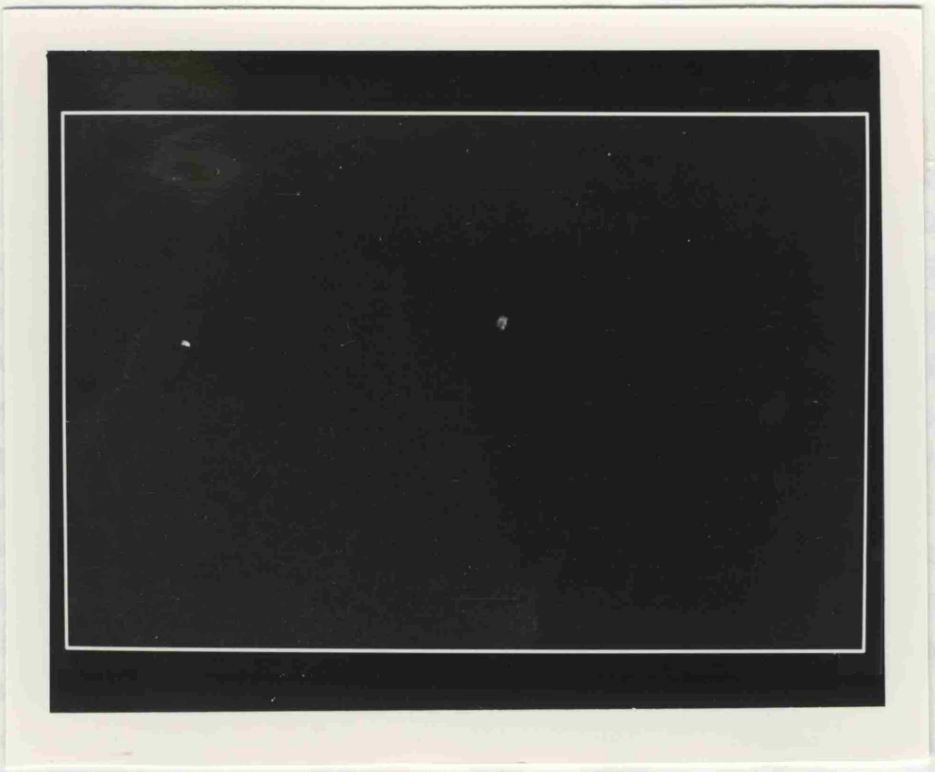

Fig 7.1c Negative control (omission of primary antibody)
A 9.5 day egg cylinder was incubated for 1 hour in the presence of FITC-labelled rabbit anti-mouse antibody at 37°C. After this, the tissue was washed in 1% saline, and positioned in a bored, glass slide and secured with nutrient agar, before examination using an epifluorescence microscope attached to a confocal laser-scanning system.

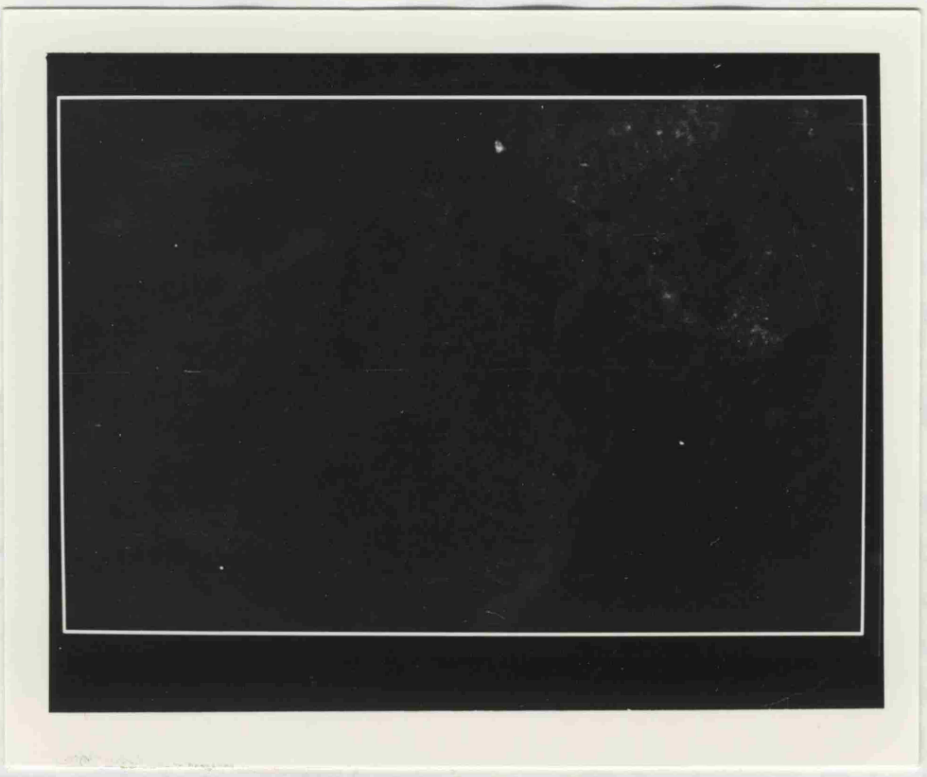

Fig 7.1d Negative control (addition of secondary antibody in the presence of a non-specific primary: mouse IgG).
A 9.5 day egg cylinder was incubated for 1 hour at 37°C in the presence of a non-specific antibody, mouse IgG, at the same concentration as αIR-3 in Fig 7.1a. The fluorescently labelled secondary was then applied for a further 60 minutes incubation. After this, the tissue was washed in 1% saline, and positioned in a bored, glass slide and secured with nutrient agar, before examination using an epifluorescence microscope attached to a confocal laser-scanning system.

Immunoprecipitation Experiment

The technique of immunoprecipitation is based upon the antibody binding to the antigen to produce a macromolecular lattice so large it falls out of solution forming an immunoprecipitate, which may be purified by centrifugation. If the antigen can be radiolabelled, the antibody-antigen precipitate can be easily detected. One approach is to metabolically label the antigen. Here, the antibody is incubated with a cell lysate preparation, which has previously been exposed to a radiolabelled factor essential for continued growth (most commonly ^{35}S methionine), in a culture medium deficient of that particular factor. The lack of unlabelled methionine forces the cells to incorporate the radiolabelled amino acid in any *de novo* protein synthesis occurring, including assembly of the protein the antibody is raised against. Thus, the antigen-antibody complex precipitated is radiolabelled and can be subsequently analysed by SDS-polyacrylamide gel electrophoresis and autoradiography.

This approach has been adapted to examine the presence of IGF-I receptors on the 11.5 day rat visceral yolk sac and embryonic tissue. At this stage of development, the embryo is rapidly growing and very metabolically active.

Method

The method used was an adaptation of the immunoprecipitation technique suggested by Oncogene Science, who supplied the αIR-3 antibody.

Embryos were explanted at 9.5 days and cultured in 75% rat serum/25% M199 for 48 hours. In addition, embryos were also explanted directly at 11.5 days to determine whether any differences existed with respect to IGF-I receptor expression between *in vivo* embryos and those which had undergone a period of culture.

Both sets of embryos, 10 in each bottle, were then placed in 6mls of incubation medium. This consisted of 3mls methionine-free Dulbecco's modified Eagle's medium (see Appendix 4) plus 3mls of rat serum, which had undergone ultrafiltration for 4 hours using a 10,000 Dalton molecular weight filter. This was to removed any substances with a molecular weight less than 10,000, which should include amino acids such as methionine (Mr=149), from the serum, while leaving other factors essential for development (Gulamhusein *et al*, 1990).

To this medium 500μCi (18.5 MBq) of ^{35}S L-methionine (ICN Flow) was added, and the embryos incubated at 37° C for approximately 6 hours.

The radioactive medium was then removed using a bent needle, and the embryos washed with PBS (phosphate buffered saline, pH7.4) (see Appendix 3).

6mls of ice cold PBSTDS (see Appendix 3) was then added to the embryos prior to incubation at 4°C for 10 minutes. After this, the embryos were separated from their yolk sacs under a dissecting microscope. The yolk sacs and embryo tissue was then disrupted by repeated aspiration through a 21 gauge needle to ensure thorough homogenisation. The resulting cell preparation was then transferred to centrifuge tubes, and spun at 1500 rpm for 15 minutes at 4°C to pellet cellular debris. The supernatant was removed and transferred to microcentrifuge tubes. These then underwent a 60 minute spin at 11000rpm in a microcentrifuge at 4°C to pellet remaining cellular debris, and the resulting supernatant collected.

The immunoprecipitation procedure can now be performed utilizing this supernatant. 15µl of Protein-A agarose (Sigma) was added to a 1.5ml Eppendorf tube. To this was added 10µl (ie 1µg) of the αIR-3 monoclonal antibody (Oncogene Science Inc.) and 1ml of the cellular extract prepared above. These tubes were then placed in a sealed plastic bag and attached to a rocking platform at 4°C overnight, to allow the antibody-antigen reaction to take place.

After this, the immunoprecipate formed was collected by centrifugation at 13000 rpm for 15 minutes in a microcentrifuge. The supernatant was then removed and small samples retained for running on the SDS gel. The resulting pellet was washed four times by the addition of 1ml PBSTDS, and centrifugation as above. After the final wash, the pellet is resuspended in 40µl of electrophoresis sample buffer.

The samples are then stored at -20°C, and subsequently analysed by SDS-PAGE.

Results

Fig 7.2 shows the results of this immunoprecipitation experiment.

Tracks 3, 5, 8 and 10 show the samples of *in vivo* and cultured embryonic and yolk sac tissue which underwent immunoprecipitation with the αIR-3 antibody, and were collected by centrifugation. Tracks 2, 4, 7 and 9 show the corresponding supernatant samples, which should not contain any immunoprecipitation products. A marker track has also been included. It can be seen that in the tracks of interest, there are no radiolabelled proteins which correspond to the molecular weights of the α and β subunits of the IGF-I receptor (130 and 95 kD respectively), which this antibody should recognise. Instead, a variety of differing molecular weight bands are apparent on some of the tracks, especially track 9. Two bands are particularly strongly radiolabelled.

These bands are approximately 200kD and 45kD respectively, the first band running at approximately the same rate as myosin heavy chain (200kD), while the second is running slightly above actin (42kD), as can be seen by comparison with the marker track (a myofibril preparation). The supernatant tracks were included to give an indication of the levels of newly synthesised proteins being produced by the tissues being examined. However, only newly synthesised protein which has been recognised by the antibody should be detected by autoradiography. Exactly what these non-specific bands represent is unclear. The smaller bands may be degradation products of the IGF-I receptor, though this is unlikely, and does not account for the larger molecular weight protein. More probable is that they are Protein A binding proteins, as Protein A agarose beads are added to bind to the antibody.

The reasons why no radiolabelled bands corresponding to the α and β subunits of the IGF-I receptor are detectable are unclear. Most likely, the number of IGF-I receptors present on the surface of these tissues is so small that it is impossible to detect them using such a metabolic labelling technique. However, enough IGF-I receptors may be present on these tissues to produce the growth retardation seen when this antibody is added to the culture medium of 9.5 day embryos.

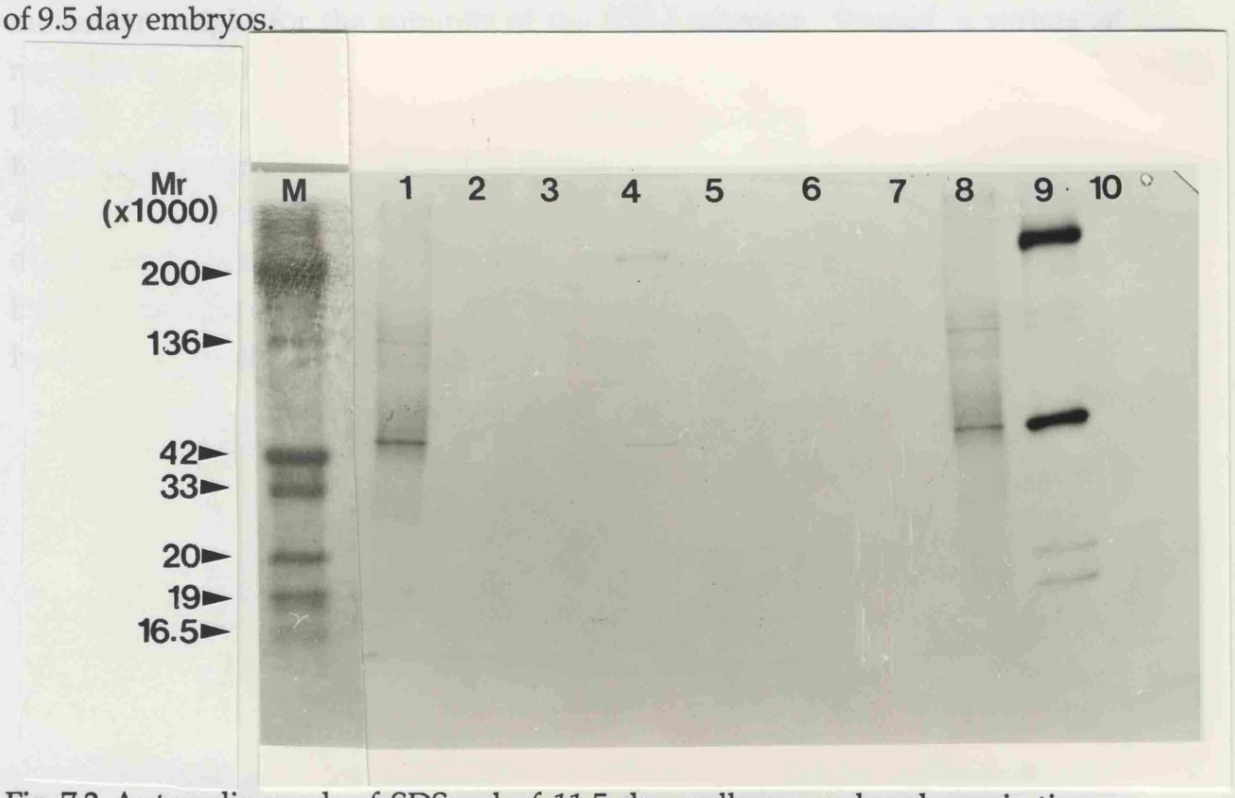

Fig 7.2 Autoradiograph of SDS gel of 11.5 day yolk sac and embryonic tissue which has undergone immunoprecipitation with a monoclonal antibody against the IGF-I receptor, indicating newly synthesised proteins which have taken up ^{35}S-methionine.
Tracks: 1=marker (myofibrils); 2=11.5 day *in vitro* embryo (supernatant); 3= 11.5 day *in vitro* embryo; 4= 11.5 day *in vitro* yolk sac (supernatant); 5= 11.5 day *in vitro* yolk sac; 6= marker (myofibrils); 7= 11.5 day *in vivo* embryo (supernatant); 8= 11.5 day *in vivo* embryo ; 9=11.5 day *in vivo* yolk sac (supernatant); 10= 11.5 day *in vivo* yolk sac. M=marker track from gel.

Discussion

The αIR-3 monoclonal was also used to try to establish where IGF-I receptors were present on 9.5 day embryonic tissue. Although only a very simple immunostaining procedure was attempted, the results were very dramatic. A large amount of immunofluorescence is detectable on the body of the egg cylinder, while none is seen on either the embryonic or the ectoplacental pole areas. The negative controls performed did not show any non-specific immunofluorescence, even when IgG was present, so it is possible to conclude that this antibody is not merely binding to the IgG receptors known to be present. The observation that this staining was also limited to the central body of the egg cylinder is also interesting. Embryonic tissue at the base of the egg cylinder inverts into this area, which eventually develops into yolk sac. This may indicate that the majority of IGF-I receptors are present on the surface of the yolk sac, rather than on the embryonic tissue.

Finally, this antibody was also used in an immunoprecipitation experiment on 11.5 day embryonic and yolk sac tissue. Although the resulting autoradiograph indicates that a variety of new proteins are being synthesised by 11.5 day tissues, unfortunately none of these correspond to the correct molecular weight for the subunits of the IGF-I receptor. Instead, a variety of non-specific binding sites became labelled. The autoradiograph shown later in Fig 6.10 demonstrates that these are non-specific sites, as they are visible in tracks were no antibody was added to the cellular preparation. Although there are data in this chapter suggesting that IGF-I receptors are present at 11.5 days of gestation, these receptors could not be detected by this technique, possibly because the rate of synthesis of IGF-I receptors is so slow, or because there is insufficient protein to detect metabolically.

CHAPTER EIGHT

EXPERIMENTS USING A POLYCLONAL ANTIBODY AGAINST THE IGF-I RECEPTOR

Introduction

The results presented in Chapter 6, in which the addition of a monoclonal antibody reported to block the IGF-I receptor produced a reduction in growth of cultured rat embryos, partially reversed by the addition of the agonist IGF-I, strongly suggested that IGF-I receptors were present during this stage of development. However, the results of the immunoprecipitation experiment described in the following chapter were somewhat disappointing, possibly because the levels of IGF-I receptors present are so small that there is insufficient protein to detect via metabolic labelling techniques. As these experiments were being carried out, another antibody raised against the IGF-I receptor became commercially available, and it was decided to investigate the effects of this antibody on rat embryonic development.

This antibody was a rabbit polyclonal, raised against a 15 amino acid sequence present in the α subunit of the human IGF-I receptor (residues 642-659). However, unlike the αIR-3 used in the previous experiments, this antibody was reported by the company producing it (Upstate Biotechnology Inc.) to cross-react with the α subunit of several other species, including the rat. The effects of this antibody were investigated in a short series of culture experiments similar to those described earlier for the αIR-3 monoclonal, plus another immunoprecipitation experiment. These results should provide further evidence whether IGF-I receptors are present, and important, during embryonic development in the rat.

Method

Rat embryos were explanted from the mother on the 9th day of gestation, as described in Chapter 2. The embryos were then placed in glass bottles, with 1ml of medium per embryo, and gassed with 5%O_2 for 1 minute. The culture medium in these experiments consisted of 75% heat inactivated rat serum, and 25% M199. The anti-human IGF-I receptor antibody was added to the embryos at this stage at a concentration of 1μg/ml, the same concentration used in the previous embryo culture experiments. The glass bottles containing the embryos were then placed in roller incubators at 37° C for the next 48 hours, after which they were assessed for morphological growth and development using the Brown and Fabro system shown in Table 6.1, and also assayed for protein.

In addition to culturing embryos in the presence of this antibody, a series of control embryos was also set up, to which no antibody had been added, as well as a series which were antibody treated but had addition IGF-I (at a concentration of 1μg/ml) in the culture medium.

Results

The results are given for control embryos, those which had the polyclonal anti-human IGF-I receptor added, and finally those which were cultured in the presence of this antibody with additional IGF-I in the culture medium. Results are given in Table 8.1, and also in the form of histograms in Figs 8.1 a,b,c,d and e.

	CONTROL n=8	EXPT n=14	+ IGF-I n=8
Morphological Score	41.75 ± 0.15 S.D.= 0.43	32.57 ± 1.83* S.D.= 2.66	38.50 ± 0.90# S.D.= 2.55
Yolk sac Diameter (mm)	3.61 ± 0.07 S.D.= 0.21	3.15 ± 0.06* S.D.= 0.24	3.48 ± 0.07# S.D.= 0.19
Crown Rump Length (mm)	3.14 ± 0.03 S.D.= 0.09	2.65 ± 0.09* S.D.= 0.35	3.10 ± 0.09$ S.D.= 0.26
Somite number	26.25 ± 0.29 S.D.= 0.83	20.57 ± 1.52* S.D.= 5.69	24.62 ± 0.56# S.D.= 1.58
Protein content (μg)	179.25 ± 6.55 S.D.= 18.53	157.57 ± 9.65* S.D.= 36.10	153.50 ± 15.08 S.D.= 42.66

Table 8.1 The effect of a polyclonal antibody to the IGF-I receptor on rat embryos grown in culture in comparison with controls, and with those with additional IGF-I in the culture medium.

Rat embryos were explanted on the 9th day of gestation and either cultured in the presence of a polyclonal antibody to the IGF-I receptor, in the presence of this antibody plus 1μg/ml IGF-I, or without the addition of an antibody. Results were analysed for significance using Student's t-test.

Significance: *= significant to control at p<0.001%
#= significant to antibody at p<0.001%.
$= significant to antibody at p<0.01%

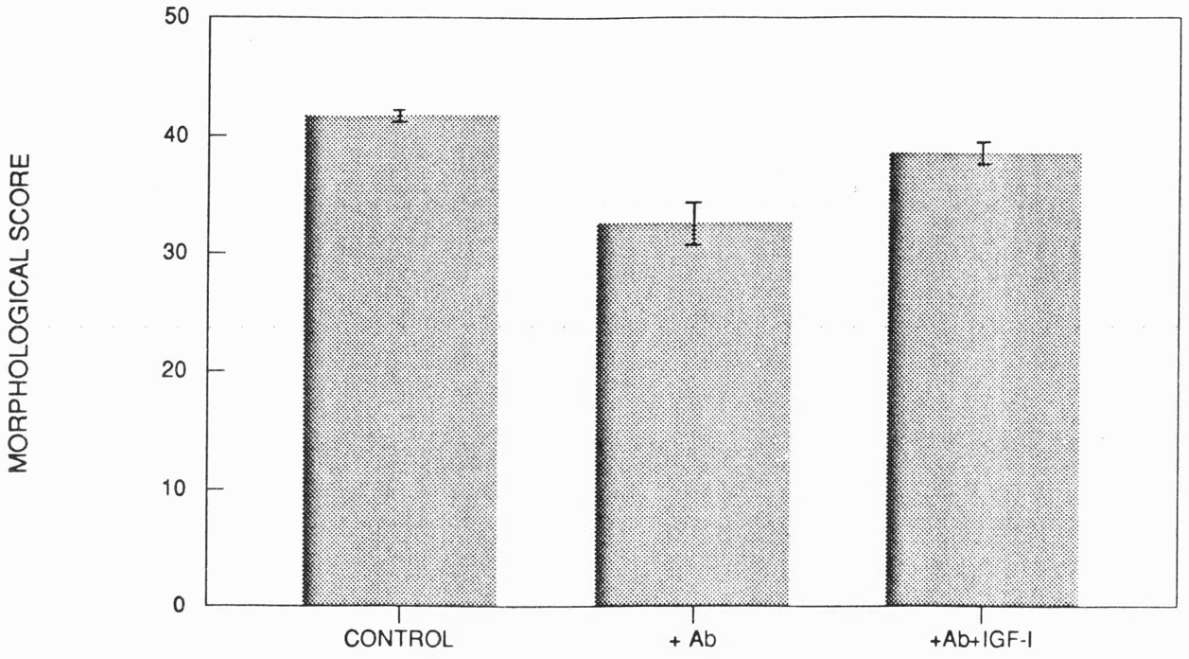

Fig 8.1a Morphological Scores of Embryos.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a polyclonal antibody to the IGF-I receptor, in the presence of this antibody with IGF-I added back to the medium, or without the addition of antibody. Results shown are the mean ± one S.E.M. (n=8 - 14).

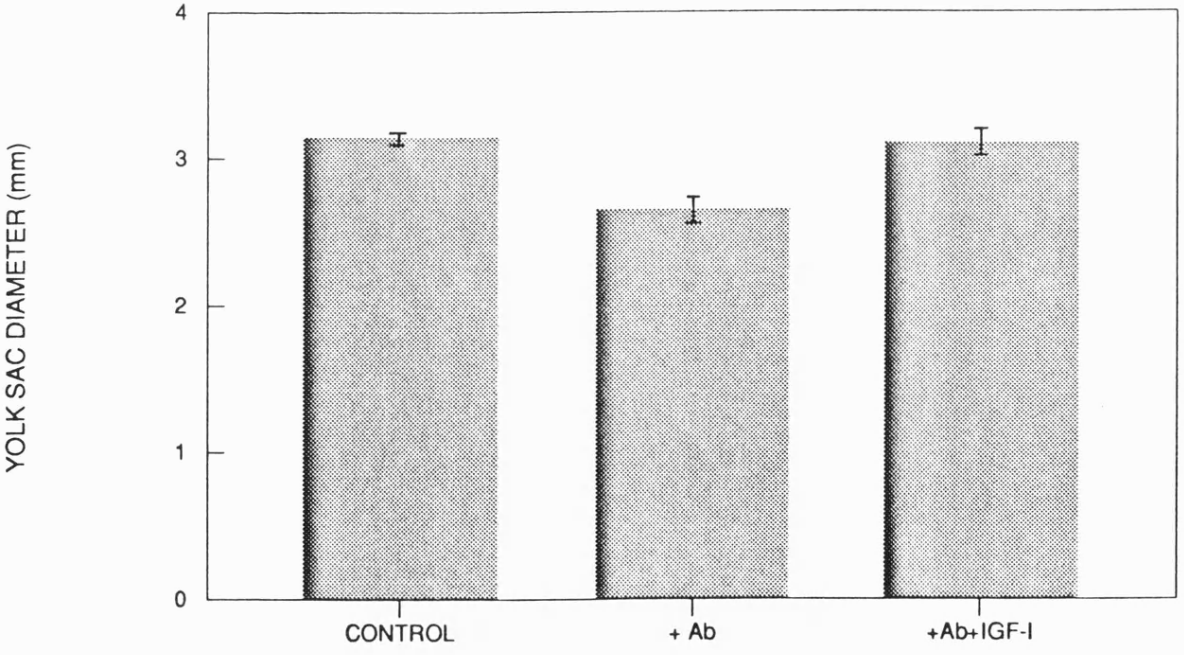

Fig 8.1b Yolk Sac Diameter.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a polyclonal antibody to the IGF-I receptor, in the presence of this antibody with IGF-I added back to the medium, or without the addition of antibody. Results shown are the mean ± one S.E.M. (n=8 - 14).

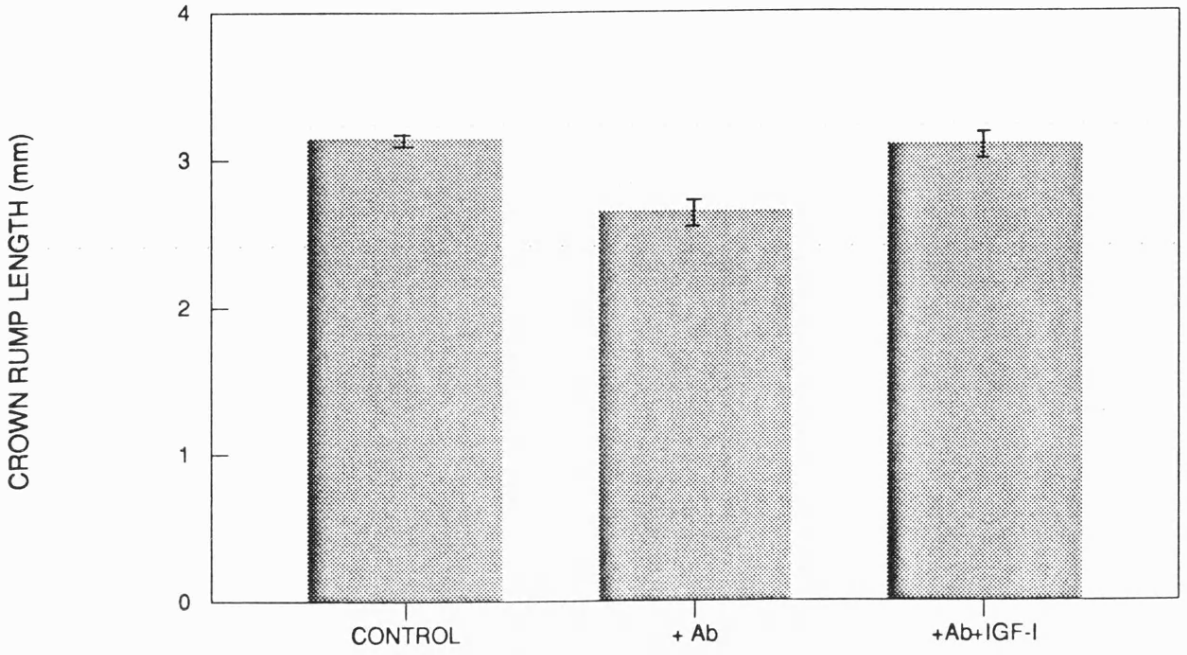

Fig 8.1c Crown Rump Length of Embryos.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a polyclonal antibody to the IGF-I receptor, in the presence of this antibody with IGF-I added back to the medium, or without the addition of antibody. Results shown are the mean ± one S.E.M. (n=8 - 14).

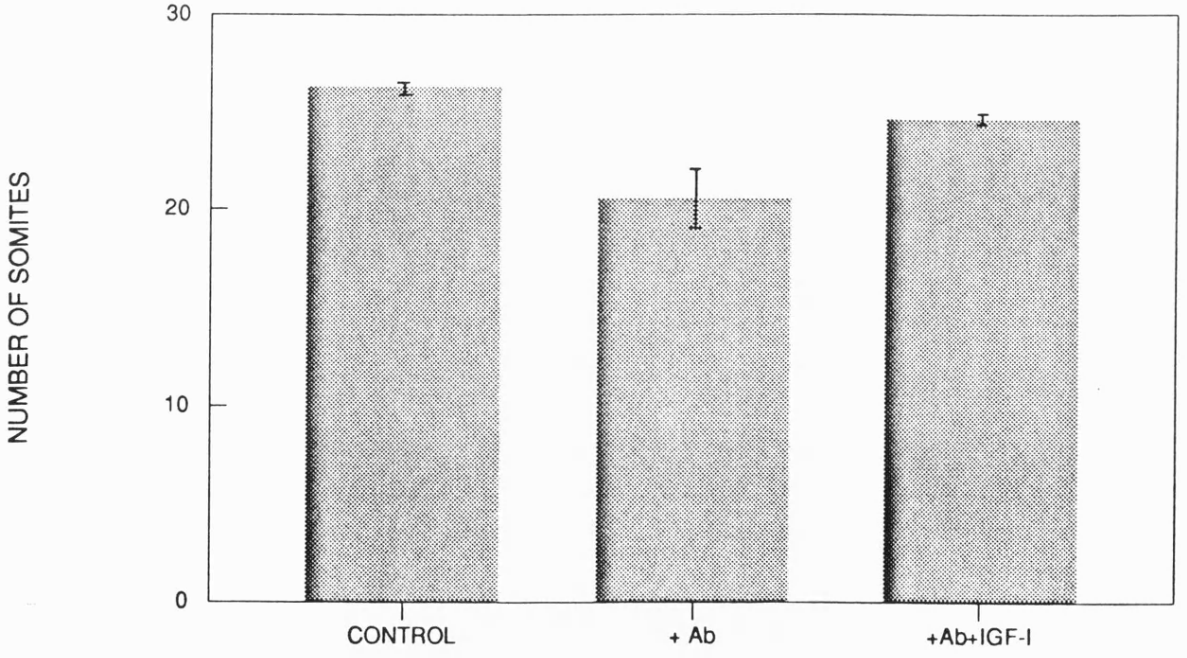

Fig 8.1d Number of Somites.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a polyclonal antibody to the IGF-I receptor, in the presence of this antibody with IGF-I added back to the medium, or without the addition of antibody. Results shown are the mean ± one S.E.M. (n=8 - 14).

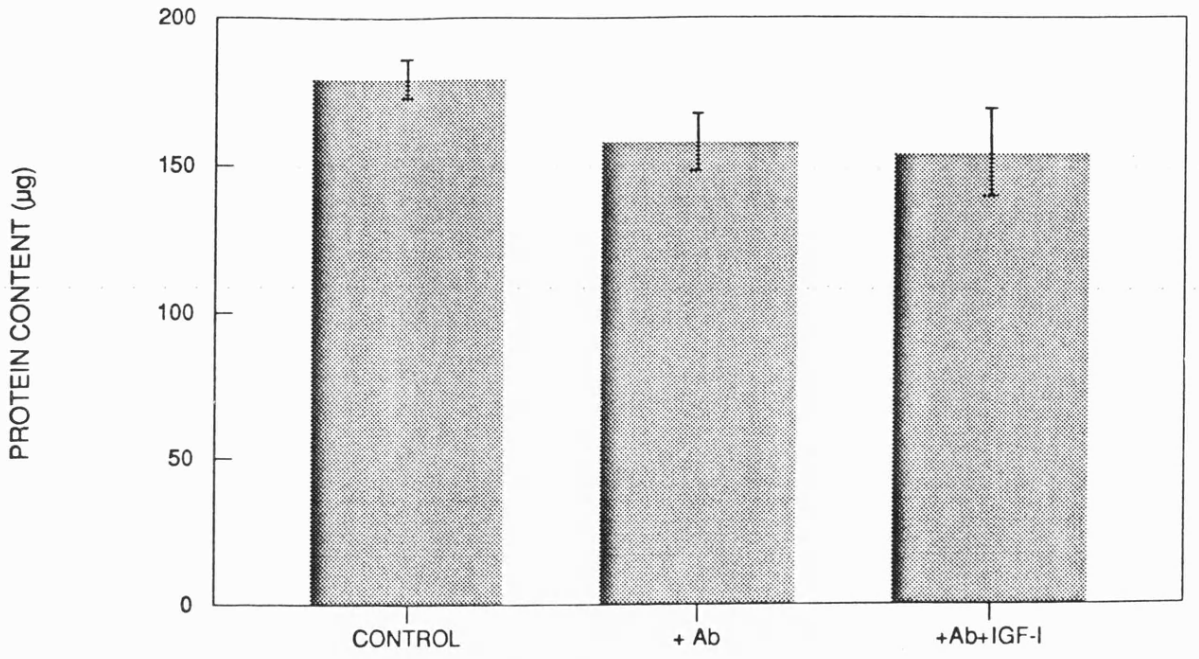

Fig 8.1e Total Protein Content of Embryos.
Embryos explanted on the 9th day of gestation were either cultured in the presence of a polyclonal antibody to the IGF-I receptor, in the presence of this antibody with IGF-I added back to the medium, or without the addition of antibody. Results shown are the mean ± one S.E.M. (n=8 - 14).

The addition of the polyclonal antibody to the IGF-I receptor produces a significant reduction in all the parameters of growth and differentiation measured. There was a 22% reduction seen in morphological score and somite number, while yolk sac diameter and crown rump length were reduced by 13% and 16% respectively. The overall protein content of these antibody treated embryos was also reduced by 13%. The presence of an additional 1µg/ml IGF-I in the culture medium appeared to significantly improve growth in a number of parameters measured. The number of morphological abnormalities seen in the presence of this polyclonal bore some resemblance to those seen with αIR-3. In particular, abnormalities in the tail area were once again prevalent, with 10 out of 14 antibody treated embryos (71%) possessing either kinked or abnormal spiralling tails.

Immunoprecipitation Experiment using a Polyclonal Antibody against the IGF-I Receptor

This experiment was performed to examine whether the rabbit polyclonal antibody against the IGF-I receptor used in the above series of culture experiments was any more sensitive than the αIR-3 monoclonal antibody previously used to detecting this receptor by immunoprecipiation.

Method

The method used for this experiment was essentially the same as that described on pages 152 and 153 of this thesis. The only differences were that the age of the tissue examined was altered, and another antibody was employed.

Rat embryos were removed from the mother on the 17th day of gestation, and their yolk sacs prepared, as described in Chapter 2.

One bottle containing 3 yolk sacs, then had 3mls of Dulbecco's modified Eagle's medium (Appendix 4) and 3mls of dialysed rat serum added, and the yolk sacs were gassed with 95%O_2 for 1 minute. After this, 500μCi (18.5 MBq) of ^{35}S L-methionine (ICN Flow) was added and the yolk sacs incubated for 6 hours at 37° C.

The incubation medium was removed and discarded, and the yolk sacs were washed thoroughly with PBS (Appendix 3). 6mls of ice cold PBSTDS (Appendix 3) was then added to each bottle of yolk sacs for 10 minutes, after which the yolk sac tissue was disrupted by repeated aspiration through a 21 gauge needle. This preparation was then spun at 1500 rpm for 15 minutes at 4° C, the supernatant collected and respun at 11000 rpm for 1 hour.

Protein-A agarose (15μl) was then placed in two 1.5ml Eppendorf tubes, and 10μl (1μg) of the αIR-3 monoclonal added to one, and 5μl (5μg) of the polyclonal added to the other. These amounts were those recommended by the companies producing these antibodies for their use in immunoprecipitation. A 1ml extract of yolk sac supernatant collected from the final spin was then added to both of the antibody-Protein-A mixtures, so that both of the antibodies were tested with this 17.5 day yolk sac tissue as a direct comparison. The two tubes were then placed in a sealed plastic bag and incubated at 4° C overnight, with constant rocking.

The immunoprecipitate was collected the next morning by centrifugation at 13000 rpm for 15 minutes in a microcentrifuge. The resulting pellet was washed four times by the addition of 1ml PBSTDS, and recentrifugation as above. The pellet was finally resuspended in 40μl of electrophoresis sample buffer, and the samples stored at -20° C prior to analysis by SDS-PAGE.

Results

The results of this second immunoprecipitation experiment are shown in Fig 8.2, which shows the resulting autoradiograph after exposure to the gel for 16 hours.

Track 2 shows a sample of the supernatant collected and which has not been exposed to the precipitating antibodies. This track does show that there is a very high rate of protein synthesis in 17.5 day yolk sacs over a six hour period. Tracks 3 and 4 show the results of immunoprecipitation with the two antibodies used (track 3 is the αIR-3 antibody and track 4 the polyclonal). Once again, no specific binding can be detected, with similar molecular weight bands to those in Fig 7.2 apparent. That this binding is totally non-specific is shown when these lanes are compared to track 1. This track was a negative control, which has not been exposed to either of the antibodies, and same bands are detectable in all three lanes. There are no differences apparent between these two antibodies, again suggesting that there is very slow turnover of the IGF-I receptor.

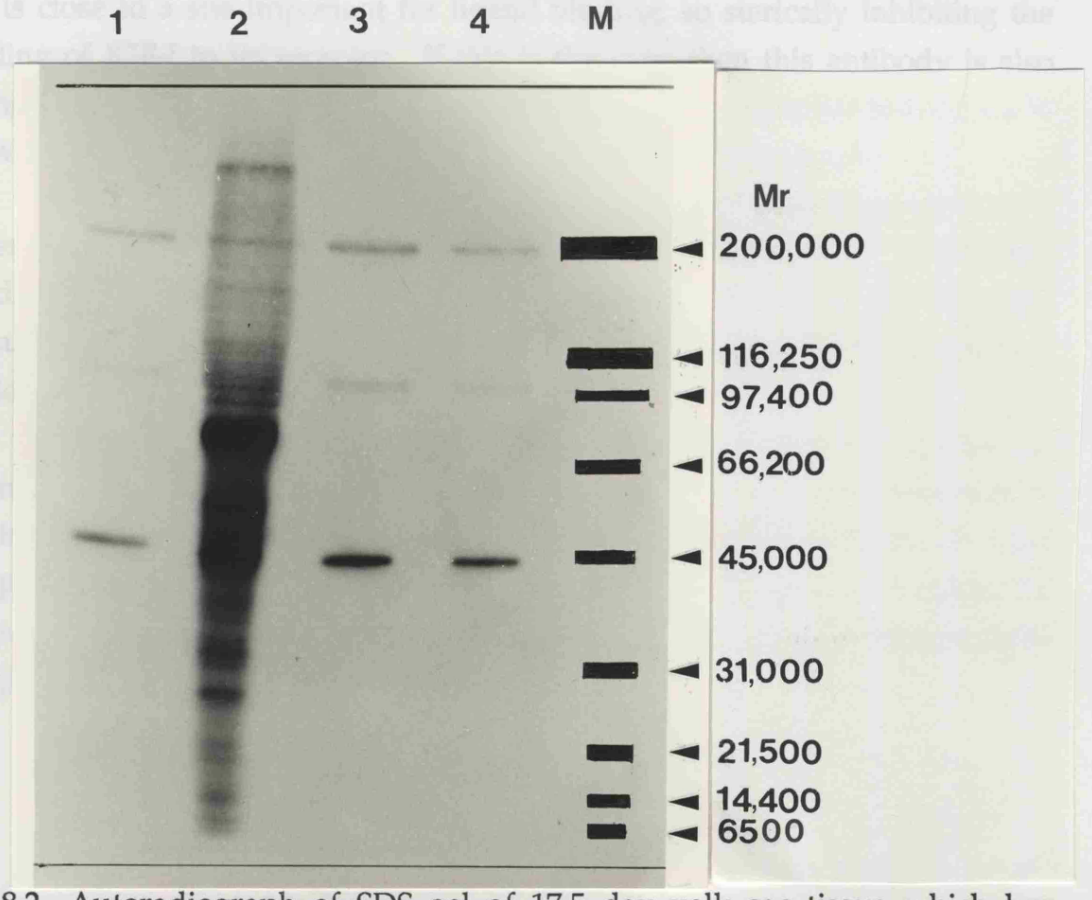

Fig 8.2 Autoradiograph of SDS gel of 17.5 day yolk sac tissue which has undergone immunoprecipitation with both a monoclonal and a polyclonal antibody against the IGF-I receptor.
Tracks: 1= negative control, extract from 17.5 day tissue not exposed to antibody: 2= supernatant; 3= + αIR-3 antibody; 4= + polyclonal antibody; M= marker (Biorad molecular weight marker).

Discussion

After the experiments with the αIR-3 antibody had been performed, another antibody, this time a polyclonal anti-human IGF-I receptor antibody became available. It was decided to use this antibody in a short series of experiments to see if any further information concerning the role of the IGF-I receptor could be determined.

The addition of this antibody to the incubation medium of embryos cultured from 9.5 to 11.5 produced a similar pattern of growth reduction to that seen with αIR-3. This result was again partially reversible by the addition of IGF-I. In particular, abnormalities are apparent in the tail area, which may demonstrate some abnormality in cartilage formation.

The reasons why this polyclonal antibody produces these effects is not clear. The antibody was raised against a portion of the α subunit. The ligand binding site is present on the α subunit, and although tyrosines 24, 31 and 60 have been implicated in the high affinity binding of the ligand to its receptor (Bayne *et al*, 1990), it is believed that several additional regions may contribute to binding (Soos *et al*, 1992). It is, therefore, possible that the antibody binding site is close to a site important for ligand binding, so sterically inhibiting the binding of IGF-I to its receptor. If this is the case, then this antibody is also acting to block binding of IGF-I to its receptor, leading to similar reductions in growth and development to those seen with the αIR-3 monoclonal.

Unfortunately, once again the results of the immunoprecipitation experiment comparing this antibody to αIR-3 are disappointing. No specific bands can be detected in the range which would correspond to this receptor. Presumably this antibody, like the αIR-3 is not able to detect IGF-I receptors at the low copy numbers at which they are synthesised.

It would seem, therefore, that IGF-I receptors are present during the period of embryogenesis in the rat, and that interference with these receptors is detrimental to the normal development of the rat. The numbers of these receptors is so low, however, that they are not readily detectable by immunoprecipitation techniques. It would next be useful to try to localise these receptors more precisely using immunocytochemistry.

CHAPTER NINE

GENERAL DISCUSSION

Discussion

The sequence of events which produces a fully differentiated organism from a fertilised ovum, are of fundamental interest and importance to biologists today. It appears that a number of peptide molecules are intricately involved with the regulation of development, and these molecules have been termed growth factors. In addition to their role in mitogenesis, these factors also appear to regulate differentiation, and are believed to act at the level of gene regulation. The number of molecules implicated as having some growth factor activity is constantly increasing, and their importance is being examined in a variety of species, tissues and cell lines.

The importance of growth factors may extend, however, beyond normal embryonic development. One possibility is that perturbations in the growth factor/ growth factor receptor system during development may result in congenital malformations or other developmental problems such as intra-uterine growth retardation. This apparently plausible explanation for some of the problems which often arise during development has not yet been fully examined.

Further, cancerous cells are often described as behaving in a manner similar to embryonic tissue, proliferating at a rate only normally seen during development. The proliferation of normal cells in culture is known to be dependant upon the presence of certain essential growth factors. Malignant cells do not possess this dependence on external factors, suggesting that they are either capable of producing essential mitogenic factors themselves, or that they can fulfil some other action which results in DNA synthesis. Examples of transformed cells (those which have lost their contact inhibition, and are able to grow freely) are known to be able to activate a growth factor gene leading to autocrine stimulation and consequent neoplasia, and it is likely that growth factors are intricately involved in the uncontrolled proliferation of malignant cells. Inhibition of their action could possibly provide a tool against this process.

This thesis has investigated the possibility that two of these growth factors, insulin and the closely related IGF-I, may play a role in the postimplantation development of one animal - the rat. This model is a good system to use because the embryonic development of the rat has been widely studied, while it is also possible to examine whole rat conceptuses *in vitro*. This makes the examination of developmental factors in isolation possible.

Following implantation of the embryo into the uterus the rate of growth dramatically increases, and the successful culture of such embryos *in vitro* requires the addition of serum to the culture medium: serum which is known to contain a variety of growth factors. One of the first differentiation events which

occurs after implantation results in the formation of the extraembryonic membranes which entirely surround the rat for the remainder of its development. The visceral endoderm and extraembryonic mesoderm together give rise to the visceral yolk sac, which is particularly important in rodents not only because it forms a physical, protective barrier, but also because it is responsible for nutrition before the placenta is fully functional.

Much of the work in this thesis describes the processing of insulin and IGF-I by this important tissue. This has been examined in a variety of ways: both radio- and fluorescently-labelled insulin and IGF-I have been examined in two differing yolk sac systems. Yolk sac tissue has been removed from the mother on the 17th day of gestation, and compared with tissue removed on E9 and subsequently cultured until it was an equivalent age. The principal advantage of the culture system is that it allows the yolk sac to develop as a closed vesicle, which in turn permits the analysis of molecules which accumulate within that vesicle. However, one disadvantage is that these yolk sacs undergo such a long period of culture, at the end of which they are somewhat metabolically depleted and physically fragile. Using both recently explanted and cultured yolk sac tissue, therefore, is important, and increases the likelihood that the results seen are not an artifact of the experimental system.

The digestion of [^{125}I]-labelled insulin present in the incubation medium occurs rapidly, such that after 4 hours of the experiment approximately 75% of the protein has been degraded. This demonstrates how efficiently the yolk sac can remove excess insulin from the culture medium. The mechanism by which this degradation is occurring has not been elucidated, however, and requires further investigation. The effects of lysosomal inhibitors upon the rate of degradation should determine whether an intracellular lysosomal pathway is involved in insulin degradation in the yolk sac, or whether degradation is principally extracellular. The fluorescence microscopy studies, which reveal FITC-labelled insulin apparently present within vesicles close to the apical surface of the cell after only a few minutes incubation, perhaps support the hypothesis that insulin is internalised. These vesicles are probably early endosomes, and may represent the first stage in the intracellular processing of insulin. An examination of the fluid within the cultured yolk sacs revealed that a small proportion of the insulin presented to the external face of the tissue was eventually found intact within this closed vesicle. It is difficult to propose a mechanism in which intact protein could gain access to the fluid in which an internalisation step is not involved. A small amount of intact insulin appears to accumulate within this fluid with time at a steady rate, despite the relative levels of intact insulin in the incubation medium falling. One possibility is,

therefore, that insulin is internalised following binding to a specific receptor, and following transfer to endosomes, a small proportion is transported intact across the yolk sac epithelium within transport vesicles. The remainder of the insulin could then either be channelled to the lysosomes to degradation, from which small molecular weight breakdown products could diffuse into both the EEC fluid and back into the incubation medium, or insulin could instead be digested within the endosomes by an as yet unidentified enzyme. If the majority of insulin digestion is occurring at the yolk sac surface, the only mechanism to explain the presence of intact insulin within the EEC fluid would be to propose that a small proportion of the hormone was internalised into transport vesicles. Once again, the actions of lysosomal enzyme inhibitors and compounds which inhibit fusion would help determine more precisely what the mechanism is.

Very similar results are seen in the case of IGF-I processing. Again, a high rate of [^{125}I]-IGF-I degradation is seen, which, like insulin degradation is reduced by low temperature while the glycolysis inhibitor sodium fluoride appeared to produce very little effect.

One problem in interpreting these results is that a degree of cross-reactivity between these ligands and their respective receptors is known to occur. Thus at high concentrations of insulin, this ligand will bind to IGF-I receptors, and similarly, IGF-I will bind to insulin receptors. It is impossible to tell with such studies whether any cross-reactivity is occurring, or to what extent.

When insulin and IGF-I are fluorescently labelled, they appear to be rapidly internalised, but are apparently channelled to different areas of the cell. While FITC-insulin can be detected within vesicles close to the apical surface of the tissue, where it remains, TRITC-IGF-I is detectable throughout the tissue and appears to be transported readily to the internal face of the yolk sac epithelium. The physiological explanation for this apparent difference in sorting mechanisms is far from clear. It may represent some method for storing insulin, perhaps because it is required as a growth factor principally on yolk sac tissue. Alternatively, endosomes may represent the major site of insulin degradation within the yolk sac. In contrast, IGF-I is transported to the inner face of the yolk sac where it could then act on embryonic receptors. Although the reasons for this difference following uptake are not clear, it is likely that it arises due to binding to specific receptors.

However, the strongest evidence that specific IGF-I receptors are present on the rat visceral yolk sac was seen when a monoclonal antibody to this receptor was investigated. Culture of rat embryos throughout the period of organogenesis in the presence of a monoclonal antibody reported to block the

IGF-I receptor (the αIR-3 antibody) resulted in a dramatic reduction in growth, plus an associated increase in morphological abnormalities. These abnormalities were not severe, but a pattern of malformation was apparent. These deficits were partially overcome by the addition of excess IGF-I, while IGF-II and insulin had little or no effect. This series of experiments provides the strongest evidence that IGF-I receptors are present from 9.5 days of gestation (or earlier), and importantly, that they play a role in the normal embryonic development of the rat.

These receptors could be present either on the extraembryonic membranes which surround the embryo, or on the embryonic tissue itself. Similarly they could bind IGF-I produced either by the mother or by the embryo. Attempts to localise this receptor using the technique of immunoprecipitation were largely unsuccessful, possibly due to the small number of IGF-I receptors present. However, when a 9.5 day egg cylinder was exposed to an immunofluorescent label, the pattern of staining seen illustrated that these receptors are almost exclusively localised on the yolk sac tissue, with little or no staining on the embryonic endoderm at this stage. From this it can be concluded that at 9.5 days at least, IGF-I receptors are present on the extraembryonic membranes where they could be responsible for uptake of maternally derived factor.

IGF-I receptors would appear, therefore, to be present to mediate processing of this growth factor throughout organogenesis. These findings strongly suggest that these factors play a role in development. However, insulin and IGF-I are only two factors to which developing embryos are exposed, and although it is useful to study the effects of such factors in isolation, it should be borne in mind that *in vivo*, the situation is considerably more complex.

It would now be useful to examine the roles of other growth factors of potential importance during rat embryonic development using similar techniques to those described in this thesis. The principal reason for employing an embryo culture system to investigate the role of growth factors, is that it becomes possible to manipulate the local environment of the embryo in a variety of ways which are not possible if that animal were developing *in utero*. Thus the intact developing embryo can be examined in detail, and in isolation from the mother. Similarly, investigations into the processing of other macromolecules by the visceral yolk sac presents us with another approach which can be used to determine factors which are likely to be important during development, as the surface of this tissue is unlikely to possess specific receptors unless that ligand is required.

In particular, monoclonal antibodies provide a powerful tool for the

identification and localisation of growth factors and their associated receptors, and if antibodies are available which specifically block a known receptor type, this presents is a useful, and comparatively simple technique to examine the roles of these receptors *in vivo*. Similar thinking lies behind the use of antisense oligonucleotides (a single stranded piece of DNA which is complementary to the mRNA encoding for a particular protein) to investigate the actions of growth factors. There have been reports in the literature of the reduction of expression of growth factor genes using antisense oligonucleotide sequences applied to the incubation medium of either cultured cells or mouse blastocysts, for example. This technology could be imported to the embryo culture/ yolk sac culture systems to directly decrease ligand and receptor activity.

APPENDICES

APPENDIX 1

HANK'S BALANCED SALT SOLUTION

SALT	CONCENTRATION(mg/ml)
Calcium chloride. $2H_2O$	O.1855
Potassium chloride	0.40
Potassium dihydrogen orthophosphate	0.60
Magnesium sulphate	0.20
Sodium chloride	8.00
Sodium hydrogen carbonate	0.70
Disodium hydrogen orthophosphate	0.0475
D-glucose	1.00
Sodium Phenol Red	0.017

APPENDIX 2

MEDIUM 199

AMINO ACID	CONCENTRATION (g/L)
L-alanine	0.050
L-arginine HCl	0.070
L-aspartic acid	0.060
L-cysteine HCl	0.0001
L-cystine	0.026
L-glutamic acid	0.1336
L-glutamine	0.100
L-glycine	0.050
L-histidine HCl.H_2O	0.02188
L-hydroxyproline	0.010
L-isoleucine	0.040
L-leucine	0.120
L-lysine HCl	0.070
L-methionine	0.030
L-phenylalanine	0.050
L-proline	0.040
L-serine	0.050
L-threonine	0.060
L-tryptophan	0.020
L-tyrosine	0.05766
L-valine	0.050

VITAMINS	CONCENTRATION (mg/L)
p-aminobenzoic acid	0.00005
ascorbic acid	0.000056
D-biotin	0.00001
calciferol	0.0001
D-Ca-pantothenate	0.00001
folic acid	0.00001
i-ionositol	0.00005
menadione	0.000016
nicotinamide	0.000025
nicotinic acid	0.00025
pyridoxal HCl	0.000025
pyridoxine HCl	0.000025
retinol acetate	0.00014
riboflavin	0.00001
thiamine HCl	0.00001
DL-Tocopherol phosphate	0.00001

INORGANIC SALTS AND OTHER COMPOUNDS	CONCENTRATION(mg/L)

As for Hank's balanced salt solution plus:

Adenine	0.010
ATP	0.001
Adenylic acid	0.0002385
Cholesterol	0.0002
Deoxyribose	0.0005
L-glutathione	0.00005
Glanine HCl.H_2O	0.0003
Hypoxanthine	0.0003
Sodium Phenol Red	0.0213
Tween 80	0.020
Ribose	0.0005
Sodium acetate	0.050
Thymine	0.0003
Uracil	0.0003
Xanthine	0.000344

APPENDIX 3

Solutions used in Immunoprecipitation Experiments

Phosphate Buffered Saline (PBS):
In 1 litre distilled water, dissolve,
- 8g sodium chloride
- 1.3g dibasic sodium phosphate
- 0.2g monobasic sodium phosphate
- Adjust pH to 7.4

PBSTDS:
Add to 1 litre distilled water, and dissolve,
- 10ml 1M dibasic sodium phosphate, pH7.2
- 9g sodium chloride
- 10ml 100% Triton X-100
- 5g sodium deoxycholate
- sodium dodecyl sulphate
- 20mg sodium azide
- 0.04g sodium fluoride
- Adjust pH to 7.25 with 1M monobasic sodium phosphate.

APPENDIX 4

DULBECCO'S MODIFIED EAGLE'S MEDIUM
(without L-cystine or L-methionine)

INORGANIC SALTS	CONCENTRATION (g/L)
Calcium chloride. $2H_2O$	0.265
Ferric nitrate.$9H_20$	0.0001
Magnesium sulphate	0.09767
Potassium chloride	0.400
Sodium chloride	6.400
Sodium phosphate monobasic	0.109

AMINO ACIDS	
L-arginine HCl	0.084
L-glutamine	0.584
L-glycine	0.030
L-histidine HCl.H_2O	0.042
L-isoleucine	0.040
L-leucine	0.105
L-lysine HCl	0.146
L-phenylalanine	0.066
L-serine	0.042
L-threonine	0.095
L-tryptophan	0.016
L-tyrosine 2Na.$2H_2O$	0.10379
L-valine	0.094

VITAMINS AND OTHER COMPOUNDS	
Choline chloride	0.004
Folic acid	0.004
Myo-inositol	0.0072
Niacinamide	0.004
Pantothenic acid	0.004
Pyridoxal. HCl	0.004
Riboflavin	0.0004
Thiamine. HCl	0.004
Glucose	1.000
Sodium phenol red	0.0159
Pyruvic acid	0.110

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